Characterizing the role and regulation of a pro-apoptotic Bcl-2 family member, Harakiri (HRK) in Glioblastoma Multiforme

by

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This is to certify that I have examined this copy of a master's thesis by

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ABSTRACT

Glioblastoma Multiforme (GBM) is the most common and aggressive type of brain tumors and the patient survival rate is approximately 12 months after diagnosis. Unfortunately, there is not any effective therapy because of poor delivery of therapeutics due to the blood brain barrier and tumor cells' resistance to the therapy. Tumor-necrosis-factor related apoptosis-inducing ligand (TRAIL) is a promising factor that induces apoptosis specifically on tumor cells; and its efficiency is preclinically tested. However, approximately %50 of GBM cell lines are resistant to TRAIL. Evidence shows that aberrant expressions of apoptotic machinery might be responsible for TRAIL resistance, but which mechanisms are particularly responsible for this innate difference is not fully understood. We have recently observed that expression of Harakiri (HRK) that is a BH3-only Bcl-2 family protein is highly increased in TRAIL-sensitive subpopulation of a human GBM cell line; and it is significantly repressed in the TRAIL-resistant subpopulation. This difference in HRK expression is remarkably higher compared to differences in other members of apoptosis.

HRK is a sensitizer BH3-only protein and regulates apoptosis by interfering with anti-apoptotic Bcl-2 and Bcl-xL proteins and blocking their function. While its function is characterized in the context of the nervous system, its implications in tumorigenesis are not well studied. Few studies show the suppressed expression levels of HRK in tumors and exogenous expression of HRK attenuates the tumor growth in some cancers. However the functional role of HRK and the relations with TRAIL have not been studied in GBM before.

In this study, we investigated the role of HRK in GBMs. We found that HRK is differentially expressed among established GBM cell lines. In order to assess the role of HRK by gain-of- and loss-of-function approaches, we generated HRK overexpression and knockdown vectors and performed experiments that tested the effects of HRK on cell viability and apoptosis. As a result, we observed that HRK overexpression itself induced apoptosis in

different GBM cells at different levels. Also, we showed that this phenotype could be blocked by forced expression of Bcl2- and Bcl-xL, suggesting the functional interaction of Bcl-2/Bcl-xL and HRK in tumor cells. Moreover, HRK overexpression showed additive activity with TRAIL in GBM cells. In addition, we identified that the agents that cooperate with TRAIL to induce apoptosis, such as the histone deacetylase inhibitor MS-275, significantly increased HRK expression levels. Also, knockdown of HRK blocked TRAIL-sensitizing effect of MS-275. This suggests that HRK induction might be one mechanism during TRAIL sensitization and apoptosis. In order to better understand how HRK gene expression is regulated and to identify novel agents with the ability to induce GBM cell apoptosis, we also developed a reporter system that monitors HRK regulation in GBM cells.

Taken together, our results suggest that HRK upregulation is associated with GBM cell apoptosis and has significant role for TRAIL-induced apoptosis. Thus, novel small compounds that can increase HRK levels in cancer cells might offer new therapeutic approaches in the future.

ÖZET

Glioblastoma Multiforme çok yaygın görülen agresif bir beyin kanseridir. GBM hastalarının yaşam süresi teşhis sonrası yaklaşık 12 aydır. Beyinin korunaklı yapısı ve kan beyin bariyerinin varlığından dolayı teröpatik ilaçlar beyine taşınamamaktadır. Ayrıca GBM hücreleri bir çok teröpatik ajana direnç göstermektedir. Bu sebeplerden dolayı etkili bir tedavi yöntemi bulunmamaktadır ve yeni tedavi şekillerine ihtiyaç duyulmaktadır. Tümör-nekroz-faktör ilişkili apoptoz indükleyici ligand (TRAIL) tümörlere spesifik özelliğinden dolayı gelecek vadeden güçlü bir aday proteindir. Klinik öncesi araştırmalarda test edilmiştir. Fakat GBM hücre hatlarına baktığımızda TRAIL'a farklı duyarlılıklara sahip hücreler olduğunu ve %50 si TRAIL'a direnç gösterdiğini görmekteyiz. TRAIL duyarlılık farklarının apoptoz mekanizmasında rol alan proteinlerin anormal ifadesinden kaynaklanabileceği çalışmalarda gösterilmiştir. Fakat hala TRAIL'a direnç mekanizması tümüyle anlaşılabilmiş değildir. Yakın zamanda, TRAIL'a farklı duyarlılığa sahip insan GBM hücre hatlarıyla yaptığımız çalışmalarda, pro-apoptotik Harakiri (HRK) geninin ifadesinin TRAIL duyarlı hücrelerde çok etkin olup, TRAIL dirençli hücrelerde baskılandığı gözlemlenmiştir. Bu gen ifadesi farklılığı, TRAIL direnç mekanizmasında olduğu bilinen diğer genlerin ifadesinden oldukça fazladır.

HRK bir pro-apoptotik proteindir. HRK anti-apoptotik Bcl-2 ve Bcl-xL proteinlerine bağlanıp, fonksiyonlarını engelleyerek apoptozu düzenler. Fonksiyonu çoğunlukla sinir sisteminde çalışılmıştır. Fakat kanserlerdeki fonksiyonu çok fazla çalışılmamıştır. Bazı çalışmalar HRK ifadesinin bazı kanserlerde baskılandığını ve HRK ifadesi arttırıldığında bazı tümörlerin büyümesinde azalma görüldüğü belirtilmiştir. Fakat GBM'lerde HRK'nin fonksiyonu ve TRAIL ile ilişkisi daha önce çalışılmamıştır.

Bu tez çalışmasında, GBM'lerde HRK'nin rolünü araştırdık. Çalışmalarımızda, HRK'nin TRAIL'a farklı duyarlılıktaki GBM hücre hatlarında farklı ifadeleri olduğunu gösterdik. HRK'nin rolünü anlamak için HRK'nin fazla ifadesini ve susturulmasını yöntem olarak kullandık. Bunun

için, HRK yüksek ifadesi vektörü ve shHRK vektörü klonladık ve HRK'nin fonksiyonunu hücre canlılığı ve apoptoz deneyleri ile test ettik. Sonuçlarımızda HRK'nin yüksek ifadesinin GBM hücre ölümüne yol açtığını ve bunun Bcl-2 ve Bcl-xL'in yüksek ifadesi ile baskılanabileceğini gösterdik. Bunu yanında, HRK'nin bazı GBM hücrelerinde TRAIL yanıtını arttırdığını ve HRK'nin susturulmasının TRAIL yanıtını azalttığını gösterdik. Ayrıca, TRAIL duyarlılığını arttırdığını bildiğimiz faktörlerin (ör: MS-275) HRK ifadesini arttırdığını gözlemledik. HRK susturulduğunda MS-275'in TRAIL duyarlılığını arttıramadığı gösterilmiştir. Bu sonuç, HRK'nin MS-275'ın TRAIL duyarlılığı arttıran mekanizmasında görevli olduğunu göstermektedir. Ayrıca GBM hücrelerinde HRK ifadesinin nasıl düzenlendiğini anlamak ve TRAIL'e duyarlılığı arttırabilecek yeni ajanlar bulmak için HRK promotör bölgesini klonladık.

Sonuç olarak, HRK ifadesinin GBM hücre ölümü mekanizmasında ve GBM'lerin TRAIL cevabında rol aldığını gösterdik. Bu sonuç göz önüne alındığında, gelecekte HRK ifadesini arttıran ajanlar bulmanın GBM tedavisi için önemli olduğunu düşünüyoruz.

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TABLE OF CONTENTS

LIST OF THE TABLES

LIST OF THE FIGURES

NOMENCLATURE

APAF-1	Apoptotic protease activating factor 1
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X protein
BBB	Blood–brain barrier
BH	Bcl-2 Homology Domain
Bid	BH3 interacting-domain death agonist
CDHN2A/p16	Cyclin dependent kinase 2a/p16
Cyt C	Cytochrome C
DISC	Death-inducing signaling complex
EGFR	Epidermal growth factor receptor
EOC	Epithelial ovarian cancer
FADD	Fas-Associated protein with Death Domain
FGF	Fibroblast Growth factor
FLIP	Flice-like inhibitory protein
GBM	Glioblastoma Multiforme
HRK	Harakiri

IAP	Inhibitor of Apoptosis protein
IDH1	Isocitrate Dehydrogenase 1
JNK	Jun N-terminal kinase
MGMT	O-6-methylguanine-DNA methytransferase
MOMP	mitochondrial outer membrane permeabilization
NGF	neuronal growth factor
PDGF	Platelet-derived growth factor
PRC2	Polycomb repressive complex 2
PTEN	Phosphatase and tensin homolog
TMZ	Temozolomide
TNF	Tumor necrosis factor
WHO	World Health Organization
XIAP	X-linked inhibitor of apoptosis

Chapter 1

Review of Literature

1. Overview of the primary brain tumors

Glioblastoma Multiforme

Gliomas form the majority of the primary brain tumors with an annual incidence of 5/100,000 individuals (1). Classification of gliomas is based on the originated cell type, histopathological appearance, locations and lineage markers. Gliomas are classified as astrocytomas, oligodendrogliomas, oligoastrocytomas, ependymomas and choroid plexus tumors (2). Astrocytomas are the most common brain tumor type. World Health Organization (WHO) classifies astrocytic tumors into four grades according to their malignancy (3). Grade I, pilocytic astrocytomas are biologically benign tumors and generally seen in young adults and children. Surgery is mainly used as treatment option. Grade II, low grade or diffuse astrocytomas grow slowly and invade brain structures. The survival rate differs from patient to patient but is approximately 4-5 years. Surgical resection, chemotherapy and radiotherapy are used for the treatment of Grade II gliomas. Grade III, anaplastic astrocytomas and anaplastic oligodendrogliomas and Grade IV, Glioblastomas are classified as malignant or high grade gliomas (2).

GBM (Grade IV) is the most common and malignant type of primary glioma with approximately 3 in 100,000 people diagnosed every year (4). The survival rate of GBM patients is approximately 1 year (5). In only 3 to 5 % of GBM patients, more than 3-year survival is reported. Although molecular and clinical factors affecting survival rate are not well known, correlation between longer survival and promoter methylation of O-6-methylguanine-DNA methytransferase (MGMT) gene or IDH1 mutation has been reported recently (6).

Recurrence of GBM is inevitable and even worse. Patients having recurrent GBM have median survival of five to seven month after optimal therapy (7) **(Figure 1).** GBMs constitute 60-70% of all gliomas (1) and can progress as primary *de novo* tumors or secondary GBMs, which are generated from the Grade II to Grade III astrocytomas (8). Features of GBM include complex chromosome abnormalities, diffuse infiltration throughout the brain parenchyma, robust angiogenesis, intense resistance to apoptosis, pseudopalisading necrosis and genomic instability (9). Vascular hyperproliferation and necrosis are the essential characteristics of GBM to differentiate it from the low grade gliomas (4). In addition, the mutation profile of primary and secondary GBMs are different. While the markers of secondary glioblastomas are TP53 mutation, Platelet-derived growth factor (PDGF), PDGF receptor (PDGFR) and Fibroblast growth factor (FGF) overexpression, loss of RB and Phosphatase and tensin homolog (PTEN), the markers of primary glioblastomas are the gain of function mutation in Epidermal Growth Factor Receptor (EGFR), loss of PTEN and Cyclin dependent kinase 2a/p16 (CDHN2A/p16) (10)(11).

The term “Multiforme” describes one of the important GBM features, which is tumor heterogeneity affecting tumor cells’ morphologies, growth rates, drug responses and gene expression levels (3),(12). For example, TP53 and EGFR mutation are observed in a subset of tumor cells. Also, MGMT is differentially expressed in GBM and MGMT silencing is related to the better response to the alkylating agent such as TMZ (3).

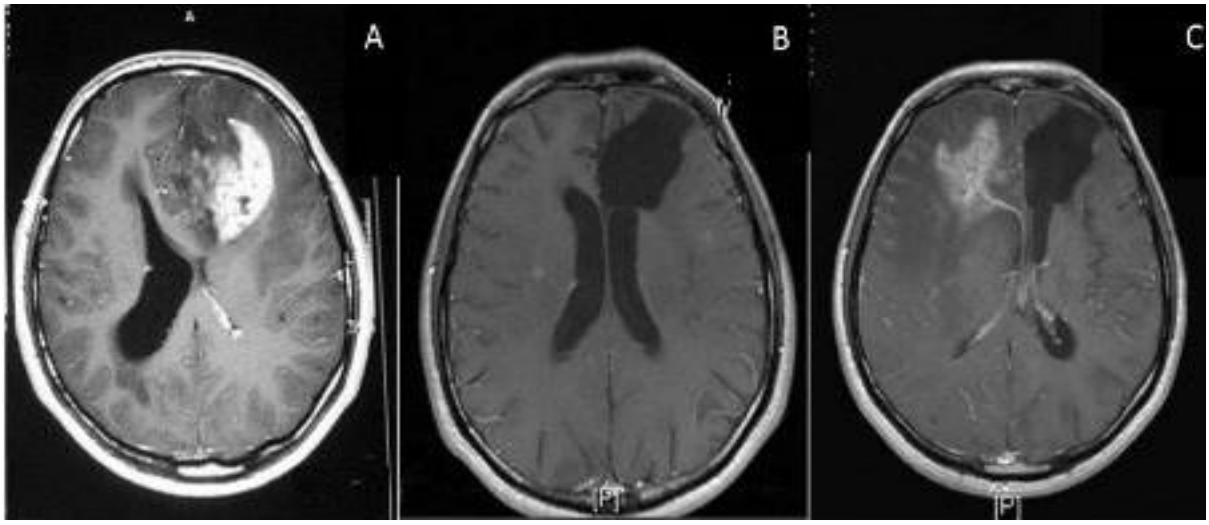


Figure 1: **MRI result of GBM patient**. **(a)** Before surgery**. (b)** After surgery. **(c)** Recurrence of tumor after 18 months of therapy (image by Prof. Dr. Ihsan Solaroglu)

Treatment options for GBM

Treating primary gliomas is one of the greatest challenges in oncology (2). Because of the tumor heterogeneity, GBM cells are resistant to conventional therapies. Moreover, repair capacity of the brain itself is very limited. Also, the low blood-brain barrier (BBB) permeability prevents achieving the desired effects of chemotherapeutics (11). Current standard treatment options are surgical resection, radiotherapy and chemotherapy (5). After diagnosis, the first step for treatment is tumor resection, but the decision for surgery is given according to tumor location and size. Following surgery, concurrent or sequential chemo-radiotherapy with Temozolomide (TMZ) is the current treatment option for GBM (9). TMZ, which is a small alkylating agent, kills cancer cells by generating O6-methyguanine in DNA, that miss-pairs with thymidine during the next cell cycle. TMZ is preferential because it shows low toxicity and can cross the BBB. Moreover, TMZ is much more able to penetrate the intra-tumoral region

compared to other drugs (1). However, resistance of tumor cells to TMZ is frequently observed. Thus, new treatment options are necessary for the GBM patients.

2. Apoptosis

Apoptosis is the process of programmed cell death that plays an essential role in embryonic development and tissue homeostasis. Apoptosis leads to morphological changes in the cells like cell shrinkage, chromatin condensation, and cytoplasmic membrane blebbing. In cancers, including GBMs, apoptotic programs are suppressed and tumor cells evade death through unique mechanisms. Deregulation of apoptosis disrupts the balance between cell proliferation and cell death, and therefore leads to the development of cancer (13).There are two key apoptosis pathways in mammalian cells; intrinsic and extrinsic pathways **(Figure 2).**

Extrinsic pathway and TRAIL

The extrinsic pathway/death receptor pathway is activated from the outside of the cell by the pro-apoptotic ligands interacting with death receptors (DRs) (14). One of the ligands activating extrinsic pathway is Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). TRAIL is a Tumor necrosis factor (TNF) superfamily member and expressed on the surface of natural killer (NK) cells, T cells, macrophages and dendritic cells. TRAIL is synthesized as pro-form with signal sequence, which is removed in the matured secreted protein and can be anchored via hydrophobic amino acids or released as a soluble protein. TRAIL proteins are functional as trimers and induce apoptosis by binding to trimeric DRs similarly to receptors like FasL or TNFα. (15). TRAIL interacts with five different DRs that are encoded by different genes in humans. Interaction between TRAIL and DR4 and DR5 leads to apoptosis. Decoy receptors 1 (Dcr1) and 2 (Dcr2) are expressed on the surface and have ligand binding domains like DR4 and DR5, however they do not have functional death domain to convey the signal. Therefore, they are unable to induce apoptosis when TRAIL binds (15),

(16). The last TRAIL receptor is osteoprotegerin (OPG) which is soluble and has a low affinity receptor for TRAIL (16).

In the extrinsic pathway, TRAIL binding to DR4 and DR5 recruits the FADD protein and pro-caspase 8 protein to the intracellular death domain of the receptor. Thereby, the death inducing signaling complex (DISC) including DR, FADD and caspase-8 are formed. Caspase-8 is oligomerized and oligomerization of caspase-8 leads its auto-activation. Active caspase-8 activates the effector caspases (caspase -3, -6 and -7) that execute apoptosis (17). TRAIL-induced apoptosis occurs in p53-independent manner, therefore TRAIL based therapies can be used as treatment options for cancers with inactivated p53 (18).

TRAIL has an ability to induce apoptosis specifically in cancer cells *in vivo* and *in vitro.* Based on its unique capacity, TRAIL-receptor agonists (TRAs) and TRAIL showing good anticancer activity in preclinical trials were developed as potential cancer therapy. However, many cancer cell lines and primary cancers are resistant to TRAIL. Resistance mechanism of TRAIL-induced apoptosis is not completely understood. However, there are some known factors having role in TRAIL resistance mechanism (19). Overexpression of DcR1 and DcR2 helps escape of cancer cells from TRAIL-induced apoptosis. Cellular FLICE-like inhibitory protein (cFLIP) is the most characterized inhibitor of TRAIL induced apoptosis and controls the DISC formation (20). cFLIP is homolog to Caspase-8 and competes with caspase-8 to bind FADD. Thereby it blocks the pro-apoptotic function of DISC complex. Other inhibitors are also known. Inhibitor of apoptosis proteins (IAP) including X-linked IAPs (XIAP), c-IAP1, c-IAP2 and survivin, block the activity of caspase-3, -7 and -9 (21). In the TRAIL resistant cells, XIAP overexpression is mostly observed. Moreover, anti-apoptotic Bcl-2 family proteins' contribution to TRAIL resistance was reported. For instance, Bcl2 overexpression caused TRAIL-resistance was observed in neuroblastoma, glioblastoma and breast cancer cells. Also high Bcl-xL and Mcl1 expression leads to TRAIL resistance in pancreas cancer and hepatocellular cancer cells, respectively (16).

Intrinsic pathway

The intrinsic pathway/mitochondrial pathway is triggered from inside the cells and controlled by Bcl2 family proteins. Chemotherapy, radiation and cellular stresses initiate the activation of intrinsic pathway. These factors lead to DNA damage by which the tumor suppressor, p53 gets activated. Activated p53 regulates the expression of pro-apoptotic Bcl-2 family members such as Puma and Noxa (22). Thereby, it facilitates the activation of Bax/Bak and release of Cytochrome C (Cyt C) and SMAC/DIABLO from the mitochondria into the cytosol. Cyt C interacts with the Apoptotic protease activating factor 1 (Apaf-1) and pro-caspase-9, leading to the formation of apoptosome complex and activation of caspase-9. Active caspase-9 activates the effector caspases (caspase -3, -6,-7) and executes apoptosis (17). SMAC/DIABLO triggers apoptosis by blocking the (23).

Crosstalk between the extrinsic and intrinsic pathways

In some cells, called type I cells, caspase-8 that becomes active in the downstream of extrinsic pathway is sufficient to trigger apoptosis. However, in some cells that are called type II cells, the amount of activated caspase-8 is not sufficient to induce the entire apoptosis process (24). Instead, a crosstalk occurs between extrinsic and intrinsic pathways through caspase-8 mediated Bid cleavage and the subsequent activation of Bax and Bak. Activated Bax and Bak induce Cytochrome C release through the mitochondrial pores and result in the activation of caspase-9 and then apoptosis. Caspase-9 provides a positive feedback loop to caspase-8 induced apoptosis (16).

3. Bcl-2 family members

Bcl-2 family members are key regulators of apoptosis in mitochondria. They are divided into three groups according to their sequence homology and function; multidomain anti-apoptotic members, multidomain pro-apoptotic members and BH3 only proteins. All Bcl-2 family members share at least one Bcl-2 homology domains which are BH1, BH2, BH3 and BH4 (25).The balance between Bcl-2 family members determines whether cells undergo apoptosis or not (26) **(Figure 3).**

Multidomain Anti-apoptotic Members

Anti-apoptotic Bcl-2 family consists of Bcl-2, Bcl-xL, Bcl-w, Mcl1, A1, and Bcl-B proteins and they keep mitochondrial outer membrane (MOM) intact by direct inhibition of pro-apoptotic members (26). Bcl-2, Bcl-xL, A1, Bcl-w and Bcl-B share four BH domains (BH1-BH4) but Mcl-1 is the only anti-apoptotic protein with three BH domains (BH1, BH2, and BH3) **(Figure 4).** Bcl-2 and Bcl-xL are located in the MOM because of their carboxyl terminal hydrophobic transmembrane tail domain but Bcl-2 can also reside in ER, nucleus or cytosol and it is translocated to MOM upon death signal (25).

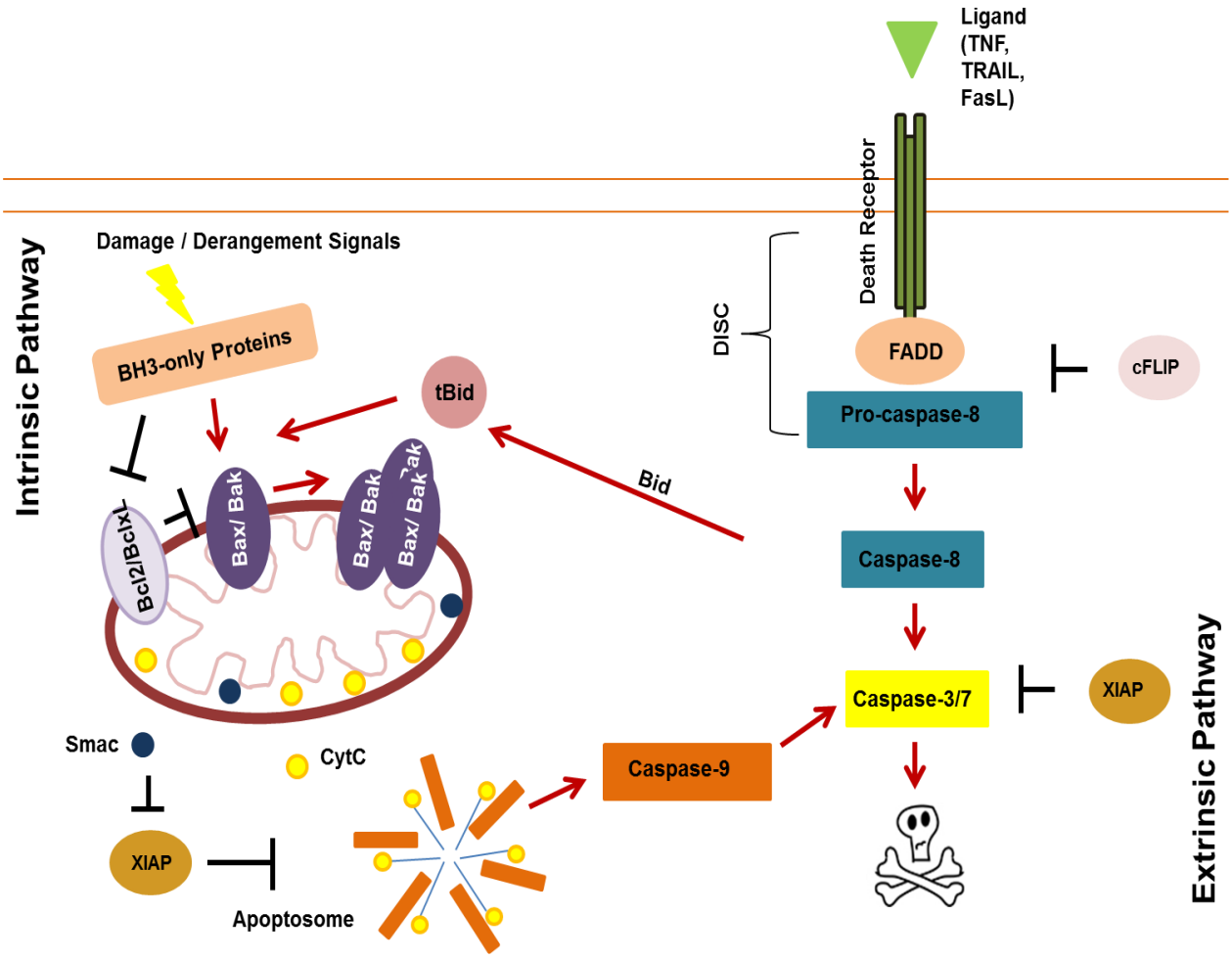


Figure 2: Schematic display of intrinsic and extrinsic pathways of apoptosis. Adapted from reference (27)**.**

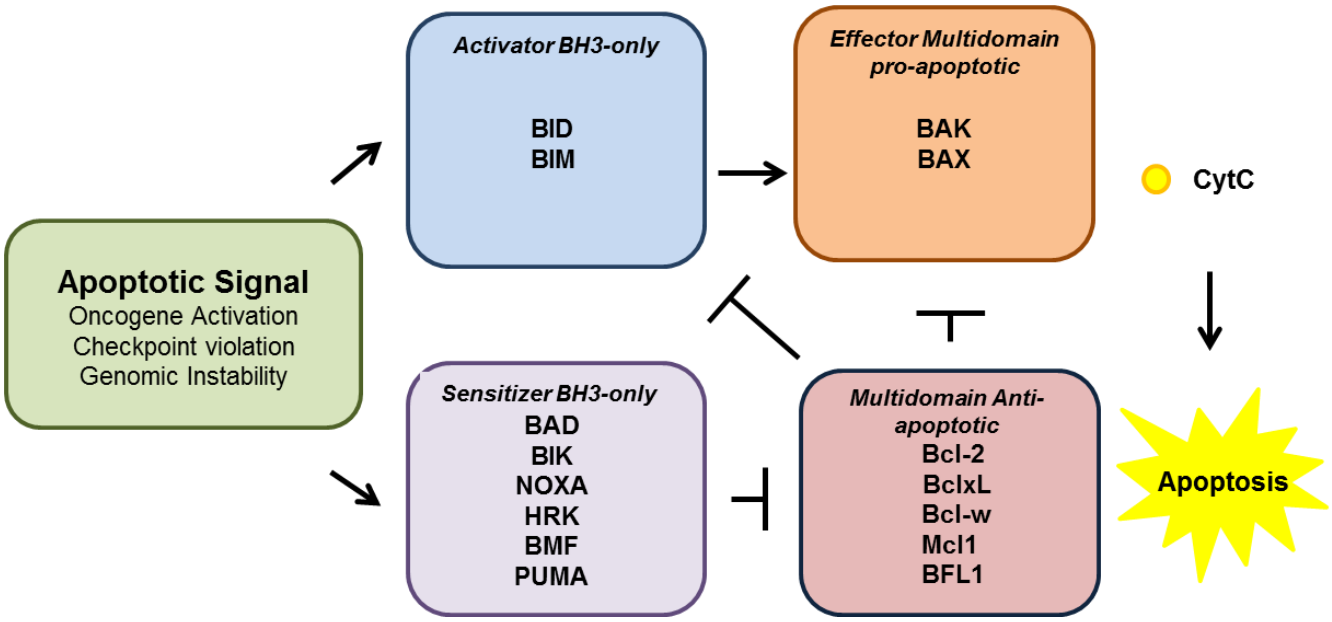


Figure 3: Schema representing Bcl-2 family control over mitochondrial apoptosis. In the presence of damage and derangement signals, activator BH3-only proteins activate effector multidomain proteins and induce MOMP and commitment to death. Anti-apoptotic proteins block activators and effectors, but the sensitizer BH3-only proteins act as antagonist of anti-apoptotic proteins by neutralizing their function selectively. Figure is adapted from reference (28).

Bcl-2 and Bcl-xL share same core structures, which include five amphipathic α-helices surrounding two central hydrophobic α-helices. BH1, BH2 and BH3 form hydrophobic groove that generates a binding site for amphipathic BH3 domain of pro-apoptotic proteins. Because the BH3-only proteins and effector proteins Bax and Bak binds anti-apoptotic proteins via their BH3 domains, there is a simple competition system. Anti-apoptotic proteins bind to effectors (Bak and Bax) and activator pro-apoptotic proteins (Bim, Bid), and prevent oligomerization of

Bax and Bak and mitochondrial outer membrane permeabilization (MOMP). However sensitizer BH3-only proteins neutralize and block function of anti-apoptotic proteins by competing with effectors and activators to trigger apoptosis as mentioned below. These interactions mostly occur on mitochondrial outer membrane where most of the Bcl-2 family members are anchored via their carboxy-terminal hydrophobic transmembrane domains (29). Anti-apoptotic members are used as therapeutic target because overexpression of anti-apoptotic Bcl-2 family members is mostly observed in cancer cells and contributes to tumorigenesis and resistance to chemotherapy (30).

Multidomain Pro-apoptotic Members

Pro-apoptotic Bcl-2 family proteins are categorized as effector proteins and BH3-only proteins according to number of BH domains. The effector proteins consist of Bak, Bax, and Bok, whihc also share four BH domains (BH1-BH4). Bak and Bax share same core structure which includes five amphipathic α-helices surrounding two central hydrophobic α-helices. When they are inactive, they state as monomers. Whereas, inactive Bak is usually resided in the MOM, inactive Bax is located in the cytosol or MOM with loose association. However, when the death signal comes, Bak and Bax become active and undergo a conformational change that exposes N-termini of the proteins. Upon activation, Bax is translocated to MOM and then Bak and Bax homo-oligomerize into the pores within MOM to induce MOMP. Induction of MOMP leads to the release of the content of mitochondrial intermembrane space (IMS) such as Smac and Cytochrome C into cytosol. Released proteins activate caspases and thereby cells undergo apoptosis. In the literature, researchers reported that MOMP cannot be induced in the absence of Bak and Bax. Therefore, MOMP inducing activity of Bak and Bax is required for mitochondrial apoptosis. However, the function of Bok is unknown (29) (26).

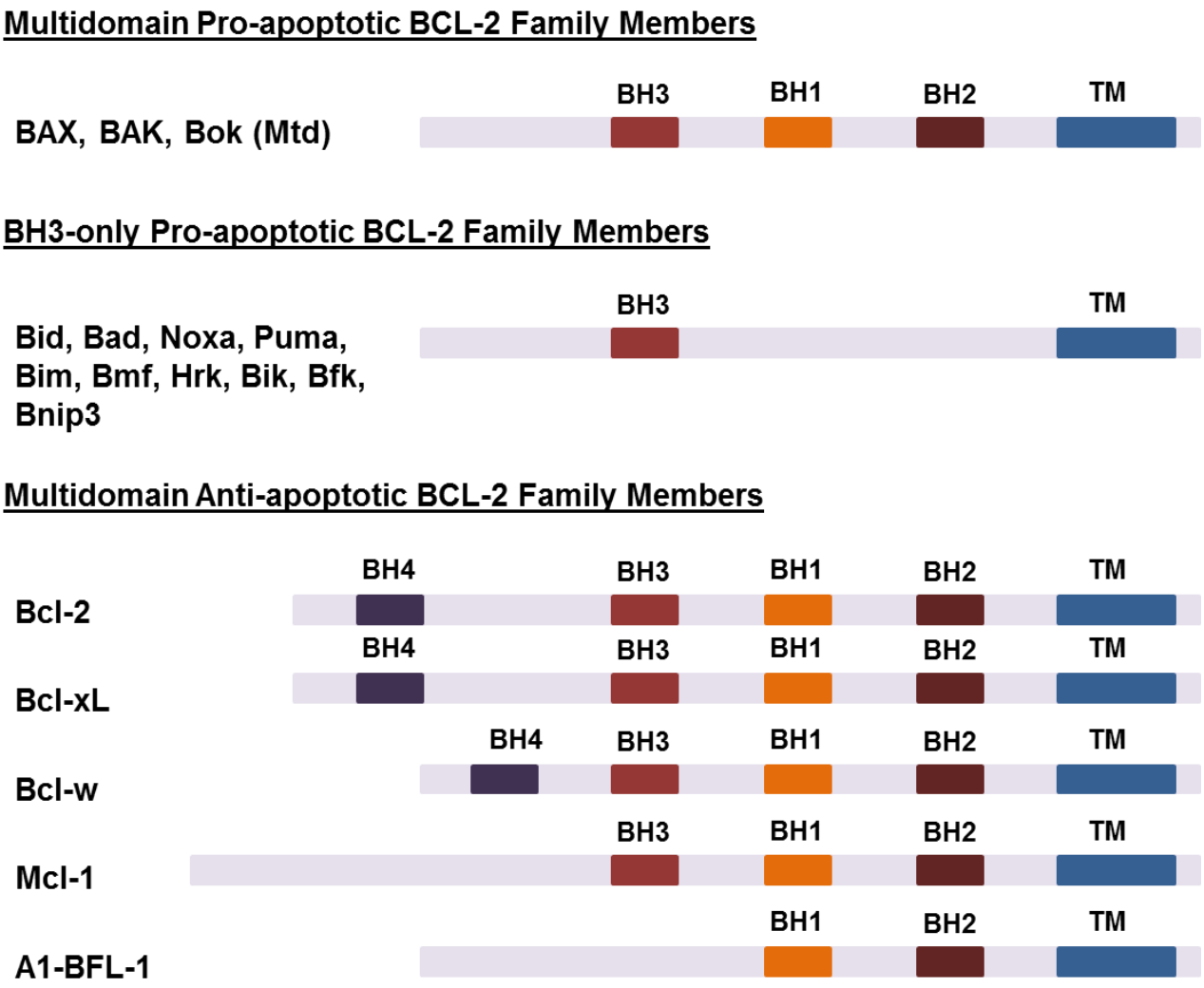


Figure 4: Representation of the sequence and structural homology of Bcl-2 family members. Figure is adapted from reference (29)

Pro-apoptotic BH3-Only Proteins

BH-3-only proteins consist of Bad, Bid Bim, Bmf, Puma, Noxa and Harakiri (HRK). They only share BH3 domain with Bcl-2 and this domain consists of an amphipathic α-helix (29) **(Figure 4).** Despite this homology, remaining sequences do not have much similarity which explains unique functions, expressions and regulation of BH-3 proteins (26) . They are important for pro-death signals. When damage or derangement signals are received, they are activated by protein stabilization, increased expression or posttranslational modifications (28) . For their pro-apoptotic activity, BH3 domain is necessary and sufficient (29).

BH3-only proteins are stimulated by cytotoxic stress signals and perform their pro-apoptotic functions with two mechanisms which are neutralization of pro-apoptotic members and direct activation of pro-apoptotic effectors Bak and Bax. According to their function and interaction ability with anti-apoptotic proteins or pro-apoptotic effector proteins, they are classified as direct activators and sensitizers. Direct activators have crucial role for Bak and Bax activation. The members of direct activators are Bim and truncated form of Bid (tBid). Recent evidence suggests that BH3-only protein Puma can also be a direct activator. Inactivated form of Bid resembles the multi BH domain members, but when it is cleaved by caspase 8, its BH3 domain is exposed and tBid is generated. Direct activators activate Bak and Bax and induce conformation change of Bak and Bax by binding to their hydrophobic groove via BH3 domain. Conformational changes include exposure of N-terminal residues and BH3 domain exposure and these changes enable Bak and Bax to oligomerize and induce MOMP. Direct activators can also bind all anti-apoptotic Bcl-2 proteins (31).

Sensitizers consist of Bad, Bmf, HRK, Noxa, and Puma. Sensitizers do not have any ability to bind Bak and Bax. Instead they bind to hydrophobic groove of anti-apoptotic proteins formed by BH1, BH2 and BH3 domains via their BH3 amphipathic helix and neutralize the function of anti-apoptotic Bcl-2 proteins. The terms “sensitizer” indicates the result of interaction between BH-3 only and anti-apoptotic proteins (31). When sensitizers bind to anti-

apoptotic proteins, they inhibit the possible interaction between anti-apoptotic proteins and pro-apoptotic proteins including activator BH3-only proteins (tBid and Bim) or monomer effectors (Bak and Bax). If anti-apoptotic proteins are already interacting with activated Bak/Bax or activators, MOMP can be induced by blocking the function of anti-apoptotic proteins by sensitizers. Even though, activator BH3-only proteins can bind all anti-apoptotic members, sensitizers specifically bind to certain anti-apoptotic proteins (29).

For the apoptosis, activation of Bak and Bax is required and controlled by two mechanisms, which are direct and indirect activation. In the direct model, activator BH3-only proteins particularly tBid and Bim and perhaps Puma induce cell death by binding to Bak and Bax directly. In indirect model, sensitizer BH3-only proteins induce cell death by neutralizing function of anti-apoptotic proteins, which are bound to activated effectors and lead to the release of active Bak and Bax. In the cell, these two mechanisms exist together. Anti-apoptotic proteins block both pro-apoptotic activators (tBid and Bim) and activated effectors (Bak and Bax). Sensitizers compete with activators, which activate effector Bak and Bax or activated effectors Bak and Bax for their oligomerization (31)(29) **(Figure 5).**

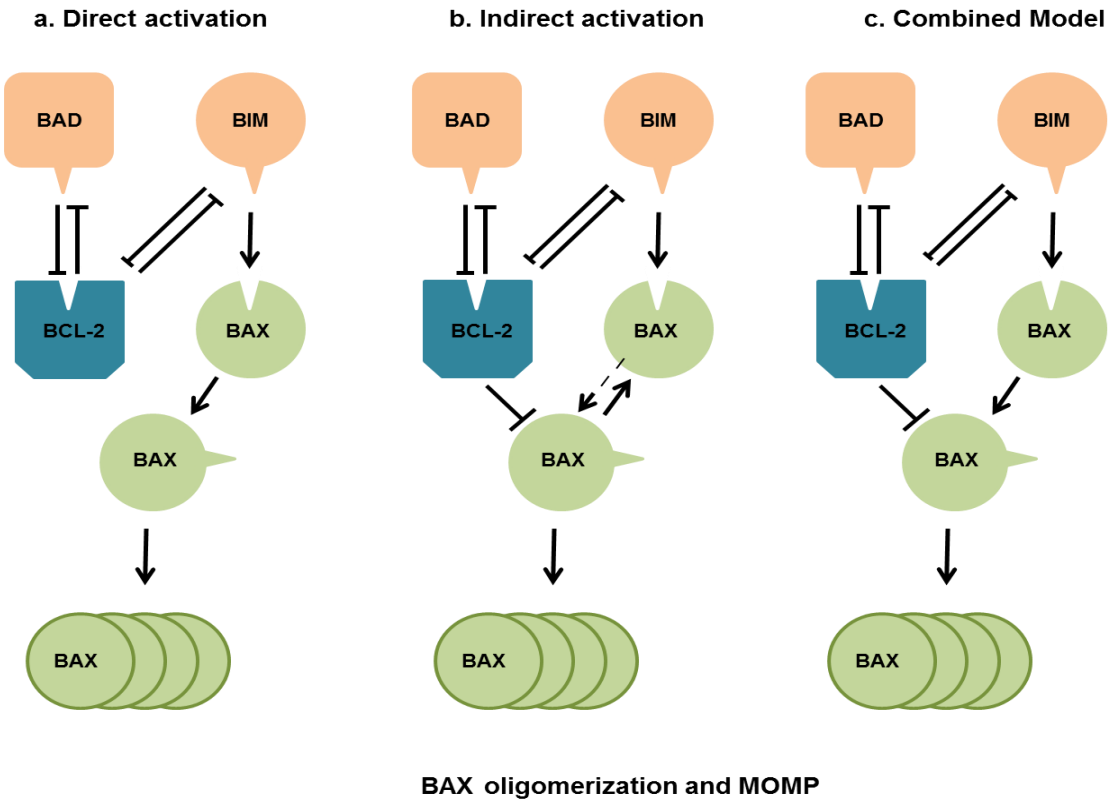


Figure 5: Representation of models for Bak/Bax activation and apoptotic switch. (a) In the direct activation model, activator BH3-only proteins (BIM, tBID, PUMA) activate effector pro-apoptotic proteins (BAX/BAK) by binding directly. Sensitizer BH3-only proteins neutralize function of anti-apoptotic proteins and induce the release of activators. **(b)** In the indirect model, anti-apoptotic proteins sequester effectors (BAX/BAK) and effectors become free to carry out MOMP only if sensitizers can neutralize all anti-apoptotic proteins. In this model, BAK and BAX are activated by unknown modifications or spontaneously at low rate. **(c)** Unified model shows all the crucial interactions occur in same situation. Anti-apoptotic proteins sequester both effectors and BH3-only proteins. The neutralizing function of sensitizer BH3-only proteins can cause the release of both activated BAX/BAK or activator BH3-only proteins. Figure is adapted from reference (31) **.**

4. BH3-only protein Harakiri (HRK)

Discovery of HRK

HRK (also known as DP5) is one of the BH3-only proteins. It was first discovered in 1997 through a yeast two-hybrid screen as a novel apoptosis regulator interacting with Bcl-2 and Bcl-xL anti-apoptotic proteins through its BH3 domain (32). On the other hand, another group also discovered HRK (DP5) gene that is strongly induced during programmed cell death of cultured sympathetic neurons (33). In addition, exogenous HRK expression triggers cell death in human kidney 293T cells and sympathetic neurons, also HRK induced cell death is prevented by the overexpression of Bcl-2 and Bcl-xL (32)(33). HRK is located at chromosome 12q13.1 (34). Its expression is primarily observed in brain and lymphoid tissues (35) and localizes predominantly in mitochondria (36). Besides the physical interaction of HRK with anti-apoptotic Bcl-2 and Bcl-xL proteins, HRK also interacts with p32 protein which is a homo-trimeric protein localizing in mitochondria and has been implicated in the regulation of splicing. Unlike Bcl-2 and Bcl-xL, interaction between HRK and p32 occurs in a BH3 domain independent manner (35). Binding of HRK to Bcl-2, Bcl-xL and p32 are essential but not sufficient for HRK's activity. However, the requirement of effector protein Bax for HRK-mediated cell death is shown (35) (37).

Role of HRK in the nervous system

HRK is mainly described in the nervous system. HRK upregulation was first observed in neuronal growth factor (NGF) withdrawal induced cell death of sympathetic neurons (33). In addition, HRK induction was also observed in cerebellar granule neurons (CGNs) deprived of potassium, toxic amount of amyloid beta treated cortical neurons, axotomized postnatal mouse motoneurons and retinal ganglion cells (38). Also, HRK overexpression induced cell

death in cerebellar granule neurons in Bax-dependent manner (37).These studies show that HRK is closely associated with programmed neuronal death (39) (40) (38).

HRK in cancer

Since the imbalance between apoptosis and cell proliferation contributes to tumor formation, regulators of apoptosis play crucial role for tumorigenesis. In some cancer cell types such as colorectal and gastric cancer, prostate carcinoma, astrocytoma and glioblastoma, HRK expression is decreased by loss of heterozygosity or hypermethylation (41) . An aberrant methylation pattern in HRK gene was observed in the region around the transcriptional start sites, CpG islands and promoter (42) (34). In parallel observation, HRK was found as a target gene of SUZ12 in epithelial ovarian cancer (EOC). SUZ12 is a polycomb repressive complex 2 (PRC2) component and essential for PRC2 mediated gene silencing by generating H3K27Me3 modification. SUZ12 is highly expressed in EOCs and its knockdown induces the upregulation of HRK and thereby apoptosis (43). Moreover, Xu et al. reported that melanoma cells acquired apoptotic resistance because of the decreased expression of HRK. Also, artificial overexpression of HRK reduced the growth of melanoma *in vivo* and *in vitro* (41). Similarly, exogenous expression of HRK attenuates the tumor growth in prostate, breast and ovarian cancers. Moreover, HRK mRNA and protein is upregulated in prostate cancer cells by (2-ME) 2-methoxyestradiol, which is an endogenous estrogen metabolite and a promising anticancer agent for some cancers (44). Thus, HRK downregulation is considered as an important factor for tumorigenesis (34). However the functional role of HRK in cancers, specifically in GBMs has not been tested.

HRK gene regulation

In the literature, certain studies showed that the HRK gene is transcriptionally regulated by the c-Jun N-terminal kinase pathway in cerebellar granule neurons. JNK inhibition blocks the upregulation of HRK in granule neurons after potassium deprivation (37). Also, the HRK gene is under the control of E2F, which a transcription factor with a role in cell cycle and apoptosis (45); and a transcriptional repressor DREAM, which binds to 3' UTR region of HRK to block its transcription (41). Moreover, a pleotropic transcription factor activating protein 2α (AP-2α) binding site was found in a HRK promoter in a melanoma model. HRK has been shown as a target of transcription factor AP-2α. AP-2α belongs to the AP-2 family and is decreased in some cancer types such as breast carcinoma, prostate carcinoma, colorectal carcinoma, glioma and melanoma. AP-2α-HRK pathway has been thought as an important pathway for tumorigenesis (41). Therefore, understanding the gene regulation of HRK is important to identify factors responsible for aberrant expression of HRK in cancers and tumorigenesis.

In summary, HRK is a sensitizer BH3-only protein and regulates apoptosis by interfering with anti-apoptotic Bcl2 and BclxL proteins and blocking their function. While its function is characterized in the context of the nervous system, its implications in tumorigenesis are not well studied. Few studies show that HRK is silenced in colorectal and gastric cancer, prostate carcinoma, astrocytoma and glioblastoma by loss of heterozygosity or hypermethylation. Furthermore, exogenous HRK expression attenuates tumor growth in prostate, melanoma, breast and ovarian cancers. However, the functional role of HRK and the relations with TRAIL have not been studied in GBM before. In this thesis, we first checked the functional role of HRK in Glioblastoma Multiforme cells. We showed for the first time exogenous HRK expression induces cell death in GBMs *in vivo* and *in vitro*. We validated the functional interaction between HRK and Bcl-2/BclxL by showing that Bcl-2/BclxL overexpression blocked HRK-induced cell death. Moreover, we showed that HRK cooperates

with TRAIL to induce cell death in GBM cells and HRK knockdown decreases the effect of TRAIL significantly. Also, we demonstrated that HRK is responsive to TRAIL sensitizing agent MS-275 and requires to its sensitizing activity. This suggests that HRK induction might be one mechanism during TRAIL sensitization and apoptosis. Second part, we developed a reporter system that monitors HRK regulation in GBM cells to understand better how HRK gene expression is regulated and to identify novel agents inducing GBM cell apoptosis.

Chapter 2

Material & Methods

Cell Culture

A172, LN18, U87MG and U373 GBM cell lines and human embryonic kidney 293T cells were obtained from American Tissue Type Culture Collection (USA). Cell lines were cultured in DMEM medium (Gibco, USA) with %10 fatal bovine serum (Gibco, USA) and %1 Penicillin-Streptomycin (Gibco, USA) in a 37^0C humidified incubator with 5% CO_2.

Production of TRAIL in 293T cells

In order to produce TRAIL, 293T cells were transfected with TRAIL vector expressing a secretable form of TRAIL (46) using Fugene Transfection Reagent (Promega, USA). Accordingly, 293T cells were seeded 2,5 $X10^6$ cells/10 cm plate. Next day afternoon, Fugene mixture containing 20 ul Fugene and 180 ul OptiMEM (Promega, USA) and DNA mixture containing 5 ug TRAIL vector and 200 ul OptiMEM were prepared for each plate. DNA and Fugene mixtures were mixed and incubated for 30 minutes. Then mixture was added to 293T cella]s drop by drop. Next day morning, medium was aspirated and replaced with 8 ml serum-free media. Approximately after 48 hours post transfection, first collection of media was performed. After removing media, it was replaced with new 8 ml serum-free media. Next day at noon, media collection was repeated. Then, TRAIL was concentrated using spin filter columns with a 15kD cut-off (Millipore, USA) and spun at 4^oC at 3800 rpm for 20 minutes. In order to measure TRAIL concentration, TRAIL Human ELISA Kit was used according to manufacturer's instructions (47). TRAIL was aliquoted and stored at -80 ^{o}C.

Cloning of mammalian HRK overexpression vector

pcDNA3.1 HRK vector, which is a mammalian expression vector, was kindly provided by Marta Miaczynska (International Institute of Molecular and Cell Biology, Poland). In order to transfer HRK cDNA sequence of pcDNA3.1 HRK vector to lenti-viral backbone, Gateway cloning method was used (Invitrogen, USA). Gateway cloning technology is a cloning method, which depends on the site-specific recombination features of bacteriophage lambda (48),(49). This method provides the transfer a DNA sequences that is cloned to entry vector into different vector systems (Destination vectors). As entry vector, pENTR1A no ccDB (w48-1) (addgene plasmid #17398) vector with bacterial kanamycin selection marker, and as destination vector pLenti-PGK-puro (w529-2) (addgene plasmid #191068) with bacterial ampicillin selection marker were used.

Gene	***Sequence***	
HRK cDNA	F	5'ACTTAGAATTCACCATGTGCCCGTGCCCCCTGCAC-3'
	R	5'-TGATACTCGAGCTACAAGTTCCGCCTGCCGAG-3'

Table 1: Primers for amplifying HRK cDNA by using pcDNA3.1 as template

First, HRK cDNA was amplified from pcDNA3.1 HRK vector using primers having compatible restriction ends (EcoRI and XhoI) with Entry vector (pENTR1a no ccDB) **(Table 1).** Amplified HRK cDNA and pENTR1A no ccDB entry vectors were digested with EcoRI and XhoI restriction enzymes using Buffer 4 (New England Biolabs,UK) for 1 hour at 37^0C. Then, the corresponding fragment of HRK cDNA was ligated into the pENTR1a no ccDB vector with ligation reaction using Quick Ligase Kit (Roche, Switzerland) and transformed into the competent Stbl3 bacteria strain. As control, only vector ligation was performed. Next day, colony picking and inoculation into Kanamycin containing LB was conducted. Next day,

plasmid purification was performed using mini-prep kit (Qiagen,Holland). Cloned vectors were validated through restriction enzyme digestion with EcoRI and XhoI.

Entry vector has attL1 and attL2 sites; and destination vectors have attR1 and attR2 sites. These sites allow recombination cloning of gene of interest into a destination vector. Therefore, in order to transfer the HRK cDNA, a recombination reaction between pENTR1A-HRK cDNA and pLenti-PGK puro Destination vector was conducted using Gateway® LR Clonase™ II enzyme mix (Invitrogen,USA). For reaction, 150 ng pENTR1A HRK cDNA vector, 150 ng pLenti PGK puro vector and 2 ul Gateway® LR Clonase™ II enzyme mix were mixed and incubated 1 hour at 25C. After incubation, reaction was terminated by adding 1ul of Proteinase K and incubating for 10 minutes at 37C. Then, 1 ul of reaction was transformed into competent Stbl3 bacteria. Next day, colony picking and inoculation into Ampicillin containing LB was conducted. Next day, plasmid purification was performed using mini-prep kit (Qiagen, Holland). Cloned vectors **(Figure 6)** were validated through restriction enzyme digestion with BamHI and XbaI (New England Biolabs, UK) and sequencing.

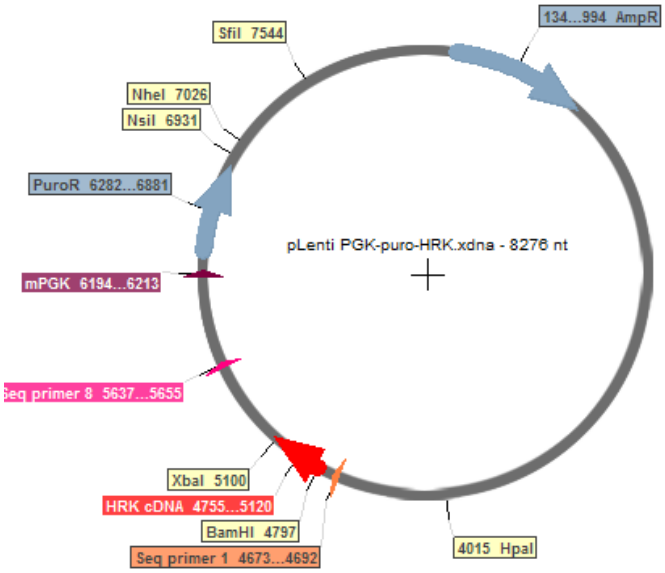


Figure 6: Plasmid map of pLenti PGK-puro-HRK vectors showing XbaI and BamHI sites and its features. Serial Cloner was used to generate vector maps.

Cloning of shHRK into MSCV-PM

In order to generate stable cell lines with HRK knockdown, shRNAs were cloned into MSCV-PM retroviral vector as a backbone. shRNA sequences targeting first exon of HRK gene were designed using RNAiCodex program (50) , and 5 different oligonucleotides were ordered. These oligos were PCR-amplified by using following primers having compatible restriction ends with backbone vector (**Table 2)**. The PCR products were purified by using PCR purification kits (Macherey-Nagel, Germany); and the concentration of products was measured by using Nanodrop (ThermoScientific, USA). PCR products and MSCV-PM vectors were digested with EcoRI and XhoI restriction digestion enzymes at 37 ^{0}C for 1 hour. For vector, after 45 min, 10x Antarctic phosphatase buffer and 1 ul Antarctic phosphatase (New England BioLabs, UK) was added to vector digestion reaction to prevent self-ligation of vector. Reaction was incubated at 37 ^{0}C for 15 minutes and 70 ^{0}C for 30 minutes. Digested PCR products and MSCV-PM vector were purified from the gel using NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Germany). Then, the inserts and vectors were ligated as 1:3 molarity ratio utilizing Quick Ligation Kit (New England Biolabs, UK). As a control, only digested vector was ligated. Then, 5 ul of reactions were transformed to competent Stbl3 bacteria. Next day, colony picking and inoculation was conducted. Next day, plasmid purification was performed using NucleoSpin Plasmid Kit (Macherey-Nagel, Germany). Cloned vectors were validated through restriction enzyme digestion with EcoRI and XhoI (New England Biolabs, UK) and sequencing and correct plasmids were detected.

Gene	***Sequence***
shHRK_cloning_For	5' GATGGCTGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCG-3'
shHRK_cloning_Rev	5'-CCCTTGAACCTCCTCGTTCGACC-3'
pSMP sequencing	5'-CCCTTGAACCTCCTCGTTCGACC-3'
shHRK_oligo_1	5’TGCTGTTGACAGTGAGCGCCGAGAAGGAAGTGGAGAGTAATAGTG AAGCCACAGATGTATTACTCTCCACTTCCTTCTCGATGCCTACTGCCT CGGA-3’
shHRK_oligo_2	5’TGCTGTTGACAGTGAGCGCCAGCTCTATATATATAAAAAATAGTGA/ GCCACAGATGTATTTTTTATATATATAGAGCTGTTGCCTACTGCCTCG GA-3’
shHRK_oligo_3	5’TGCTGTTGACAGTGAGCGAAGCGATCGTAGAAACACAGAATAGTG/ AGCCACAGATGTATTCTGTGTTTCTACGATCGCTCTGCCTACTGCCT GGA-3’
shHRK_oligo_4	5’TGCTGTTGACAGTGAGCGCCGAGAAGGAAGTGGAGAGTAATAGTG AAGCCACAGATGTATTACTCTCCACTTCCTTCTCGATGCCTACTGCCT CGGA-3’
shHRK_oligo_5	5’TGCTGTTGACAGTGAGCGCACAGCTCTATATATATAAAAATAGTGA/ GCCACAGATGTATTTTTATATATATAGAGCTGTATGCCTACTGCCTCG GA-3’

Table 2: List of the cloning primers and shOligos used for shHRK cloning

Viral Packaging and transduction

Viral production is performed via transfection of three plasmids, which are transfer vector, packaging vector and envelope vector to 293T cells. On Day 0, Cells ($2.5x10^6$) were seeded into 10 cm cell culture plates in DMEM with 10% FBS. Next day, transfer of plasmid DNA (2,5 ug), packaging plasmids (2,25 ug) which are pUMVC for retroviral packaging and 8.2dVPR for lenti-viral packaging, and glycoprotein expressing plasmid VSVG (2,5 ug) were transfected to 293T cells using Fugene HD Transfection Reagent (Promega, USA). GFP vector was used as control for viral production. After 48 and 72 hours post transfection, media was collected and filtrated using 0.45 µm filters. Virus containing media was aliquoted and stored at -80 freezers. Before transduction, 293T cells were infected gradually increasing amount of fluorescent labelled viruses and viral titer was calculated by checking the percentage of marker positive cells over total cells. Required amount of viruses were calculated for the %100 infection.

For transduction, cells were seeded as 10.000 in black-bottom 96-well plates (Corning Costar, clear bottom black side). Next day, cells were infected with 80 ul viruses with medium containing protamine sulfate (10ug/ml). Approximately 16 hours later, mediums were replaced with fresh mediums. Next day, for the viruses having puromycin selection marker, cells' medium were changed with puromycin (1 ug/mL) containing fresh medium and incubated for selection for 3 days.

Determination of Cell Viability

Cell viability was determined using Cell Titer-Glo® (CTG) Luminescent Cell Viability Assay (Promega, USA). CTG is an indicator of metabolically active cells by measuring the ATP amount in the cell. It lyses the cells and releases the ATP and block the ATPase the while providing luciferin substrate, luciferase and other reagents than measure the

bioluminescence. CTG assays were performed in the 96 well plates. Cells were seeded into 96 well black bottom plate as 10.000/per well. Next day, cells were transduced with HRK viruses. Next day morning, viruses were removed and replaced with fresh medium. After approximately 36 hours post transduction, TRAIL treatment was performed for the combination effect. Next day, after removing the media, 4 ul CTG substrate is added to 40 ul DMEM medium for each well. Following CTG addition, the plate was put into Synergy H1 Reader (Biotek, USA) for 2 minutes of shaking and 8 minutes of incubation at dark luminometric read is performed at 560 nm wavelength. Survival was described as the percentage of viable cells in the treated sample compared to untreated control samples. All experiments were performed in triplicates.

Caspase 3/7 Activity Assay

Caspase 3/7 Activity Assay (Promega, USA) is a luminescence assay measuring the active caspase-3 and caspase-7. Caspase 3/7 activity assay kit provides a proluminescent caspase-3/7 substrate containing the tetrapeptide sequence DEVD (51). When this substrate is cleaved by active caspases, aminoluciferin, which is a substrate of luciferin, is released and light is produced. The signal shows the caspase 3/7 activity. Cells were seeded into 96 well black bottom plate as 10.000/per well. Next day, cells were transduced with HRK viruses. Next day morning, viruses were removed and replaced with fresh medium. After approximately 36 hours post transduction, TRAIL treatment was performed for the combination effect. After 3 hours incubation, 17 ul of the Caspase-Glo® 3/7 reagent was added to each well and caspase activity was measured at 560 nm by Synergy H1 Reader (Biotek, USA). All experiments were performed in triplicates.

Caspase 8 Activity Assay

Caspase 8 Activity Assay (Promega) is a luminescence assay measuring the active caspase-8. Caspase 8 activity assay kit provides luminogenic caspase-8 substrate in an optimized buffer system for cell lysis, luciferase and caspase activity. When this substrate is cleaved by active caspase-8, aminoluciferin, which is a substrate of luciferin is released and light is produced. The signal shows the caspase 8 activity. Cells were seeded into 96 well black bottom plate as 10.000/per well. Next day, cells were transduced with HRK viruses. Next day morning, viruses were removed and replaced with fresh medium. After approximately 36 hours post transduction, TRAIL treatment was performed for the combination effect. After 3 hours incubation, 17 ul of the Caspase-Glo® 8 reagent and MG-132 inhibitor mix (1000:3 ratios) was added and caspase activity was measured at 560 nm by Synergy H1 Reader (Biotek, USA). All experiments were performed in triplicates.

Caspase 9 Activity Assay

Caspase 9 Activity Assay (Promega) is a luminescence assay measuring the active caspase-9. Caspase 9 activity assay kit provides luminogenic caspase-9 substrate in an optimized buffer system for cell lysis, luciferase and caspase activity. When this substrate is cleaved by active caspase-9, aminoluciferin which is a substrate of luciferin is released and light is produced. The signal shows the caspase 9 activity. Caspase 9 activity was performed as described above.

Quantitative RT-PCR

In order to detect the mRNA expression, NucleoSpin RNA isolation kit (Macherey-Nagel, Germany) was used by following the manufacturer's instructions to isolate mRNA from the GBM cells. RNA concentrations were measured using Nano Drop. For the cDNA

synthesis, 500 ng or 1000 ng RNA, 2,5 ul of 2 mM dNTPs (Life Technologies, USA), 1 ul of Hexanucleotide mix (Invitrogen, USA) and ddH_2O were assembled in 16,5 ul reaction and incubated at 65^{o}C for 5 min. After 5 min, tubes were quickly put ice. The RT mix consists of 5ul 5x First Strand Buffer (Invitrogen, USA), 2ul of 0,1 M DTT (Invitrogen, USA) and 0,5 ul of RNasin (Promega, USA). For each reaction, RT mixture was added to each tube and incubated for 10 minutes. Then, 1 ul of MMLV-RT enzyme (Invitrogen, USA) was added to each reaction and incubated at 37^{o}C for 1 hour and then 70^{o} C for 15 minutes. At the end, 75 ul of RNase-free ddH2O was added to each reaction. Relative mRNA expression levels were detected as follows: 10 ul of 2X LightCycler® 480 SYBR Green I Master (Roche, Germany), 1ul of 5 uM primer mix, 7 ul nuclease free water and 2 ul of cDNA was mixed for each reaction and loaded to 96 well opaque white plate (Roche, Switzerland). After the 2 minutes of shaking, plate was placed to Roche's LightCycler 480 Instrument II and delta Ct values were detected. Used primers are listed below **(Table 3).**

Gene	***Sequence***	
hGAPDH	F	5'-AGCCACATCGCTCAGACAC-3'
	R	5'-GCCAATACGACCAAATCC-3'
HRK	F	5'-AGGTTGGTGAAAACCCTGTG-3'
	R	5'-GCATTGGGGTGTCTGTTTCT-3'
BAX	F	5'- GGGTGGTTGGGTGAGACTC-3'
	R	5'-AGACACGTAAGGAAAACGCATTA-3'
BAK	F	5'-GTTTTCCGCAGCTACGTTTTT-3'
	R	5'-GCAGAGGTAAGGTGACCATCTC-3'
BCL-2	F	5'-CAGGGCGATGTTGTCCACC-3'
	R	5'-GGGGAGGATTGTGGCCTTC-3'

BCL-xL	F	5'-GGTCGCATTGTGGCCTTTTTC-3'
	R	5'-TGCTGCATTGTTCCCATAGAG-3'
DR4	F	5′-ACCTTCAAGTTTGTCGTCGTC-3′
	R	5′-AACTC TCCCAAAGGGCTATGT-3′
DR5	F	5′-AAGACCCTTGTGCTCGTTGT-3′
	R	5′-AGGTGGACACAATCCCTCTG-3′

Table 3: List of qRT-PCR primers and sequences

Western Blotting

Cells were seeded at 6 well plate (3*10^5 cells/well) and transduced with HRK overexpression viruses.1 day after transduction, cells were treated with TRAIL (at different concentrations for each cell line). After 3 hours of incubation, cells were detached by trypsin treatment and were precipitated by centrifugation at 1500 rpm for 5 minutes. Pellets were lysed using lysis buffer containing 1% Nonidet P40 (NP-40), 150 mM NaCl, 1mM EDTA, 50 mM Tris-HCl (pH 7.8), 1 mM NaF, 0.5 mM PMSF, 1X phosphatase inhibitor cocktail (PhosSTOP, Roche, Switzerland) and 1X protease inhibitor cocktail (cOmplete Protease Inhibitor Cocktail Tablets, Roche, Germany) for 30 minutes on ice by vortexing at 10 minutes intervals. Lysates were centrifuged at 13.000 rpm for 10 min at 4 ^{o} C to get the supernatants containing whole protein extract. Then protein extract were boiled with 1x final concentration of 4x loading dye containing 900 ul of 4X Laemmli sample buffer (Biorad, USA) and100 ul of beta-mercaptoethanol (Biorad, USA) at 95 ^{o} C for 15 minutes and can be stored at -20 ^{o} C freezer. Protein quantification was performed with BCA Protein Assay kit (Life Technologies).

For immunoblotting, 20-25 ug proteins were run on SDS-PAGE gel for their separation and electrophoretically transferred to the PVDF membrane Trans-Blot® Turbo™ RTA Mini

PVDF Transfer Kit (#170-4272, Biorad, USA). After transfer was completed, membrane was stained with Ponceau S Red (Biorad, USA) to detect the transfer efficiency. By washing the membrane with ddH_2O, the separation of whole proteins and wells was observed. Then membrane was cut according to size of the protein of interest. Then membrane pieces were blocked in 5% non-fat dry milk (Biorad, USA) in 1xTBS-T (20 mM TrisHCl, pH 7.8, 150 mM NaCl, 0.1%, v/v Tween-20) blocking buffer at room temperature for 1 hour. After discarding the blocking buffer, membranes were incubated with primary antibodies in primary antibody solution (2% BSA, 0.02%NaN3 in TBST) overnight at 4^0C. Primary antibodies and their dilutions are listed at **Table 4**. Next day, membranes were washed 4 times with TBST for 5, 10,15 and 20 minutes. Then, membranes were incubated with secondary antibodies conjugated to HRP in blocking buffer for 1 hour at room temperature. Membranes were washed 4 times with TBST for 5, 10,15 and 20 minutes. Finally, proteins were visualized via chemoluminescence detection method using Pierce ECL Western Blotting substrate (Life Technologies) or Clarity™ Western ECL Substrate (Biorad, USA) and the light were captured in CL-XPosure Film (Thermo-Scientific, USA).

Antibody	***Catalog Number***	***Brand***	***Dilutions***
α-Tubulin	T9026	Sigma-Aldrich	1:4000
HRK	6972	Santa Cruz Biotech	1:250
Cleaved PARP	5625	Cell Signaling	1:1000

Table 4: List of primary antibodies and dilutions

HRK promoter cloning

To clone the putative promoter region of HRK, sequence of promoter was found by UCSC Genome Browser (52). Primers **(Table 5)** having compatible restriction ends (KpnI and BglII) with backbone vector pGL3-basic were designed to amplify 329 bp and 1000 bp sequence of HRK promoter. Genomic DNA was isolated from the human fibroblast cells, which were kindly provided by Dr. Tamer Onder (Koc University, Turkey). Putative promoter regions were amplified by PCR reactions. Amplified pHRK sequence were cleaned up (Qiagen, Holland) and cut by KpnI and BglII restriction enzymes (New England BioLabs, UK). pDR5-pGL3 vectors were cut by KpnI and BglII (New England BioLabs, UK) to extract the pDR5 sequence at 37 ^{0}C for 1 hour. For vector, after 45 minutes incubation, 10x Antarctic phosphatase buffer and 1ul Antarctic phosphatase (New England BioLabs, UK) were added into vector digestion reaction to prevent self-ligation of vector. Reaction was incubated at 37 ^{0}C for 15 minutes and 70 ^{0}C for 30 minutes. Digestion reaction was run on the gel and pGL3 backbone was isolated from the gel by using gel extraction kit (Qiagen, Holland). pGL3 vector and pHRK were ligated with 1:3 molarity ratio utilizing Quick Ligation Kit (Roche, Switzerland). As a control, only digested vector was ligated. Then, 5 ul of reactions were transformed to competent Stbl3 bacteria. Next day, colony picking and inoculation in ampicillin containing LB was conducted. Next day, plasmid purification was performed using NucleoSpin Plasmid Kit (Macherey-Nagel, Germany). Cloned vectors were validated through restriction enzyme digestion with KpnI and BglII (New England BioLabs, UK).

In order to clone pHRK-Fluc sequence (1981 bp) into lenti-viral vector, pHRK-Fluc was amplified from the pHRK-pGl3 vector as template and primers having BamHI and EcoRV restriction ends **(Table 5).** A visiting student, Asli Azizoglu, kindly assisted the cloning experiments. PCR product was cleaned up and digested with BamHI and EcoRV restriction enzymes (New England BioLabs, UK). As a lenti-viral backbome, pDR5-Fluc-CMV-GFP vector was used (46). It was digested with BamHI and EcoRV at 37 ^{0}C for 1 hour to extract

pDR5-Fluc sequence. For vector, after 45 minutes incubation, 10x Antarctic phosphatase buffer and 1ul Antarctic phosphatase (New England BioLabs, UK) were added into vector digestion reaction to prevent self-ligation of vector, and incubated at 37 ^{0}C for 15 minutes and 70 ^{0}C for 30 minutes. Digestion reaction was run on the gel and CMV-GFP backbone was isolated from the gel by using gel extraction kit (Macherey-Nagel, Germany). CMV-GFP vector and pHRK-Fluc insert were ligated with 1:3 molarity ratio utilizing Quick Ligation Kit (New England BioLabs, UK). As a control, only digested vector was ligated. Then, 5 ul of reactions were transformed to competent Stbl3 bacteria. Next day, colony picking and inoculation in ampicillin containing LB was conducted. Next day, plasmid purification was performed using NucleoSpin Plasmid Kit (Macherey-Nagel, Germany). Cloned vectors were validated through restriction enzyme digestion with BamHI and EcoRV (New England BioLabs, UK).

In order to normalize the Fluc activity regulated by pHRK, pHRK-Fluc sequence (1981 bp) was cloned into a lenti-viral vector containing Rluc-mCherry (with help of Asli Azizoglu). pDR5_Rluc_DsRed vector and pHRK-Fluc-CMV-GFP vector were digested with NheI and BamHI, in CutSmart Buffer (New England BioLabs), for 4 hours at 37C. Antarctic phosphatase was added to vector at the beginning of the 4th hour. Rluc-mCherry vector and pHRK-Fluc insert were ligated with vector:insert molarity ratio of 1:10 using 1ul of T4 ligase (New England BioLabs, UK). Then, 5 ul of reactions were transformed to competent Stbl3 bacteria. After, colony picking, inoculation in ampicillin containing medium and mini-prep (MN, Germany), vectors were validated by NheI and BamHI digestion.

Gene	*Sequence*
pHRK_For1_KpnI	5'-AGCGGTACCTGTCAGGGAGAGAGAGGTCC-3'
pHRK_Rev_BglII	5'-AGCAGATCTCCTCCCCTGGACACCAAGT-3'
pHRK_For9_KpnI	5'-AGCGGTACCAATCGGGGTCATCGCAGAAA-3'
pHRK_For1_BamHI	5'-AGCGGA TCCAGGAGAACAACCTTCCCTCG-3'
Fluc_Rev_EcorV	5'-AGCGATATCTTACACGGCGATCTTTCCGC-3'
pHRK_For9_BamHI	5'-AGCGGATCCAATCGGGGTCATCGCAGAAA-3'

Table 5: List of the primers used for pHRK cloning

Cloned Vectors	*Selection markers*
pENTR1A-HRKcDNA	**-**
PGK-puro-HRK	**puromycin**
PGK-neo-HRK	**neomycin**
CW.57.1-HRKcDNA	**puromycin**

Plix 402-HRKcDNA	**puromycin**
MSCV-shHRK1	**puromycin**
MSCV-shHRK3	**puromycin**
MSCV-shHRK4	**puromycin**
pHRK-pGL3	**-**
pHRK-Fluc-CMV-GFP	**-**
pHRK-Fluc-CMV-RlucDsRed2	**-**
pHRK_longform-pGL3	**-**
pHRK_longform-Fluc-CMV-GFP	**-**
pHRK_longform-Fluc-CMV-FlucDsRed2	**-**

Table 6: List of the cloned vectors

Used Vectors
LV-GFP

pBabe-GFP-puro
LV-GFP-Fluc
pDR5-pGL3
pDR5-Fluc-CMV-GFP
pDR5-Fluc-CMV-RlucDsred2
pHRK-Fluc-CMV-GFP
pHRK-Fluc-CMV-RlucDsRed2
pHRK_longform-pGL3
pHRK_longform-Fluc-CMV-GFP
pHRK_longform-Fluc-CMV-FlucDsRed2
pHRK-pGL3
MSCV-shHRK1
MSCV-shHRK3
MSCV-shHRK4

MSCV-shFF
pcDNA HRK3.1
pENTR1A-HRKcDNA
PGK-puro-HRK
PGK-neo-HRK
CW.57.1-HRKcDNA
Plix 402-HRKcDNA
Plix-402-EGFP
PMIG-Bcl2
PMIG-BclxL
Fluc-mCherry

Table 7: The list of vectors that were used throughout the thesis

Measurement of Fluc/Rluc activity *in vitro*

Cells were seeded into 24-well plate as 25.000. Next day, cells were transfected with LV-GFP, LV-GFP-Fluc, pDR5-Fluc-CMV-GFP and pHRK-pGL3 or pHRK-Fluc-CMV-GFP vectors using Fugene HD Transfection reagent (Promega, USA) using manufacturer's protocol (53). Next day, mediums were replaced with fresh medium. Next day, after observe the fluorescence labeled cells, cells were removed and seeded at 96-well black bottom plates as 10.000/well. For Fluc measurement half of the cells were treated with 1000 ug/ml D-Luciferin substrate and for the Rluc measurement half of the cells were treated with 0,2 uM coelanterazine. Firefly Luciferase (Fluc) and Renilla Luciferase (Rluc) activity was measured by BioTek2s Synergy H1 Plate reader and analyzed by Gen5 data analysis software.

Real-Time Cell Growth Analysis

For the long-term analysis of cell growth, xCELLigence RTCA Station and analyzer (ACEA Bioscience, USA) were used. Disposable, single use, electrode array covered E-plate 96 was used to monitor cell growth in this system. E-plate 96 was loaded with 100 uL cell culture medium and put into the RTCS SP Station to measure the background impedance. Then, plate was removed from the station and 2500 cell/per well in 100 uL medium was added into the E-plate 96 and then incubated for 30 minutes for settlement of cells. Then, plate was put again in RTCS SP Station and impedance of each well was measured with 30 minutes intervals for 140 hour. For the TRAIL and HRK combination, 2 days after virus removal, LN18 and U87MG cells were treated with 25 ng and 50 ng TRAIL respectively and impedance of each well was measured with 30 minutes intervals for 140 hour. The data was presented by using xCELLigence real time cell analyzer.

In vivo experiments

In this project, SCID mice housed and cared in appropriate conditions of Koç University Animal Facility were used and all protocols were approved by the institution boards of Koç University (HADYEK#2014-22). All animal experiments were performed in collaboration with Ahmet Cingoz. Firefly Luciferase (Fluc) and mCherry expressing stable U87MG cells were generated by viral transduction. Initially, mCherry expression was checked under the fluorescence microscopy and their Fluc activity was validated by utilizing in vitro luminescence assay. To this end, increasing number of Fluc-mCherry expressing U87MG cells (0-50.000/well) were seeded on 96-well black-bottom plate and treated with 1000 ug/ml D-Luciferin. Bioluminescence measurements were taken by BioTek2s Synergy H1 Plate reader and Gen5 data analysis software.

Fluc-mCherry expressing U87MG cells were seeded as 2 $x10^6$ cells into six 10 cm cell culture plates. After 2 days, half of the plates were transduced with pLenti-PGK-HRK-puro viruses, and untransduced cells were used as control. Next day, viruses were removed and cells were counted. For tumor implantation, 5 mice were used and both 2x 10^6 HRK expressing and 2x10^6 control U87MG cells per mouse were injected in 100 ul PBS subcutaneously. After injection, tumor cells were visualized by using IVIS Lumina III equipment. Accordingly, Fluc activity of tumors was measured by injecting mice with 150 ug/g body weight of D-Luciferin intraperitoneally. Tumor injections and Fluc imaging experiments were performed on mice anesthetized by isoflurane. Progression of tumor was monitored subsequently for 12 days and sum of the photon counts of tumor regions were obtained. This experiment is still proceeding.

Statistical Analysis

Student t-test was used for analysis of data by comparing two groups. Data were plotted as mean +_ SEM and differences were considered significant at p< 0, 05. *, **, *** denote p< 0, 05, p<0, 01, p<0,001 on the figures. Anova was used to calculate significance of *in vivo* data.

Chapter 3

Results

1. GBM cells have differential responses to TRAIL

Although TRAIL selectively kills cancer cells, the response to TRAIL is very different among different cancer cell lines.. In order to examine the effects of TRAIL on established GBM cell lines (A172, LN18, U87MG and U373), we treated these cells with differential doses of TRAIL (0, 5, 10, 20,100, 200 ng/mL) and determined the viability of the GBM cells by using Cell Titer Glo assay after 24 hour treatment. Thus, cells were categorized according to their TRAIL response as sensitive (A172), mid-sensitive (U87MG and LN18) and resistant (U373) **(Figure 7)**.

2. HRK has significantly higher expression in TRAIL-sensitive subpopulation of a primary GBM cell line

We have preliminary data from Dr. Bagci-Onder's previous studies that generated a TRAIL-resistant and TRAIL-sensitive primary GBM cell line pair from a GBM patient (data not shown). Accordingly, GBM cells that were cultured under different conditions exhibited different responses to TRAIL when they were grown in EF (EGF, FGF rich) or FBS containing mediums.

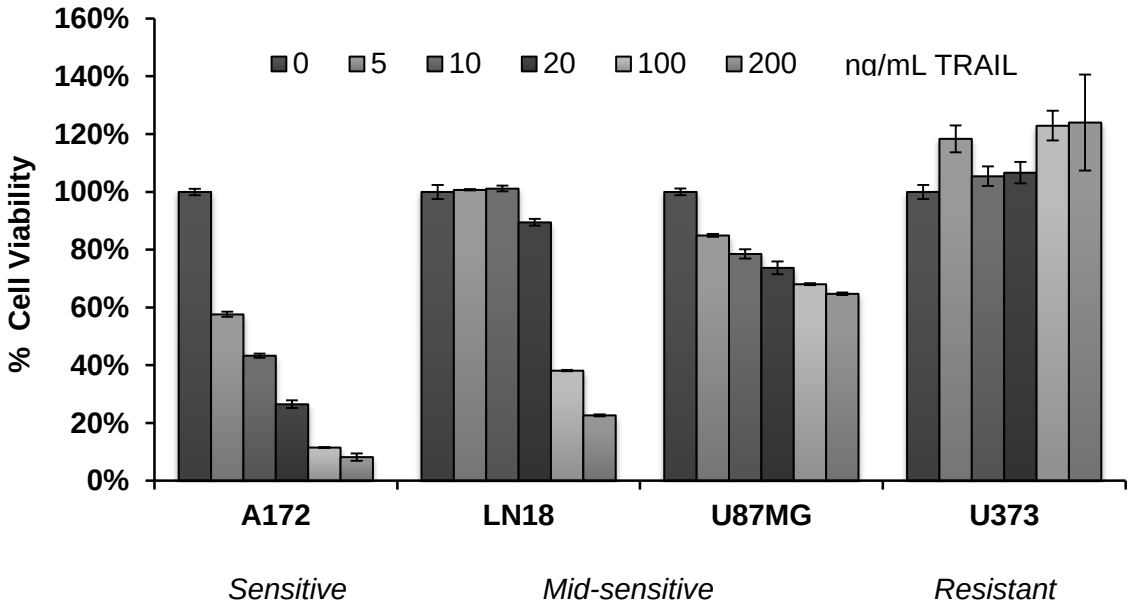


Figure 7: GBM cells have differential response to TRAIL. 4 different GBM cell lines were treated with different concentrations of TRAIL (0, 5, 10, 20,100 and 200 ng/ml). Cell viability was determined via Cell Titer Glo assay after 24 hours.

Whereas cells growing at EF containing medium were TRAIL-sensitive, cells growing in FBS containing medium were TRAIL-resistant **(Figure 8A).** In order to examine the mechanism behind these different responses, Dr. Bagci-Onder examined the apoptotic gene expression levels between TRAIL-resistant and sensitive-subpopulation of GBMs. qRT-PCR analysis for TRAIL receptors, regulator and inhibitors of apoptosis, anti-apoptotic Bcl-2 family members and pro-apoptotic Bcl-2 members was performed. We observed that anti-apoptotic Bcl-2 family members and inhibitors were downregulated and pro-apoptotic members and TRAIL receptors were upregulated in TRAIL-sensitive subpopulation compared to the TRAIL-resistant subpopulation of GBMs. Especially, HRK gene was significantly upregulated in TRAIL-sensitive subpopulation of GBM cells **(Figure 8B),** which formed the basis of the next experiments in this thesis.

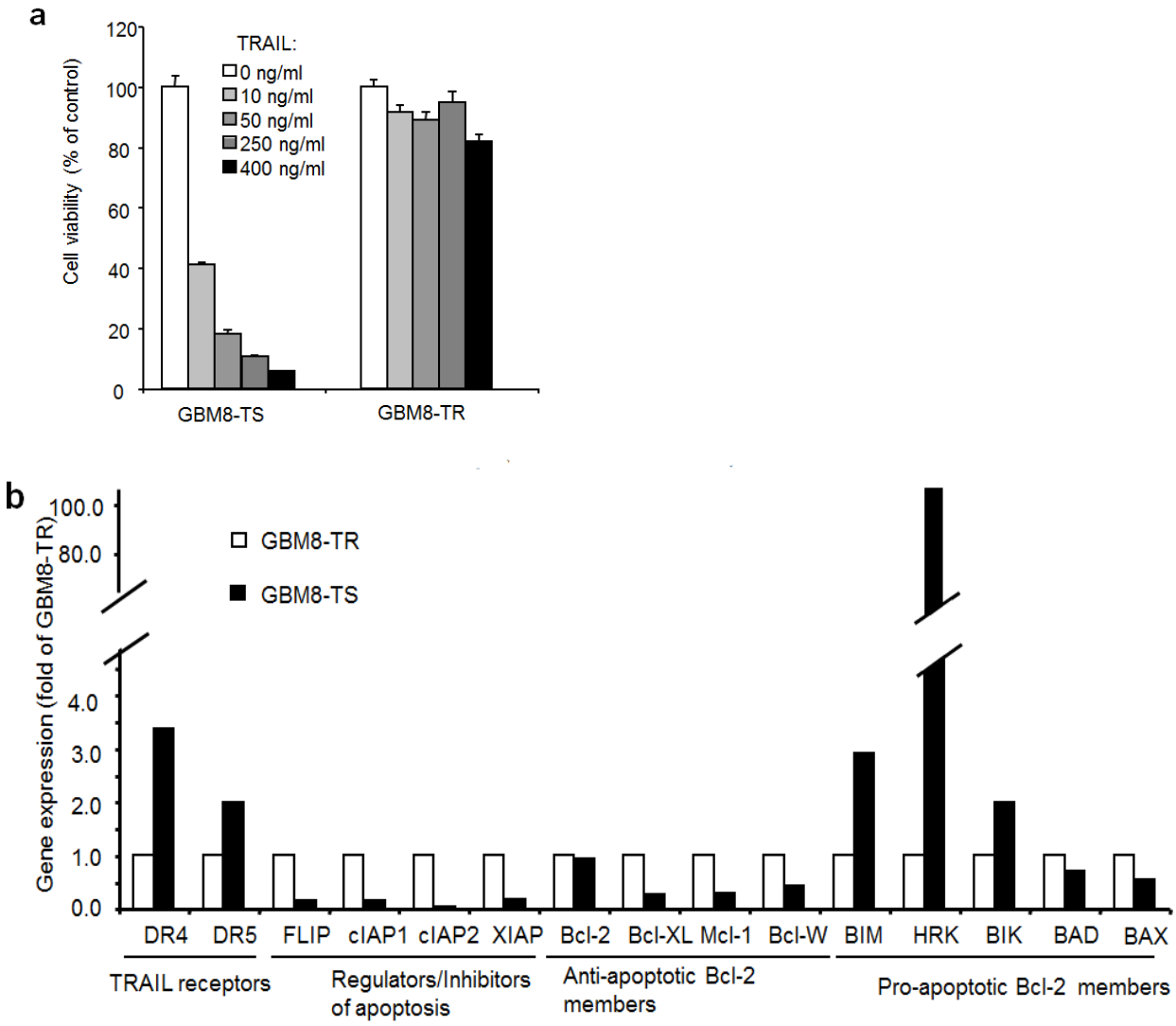


Figure 8: HRK has significantly higher expression in TRAIL-sensitive subpopulation of a primary GBM cell line. (a) TRAIL response of an isogenic GBM cell line pair selected for TS (TRAIL sensitive) and TR (TRAIL resistance) subpopulations that were exposed with differential doses of TRAIL (0, 10, 50, 250, 400 ng/mL) were assessed. **(b)** qRT-PCR analysis of apoptotic members in GBM-TS and GBM-TR subpopulations was performed. (Performed by Dr. Bagci-Onder, unpublished data)

3. HRK is differentially expressed in GBM cells

Since HRK expression was significantly upregulated in TRAIL-sensitive subpopulation of GBM cells selected from a patient, we wished to examine the endogenous HRK expression levels among a panel of our established GBM cell lines (A172, LN18, U87MG and U373) as well. . Endogenous HRK expression of the TRAIL-sensitive cell line, A172, was ~50 fold higher than U87MG, LN18 and U373 cells **(Figure 9).** The qRT-PCR results suggested a correlation between TRAIL sensitivity and HRK expression.

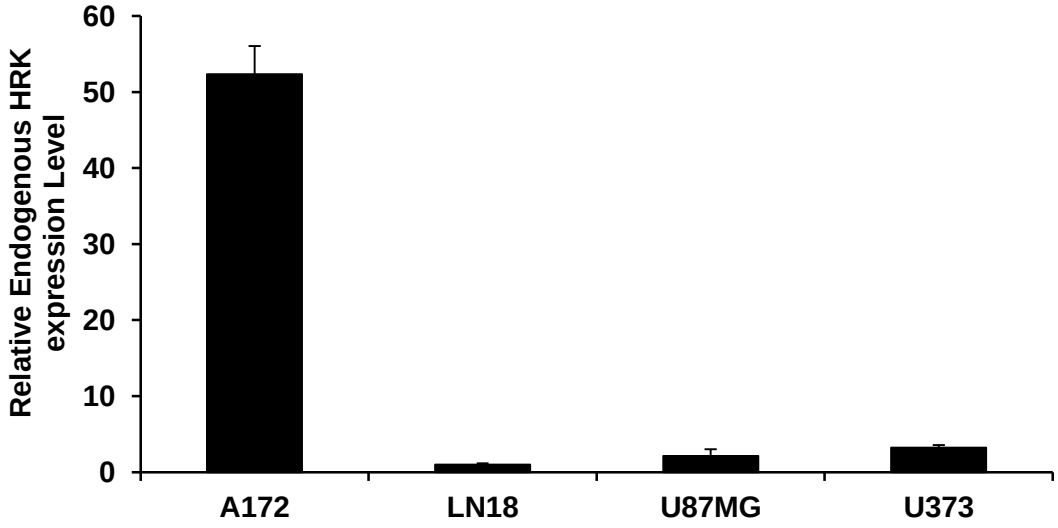


Figure 9: qRT-CR analysis of endogenous levels of HRK in GBM cell lines. HRK mRNA level relative to GAPDH mRNA level were measured and ΔΔCt method (54) was used to detect relative expression.

4. HRK can be overexpressed in GBM cell lines by lenti-viral expression vectors

Since the functional role of HRK has not been studied in GBMs and the endogenous expression of HRK is different among GBM cell lines, we wished to test the role of HRK by overexpressing it in GBM cells. To this end, we generated a HRK overexpression vector in a lenti-viral backbone using Gateway cloning technique for the characterization of HRK function in GBM cell lines (please see Materials and Methods for details). First, we amplified the HRK cDNA from the pcDNA3.1 HRK vector and cloned it into pENTR1A entry vector. Then, we cloned the HRK cDNA sequences into the lenti-viral PGK-puro destination vector through recombination reaction. PGK-puro-HRK vector was validated by restriction digestion and sequencing. We produced viral particles containing HRK gene and also GFP as negative control through viral packaging. We then infected the four established GBM cell lines with HRK and GFP viruses. Western blot analysis validated the HRK overexpression in GBMs compared to the GFP control **(Figure 10)**, suggesting that successful HRK overexpression was possible in GBM cells.

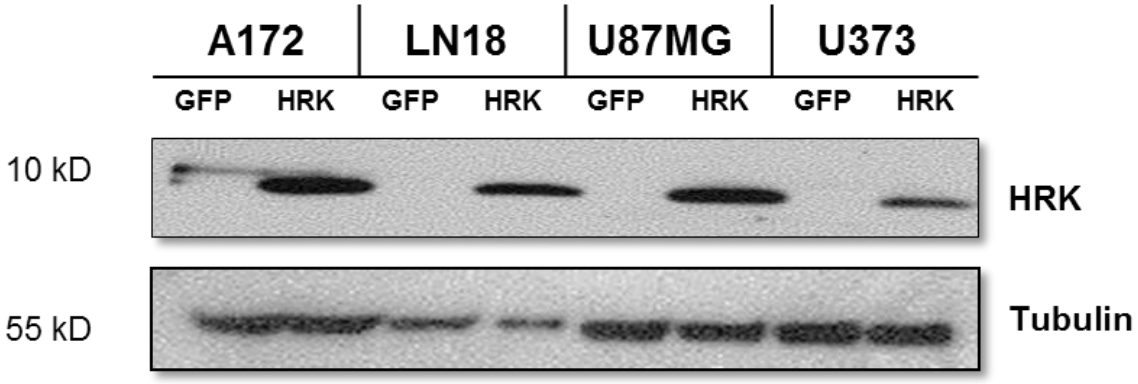
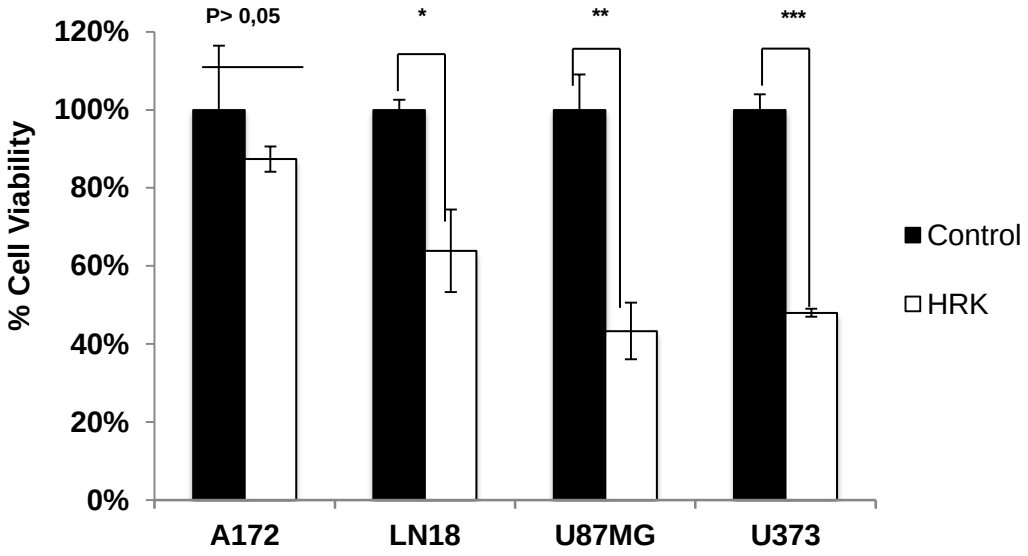


Figure 10: Validation of ectopic HRK overexpression in GBM cells. Western blot analysis of HRK overexpression in the whole cell lysate of HRK and GFP overexpressed GBM cell lines.

5. HRK overexpression reduces cell viability and activates effector caspases in GBM cells

In order to test the functional effect of HRK expression on GBM cells, we first performed cell viability assays. Upon 24 hours after HRK infection, GBM cells were tested for their viability. Our experiments with Cell Titer Glo assays revealed that HRK overexpression triggered cell death significantly in LN18, U87MG and U373 but not in A172 cells **(Figure 11A).** To assess whether Caspase activation was also involved in observed reduction in cell viability, we measured the active caspase 3/7 using Caspase 3/7 Glo assays. We demonstrated that HRK significantly increased caspase 3/7 activity in all GBM cell lines tested **(Figure 11B**), however two different degrees. LN18, U87MG and U373 cells had higher caspase activation compared to A172 cells in consistency with the viability results.

a



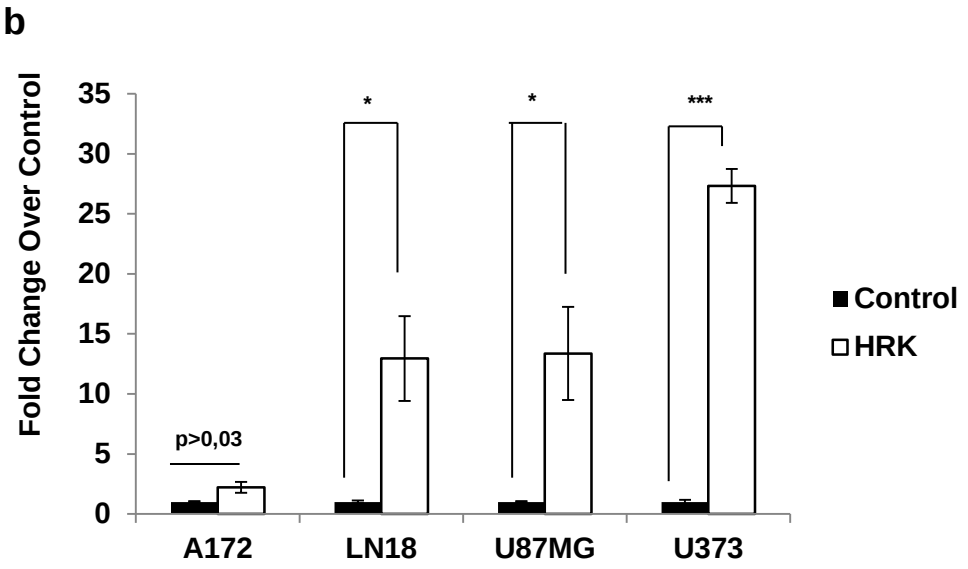


Figure 11: HRK overexpression decrease cell viability and increase the active caspase 3/7. (a) Viability analysis of GBM cells testing the effect of HRK overexpression versus GFP overexpression. **(b)** Caspase 3/7 activity analysis of HRK and GFP overexpressed GBM cells. (*, **, *** denote p< 0, 05, p<0, 01, p<0,001, t-test)

6. Caspase inhibition blocks the HRK induced caspase activity

To test whether caspase activity was functionally necessary for HRK-induced cell death, we blocked caspase activation using specific inhibitors. We used 20 uM general caspase inhibitor targeting caspase-8 -9 -3 and -7 (Z-VAD-FMK), and used a negative caspase inhibitor (Z-FA-FMK) as controls. General Caspase inhibitor treatment for 24 hours prior to and during HRK infection markedly blocked the HRK-induced caspase activity compared to negative caspase inhibitor treatment **(Figure 12).** These results suggest that caspase activation was necessary for the HRK-induced changes in cell viability.

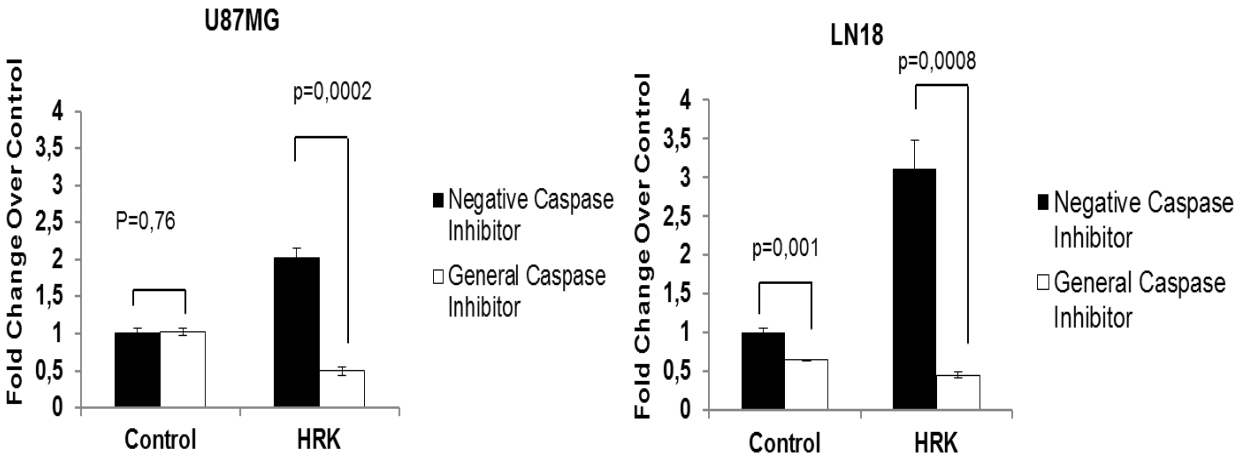


Figure 12: HRK induced caspase activity is blocked by General Caspase Inhibitor. 20 uM general caspase (Z-VAD-FMK) and negative caspase inhibitor were used (Z-FA-FMK).

7. Apoptotic marker, cleaved PARP is increased by HRK overexpression in GBM cells

In order to gain more insight about the HRK-induced cell death, we checked one of the most widely used apoptotic marker; cleaved PARP, by performing western blot. Western blot analysis showed that HRK overexpression increased PARP cleavage significantly in LN18 and U373 cells but not in U87MG and A172 cells (**Figure 13**). This result suggested that HRK plays a role GBM cell apoptosis, and the downstream apoptotic events, such as PARP cleavage is modulated by HRK expression.

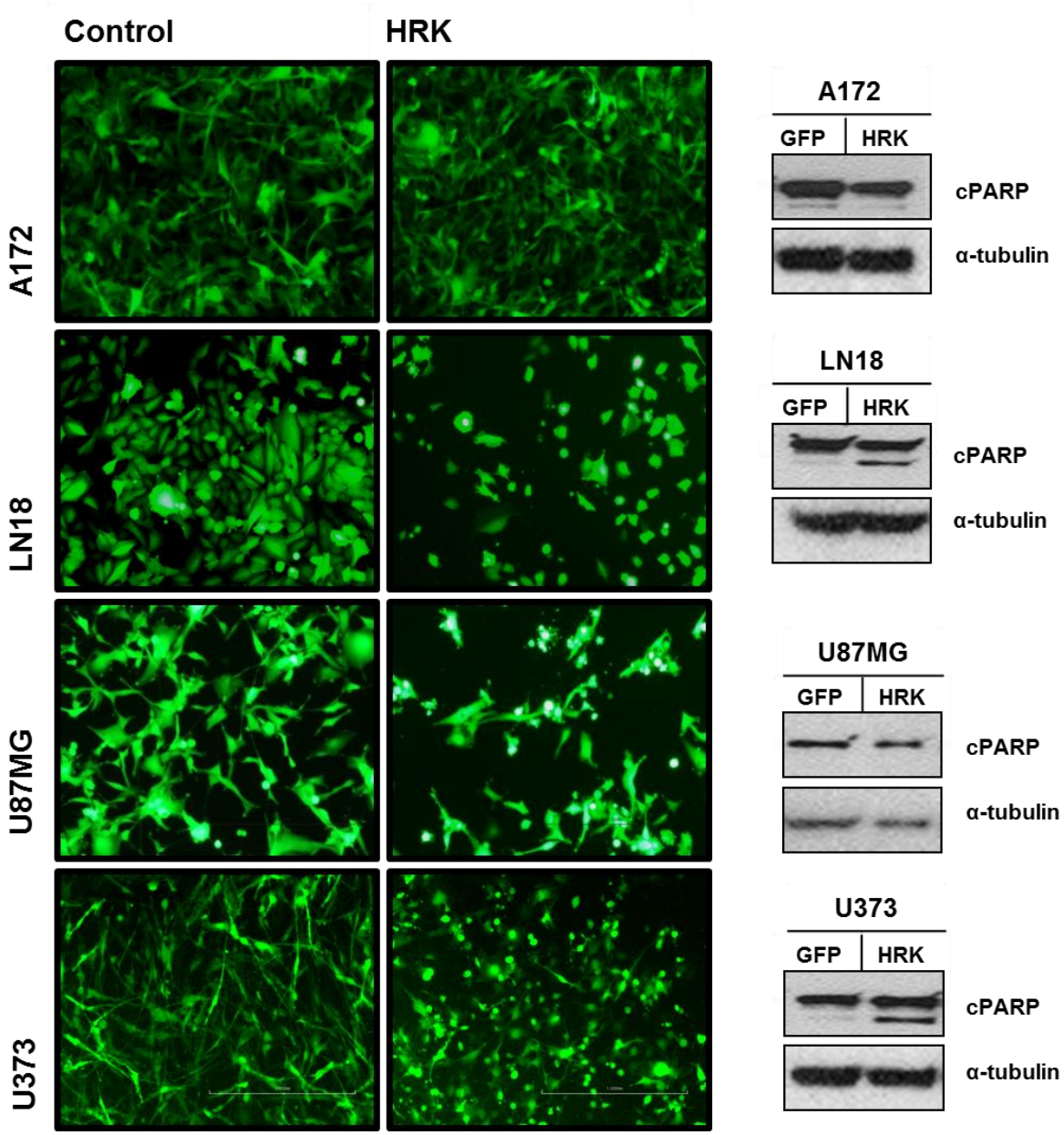


Figure 13: HRK overexpression induces PARP cleavage in GBM cells. Western Blot analysis of cleavage of PARP in HRK and GFP overexpressed GBM cells.

8. HRK overexpression decreases cell growth in real-time

In order to assess the long-term effects of HRK overexpression on GBM cell growth and proliferation, we performed a real-time cell growth analysis. In this experiment, we seeded GBM cells into a special plate that allows the quantification of cells at the surface of the plates over time through measuring cell impedance in a special equipment (Xcelligence). We first seeded the cells (t0) and let them grow for 24 hours. Later, we transduced GBM cells with HRK and GFP viruses at t1. After 24 hour of incubation with viruses, viral media was removed and replaced with fresh media at t2 time point. We then observed cell growth for an additional 72 hours. Together, this cell growth analysis showed that HRK expression markedly reduced cell proliferation in long term **(Figure 14),** as the kinetics of cell growth were different in LN18, U87MG, and U373 cells. In consistent with our previous experiments, A172 cells responded the least to HK expression in this setting as well.

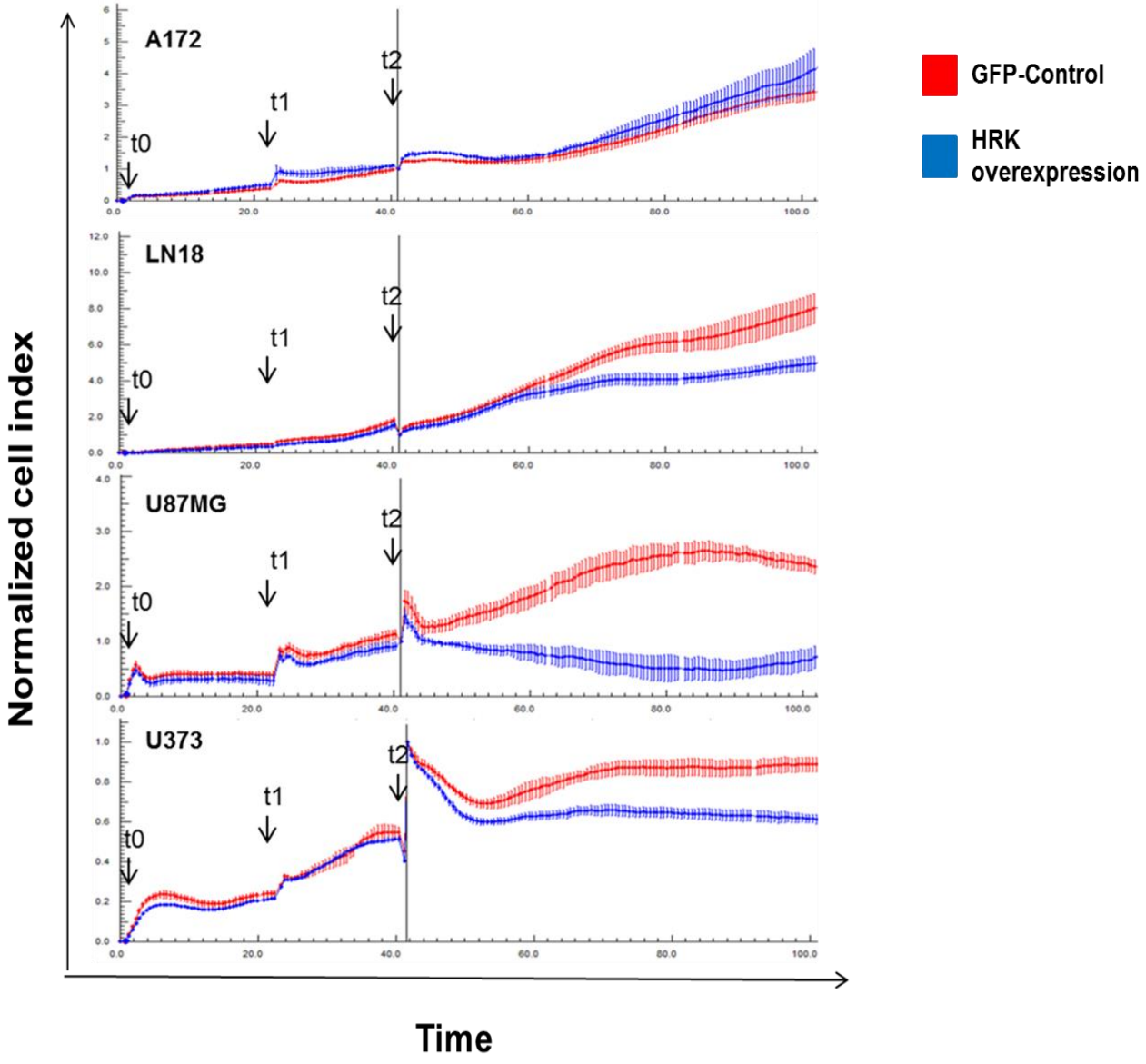


Figure 14: HRK expression reduces the rate of cell growth in long-term. Real-time cell growth analysis of HRK and GFP transduced A172, LN18, U87MG and U373 cells. Red shows GFP expressing and blue shows HRK expressing GBM cells. (t0 indicates cell seeding, t1 indicated viral transduction and t2 indicated virus removing).

9. Bcl-2 and Bcl-xL overexpression inhibits HRK induced cell death

HRK is known as a sensitizer BH3 only protein and triggers apoptosis by neutralizing the anti-apoptotic Bcl-2 and Bcl-xL proteins (55) However the apoptosis regulatory role of HRK in cancer cells has not been studied before. In order to test the functional interaction between Bcl2, BclxL and HRK in GBM cells, we first generated GFP, Bcl-2 and/or Bcl-XL overexpressing GBM cells through retroviral mediated gene transduction. The retroviral vectors we used for overexpressing these proteins contained a GFP cassette; therefore we could monitor transduction efficiency with GFP fluorescence. We then overexpressed HRK protein in Bcl-2 and/or Bcl-xL and GFP overexpressing GBM cells and assessed the effect of HRK. Cell viability assays demonstrated that Bcl-2 or/and Bcl-xL overexpression inhibited-HRK induced apoptosis and led to phenotype recovery at LN18 **(Figure 15B),** U87MG **(Figure 15C, 16C),** U373 **(Figure 15D, 16B)** but not at A172 cell as expected **(Figure 15A)**.

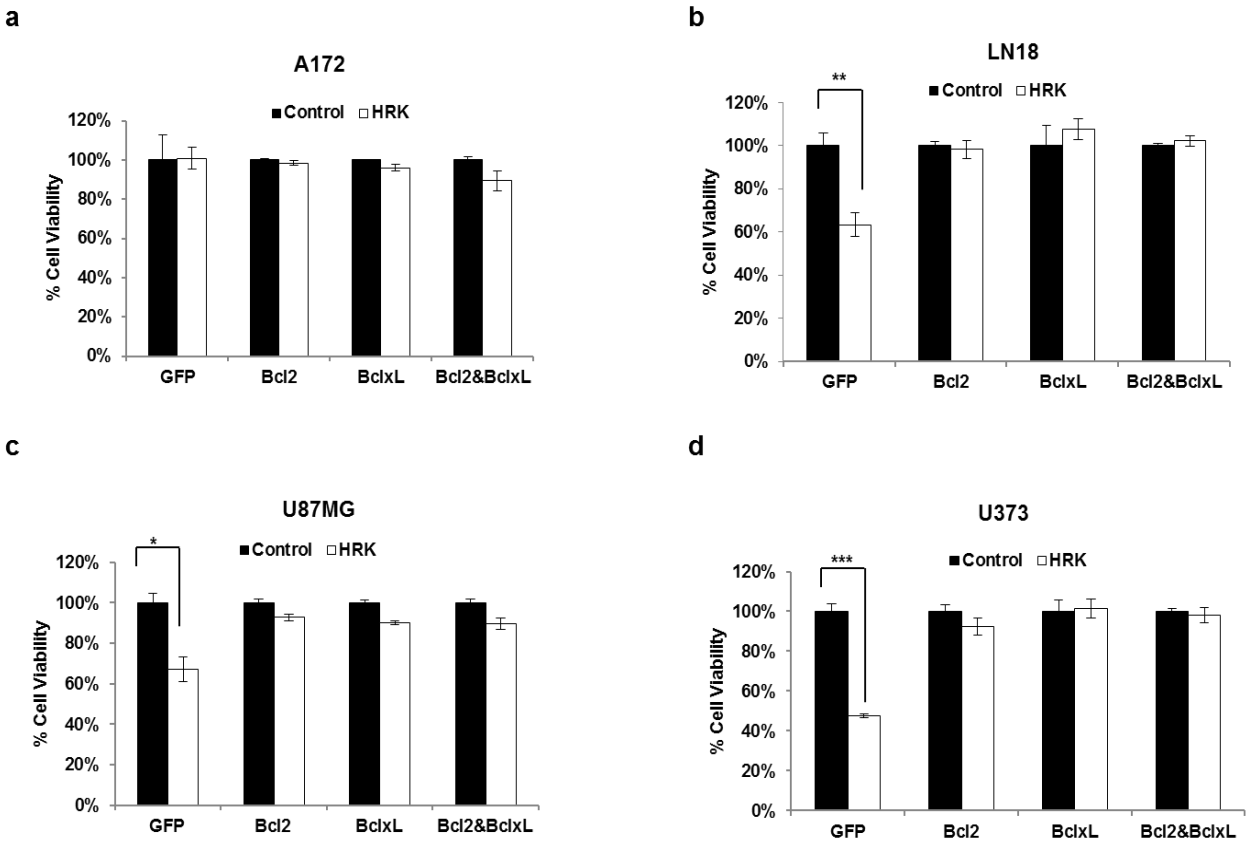


Figure 15: Bcl-2 and/or Bcl-xL overexpression inhibits HRK-induced cell death. Cell viability analysis of HRK overexpression in GFP, Bcl-2 and/or Bcl-xL overexpressed **(a)** A172, **(b)** LN18, **(c)** U87MG, **d)** U373. (*, **, *** denote p< 0, 05, p<0, 01, p<0,001, t-test).

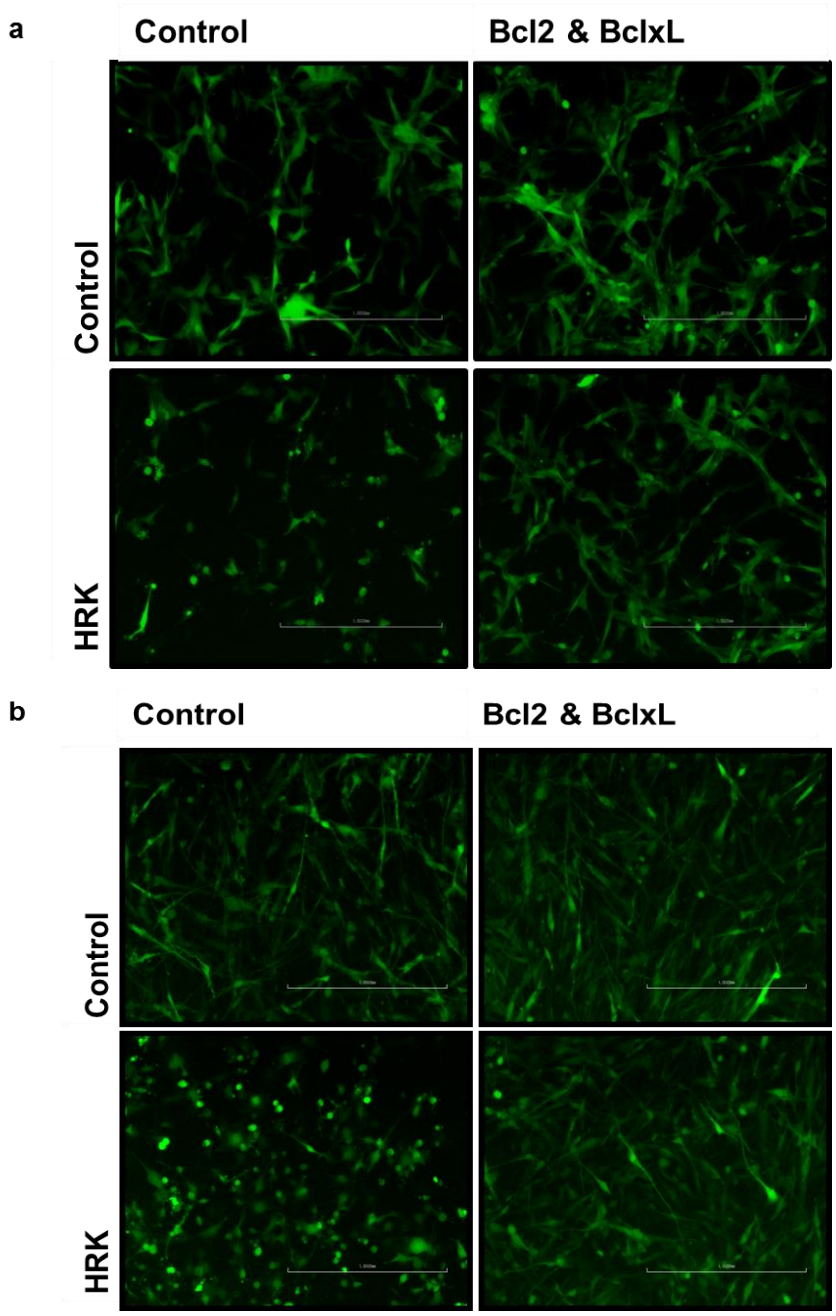


Figure 16: Representative fluorescence images of Bcl-2 and Bcl-xL overexpression effects on HRK expressing (a) U87MG and (b) U373 cells.

10. HRK overexpression cooperates with TRAIL in U87MG and LN18 cells

In order to examine the combinational effect of HRK overexpression and TRAIL in GBM cells, we treated HRK and GFP infected GBM cells with TRAIL (50 ng). Cell viability analysis showed that whereas TRAIL reduced the cell viability of TRAIL sensitive A172, HRK did not add an additional killing, consistent with previous results (**Figure 17A**). In TRAIL-resistant U373 cells, cell viability was decreased by HRK overexpression but it was not affected by TRAIL treatment (**Figure 17B**). In contrast, HRK overexpression cooperated with TRAIL in U87MG and LN18 TRAIL mid-sensitive GBM cell lines and HRK overexpressing cells had better response to TRAIL treatment (**Figure 17C, 17D**).

For further examination, we measured caspase 3/7 activities in HRK overexpressing A172, LN18, U87MG and U373 cells that were also treated with TRAIL for 3 hours. Caspase 3/7 activity measurements and cell viability analysis gave similar results and showed that HRK and TRAIL collaborated to induce apoptosis in U87MG and LN18 GBM cell lines **(Figure 18C, 18D)** but not in A172 **(Figure 18A)** and U373 cells **(Figure 18B).**

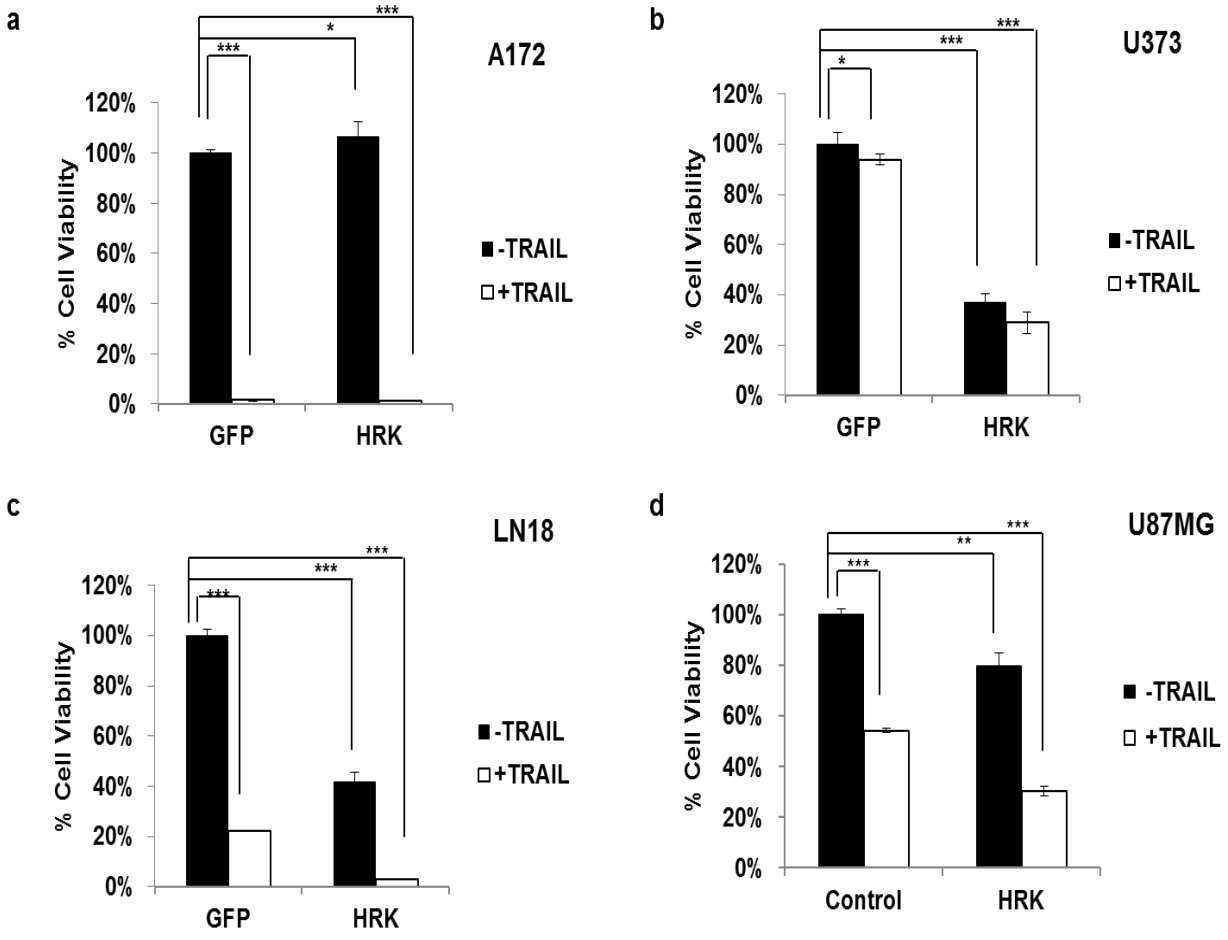


Figure 17: The combined effect of HRK overexpression and TRAIL on GBM cells viability. Cell viability analysis of GFP and HRK overexpressed **(a**) A172, **(b)** LN18, **(c)** U87MG and **(d)** U373 cells upon TRAIL treatment (50 ng). (*, **, *** denote p< 0, 05, p<0, 01, p<0,001, t-test)

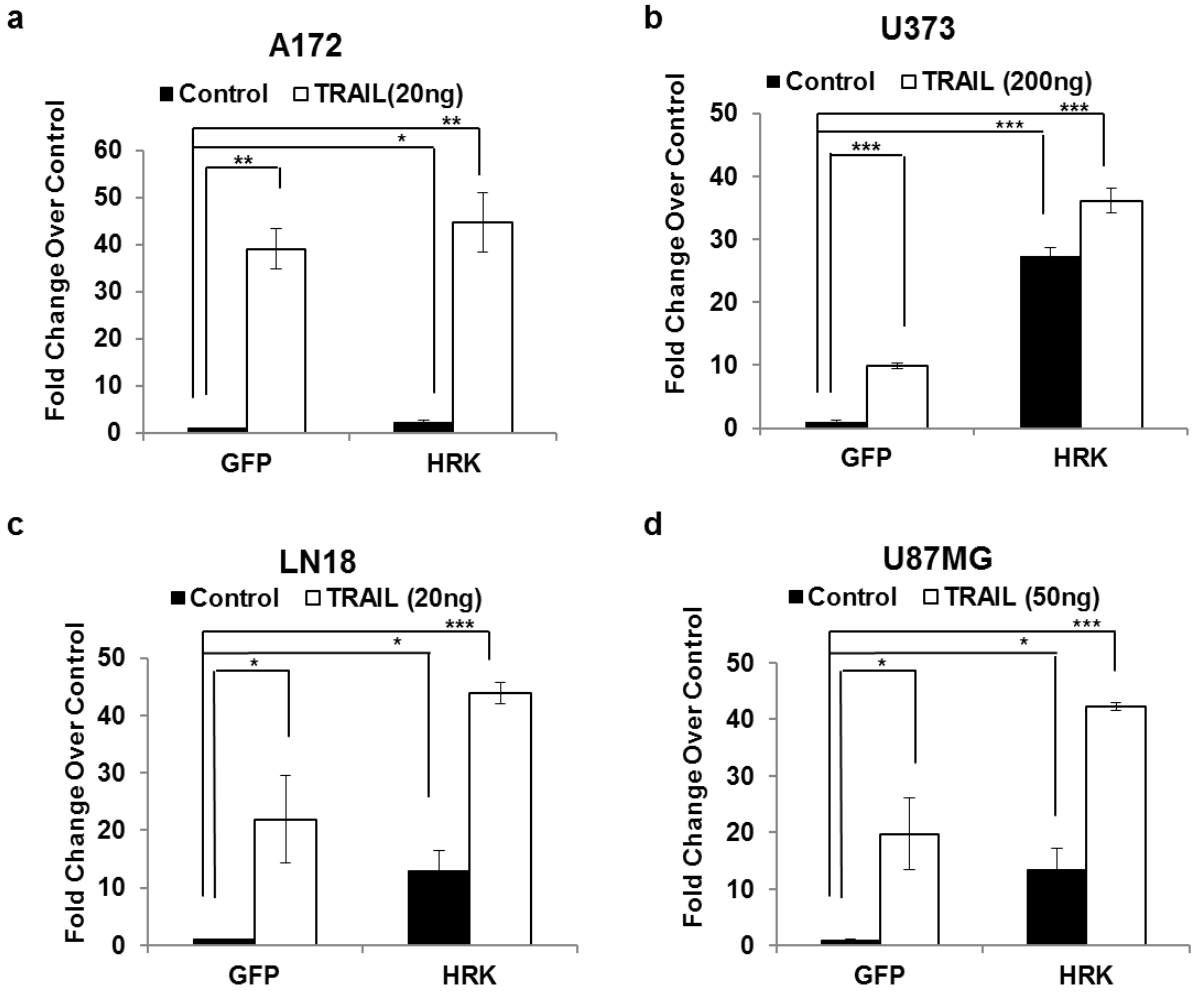
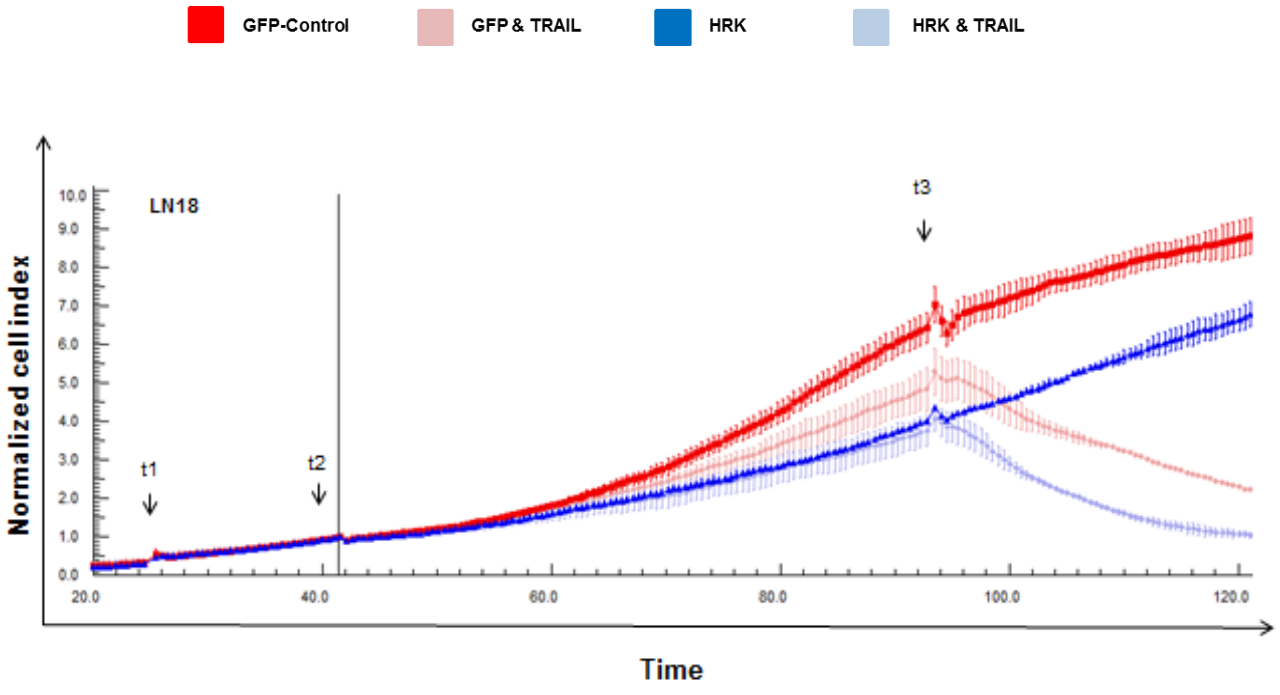


Figure 18: The combined effect of HRK overexpression and TRAIL on caspase activity in GBM cells. Caspase 3/7 activity analysis of GFP and HRK overexpressed **(a)** A172, **(b)** U373, **(c)** LN18 and **(d)** U373 GBM cell after 3 hours TRAIL treatment (20 ng, 200 ng, 20 ng and 50 ng respectively), performed by Caspase-Glo assays (Promega). (*, **, *** denotes p< 0, 05, p<0, 01, p<0,001, t-test)

11. HRK and TRAIL cooperation in LN18 and U87MG cells' long term viability

In order to determine the long-term effect of TRAIL and HRK cooperation in LN18 and U87MG cell, we employed real-time cell growth assays. In this experiment, we seeded GBM cells into special plates as 2500/well (as described above). Approximately 24 hours later, we transduced GBM cells with HRK and GFP viruses at t1 time point. After 24 hours incubation, media was removed and replaced with fresh media at t2 time point. After 40 hours, 25 ng TRAIL for LN18 **(Figure 19 A)** and 50 ng TRAIL for U87MG cell **(Figure 19 B)** treatment was performed. As seen in the plots, TRAIL treatment decreased the number of measurable live cells in this system, and TRAIL and HRK together led to reduced cell number in both LN18 and U87MG cells.



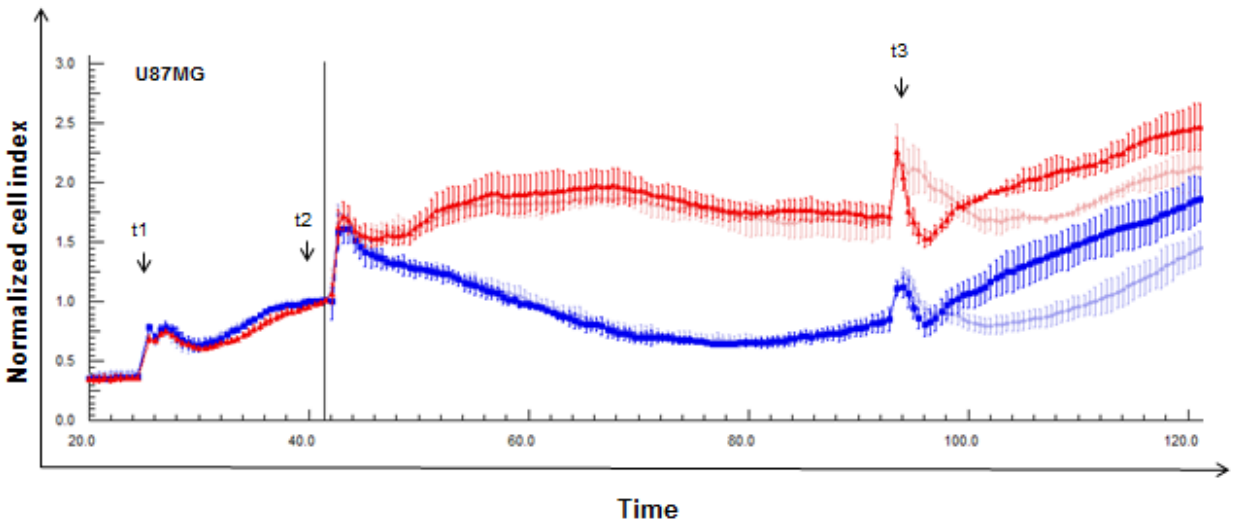


Figure 19: Effect of HRK and TRAIL cooperation on the growth of LN18 and U87MG cells in long-term. Real-time cell growth analysis of HRK and GFP transduced LN18 and U87MG cells upon 25 ng and 50 ng TRAIL treatment respectively. Red shows GFP expressing and blue shows HRK expressing GBM cells. Lighter colors indicate cells with TRAIL treatment (t1 indicated viral transduction and t2 indicated virus removing, t3 indicated TRAIL treatment).

12. HRK and TRAIL cooperation in apoptosis of GBM cells

In this research, HRK and TRAIL are players to induce intrinsic and extrinsic pathway respectively. In addition, whereas caspase-8 is an initiator caspase for extrinsic pathway, caspase 9 is the one for intrinsic pathway. Both activate the effector caspase 3 and-7 leading to apoptosis. Therefore, we assessed the effects of TRAIL and HRK cooperation on caspase -8 -9 and -3/7 activity. To this end, we first transduced cells with HRK or GFP vectors for 24 hours and then added TRAIL (10 ng) for 3 hours. We measured active caspase -8, -9 besides caspase 3/7 in HRK overexpressing LN18 **(Figure 20A)** and U87MG **(Figure 20B)** cells upon

TRAIL treatment. Caspase 3/7 assays showed that TRAIL and HRK together lead to more activation of caspase 3/7 in U87MG and LN18 cells. As expected, caspase-8 activation was induced by TRAIL whereas caspase-9 activation was triggered by HRK overexpression. Nevertheless, their combination is more effective for both caspase -8 and -9 activities.

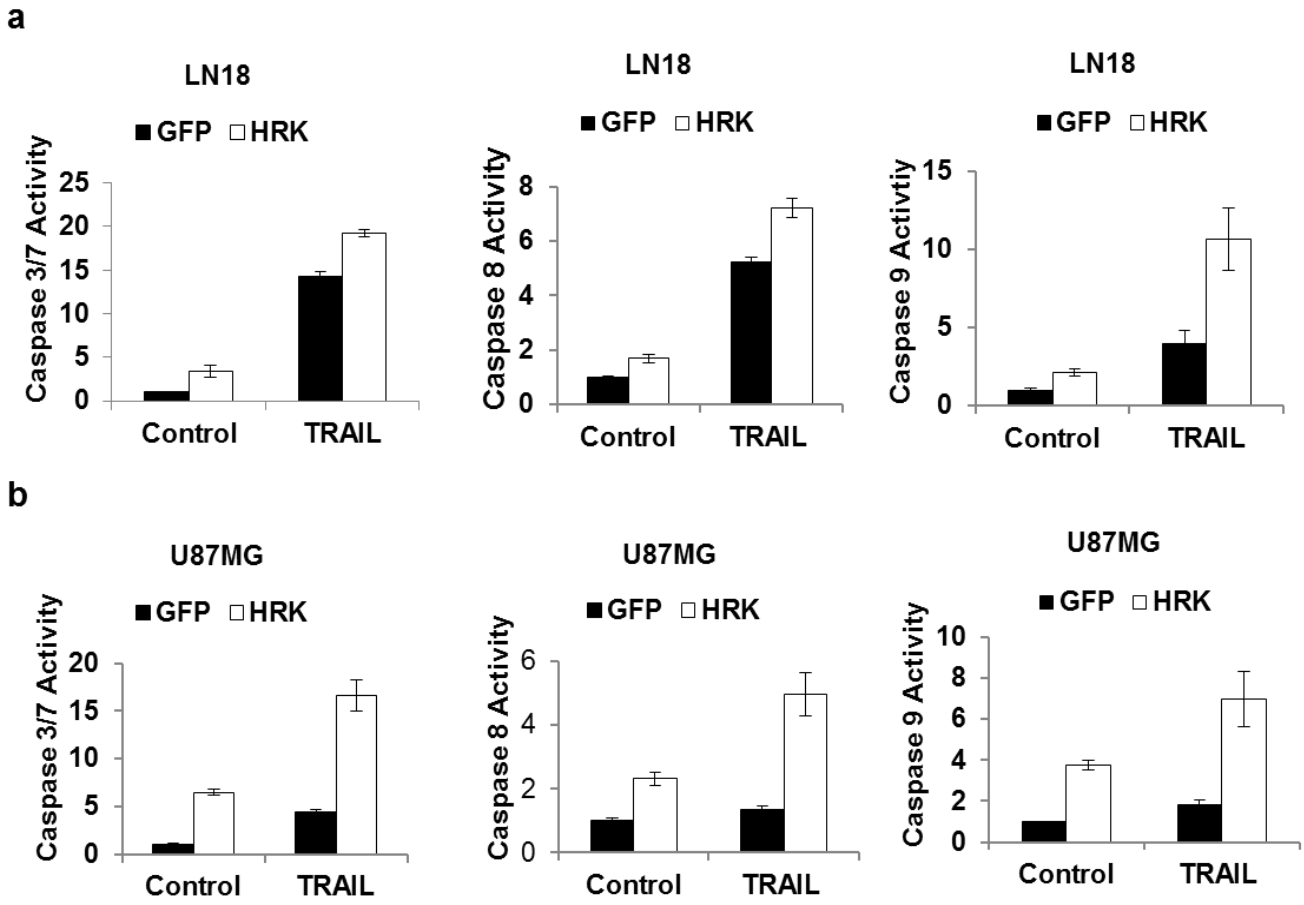


Figure 20: Effect of HRK and TRAIL combination on Caspase 3/7, caspase -8 and caspase -9 activities in LN18 (a) and U87MG (b) cells.

In order to gain more insight about HRK cooperation with TRAIL in GBM cell apoptosis, we first observed cells' killing dynamics under microscope and noticed that at 24 hours after TRAIL treatment of HRK-overexpressing cells, most cells die (as seen in **Figure 15 A**). Therefore, to check for markes of apoptosis with western blot analysis, we applied TRAIL (50 ng) for 3 hours and collected cell lysates to check for PARP cleavage in HRK overexpressing LN18 and U87MG cell.. Analysis showed that HRK overexpression with TRAIL treatment increased the cleaved PARP compared to the HRK overexpression only and TRAIL treatment only in LN18 **(Figure 21A)** and U87MG **(Figure 21B).**

13. HRK silencing partially blocks TRAIL-induced apoptosis

We showed that HRK and TRAIL cooperate to induce apoptosis in U87MG and LN18 cells. Further, in order to elucidate the requirement of HRK for TRAIL induced apoptosis, we generated a silencing shHRK vector targeting the first exon of HRK gene and also non-targeting shControl in a lenti-viral backbone (please refer to materials and methods). In order to check the knockdown efficiency, we performed qRT-PCR analysis in shHRK and shControl transduced U87MG cells. Analysis demonstrated that shHRK reduced HRK mRNA expression to ~50% compared to shControl cells **(Figure 22A).** We selected GBM cells that express shHRK vector through Puromycin selection and generated stable cells. Then, to investigate the requirement of HRK in TRAIL induced apoptosis, we performed cell viability analysis in shHRK and shControl transduced U87MG cells upon TRAIL treatment (75 ng). Analysis showed that HRK knockdown partially prevents TRAIL induced apoptosis in U87MG cells **(Figure 22B).** These results suggest that HRK is partly involved in TRAIL-induced apoptosis of GBM cells.

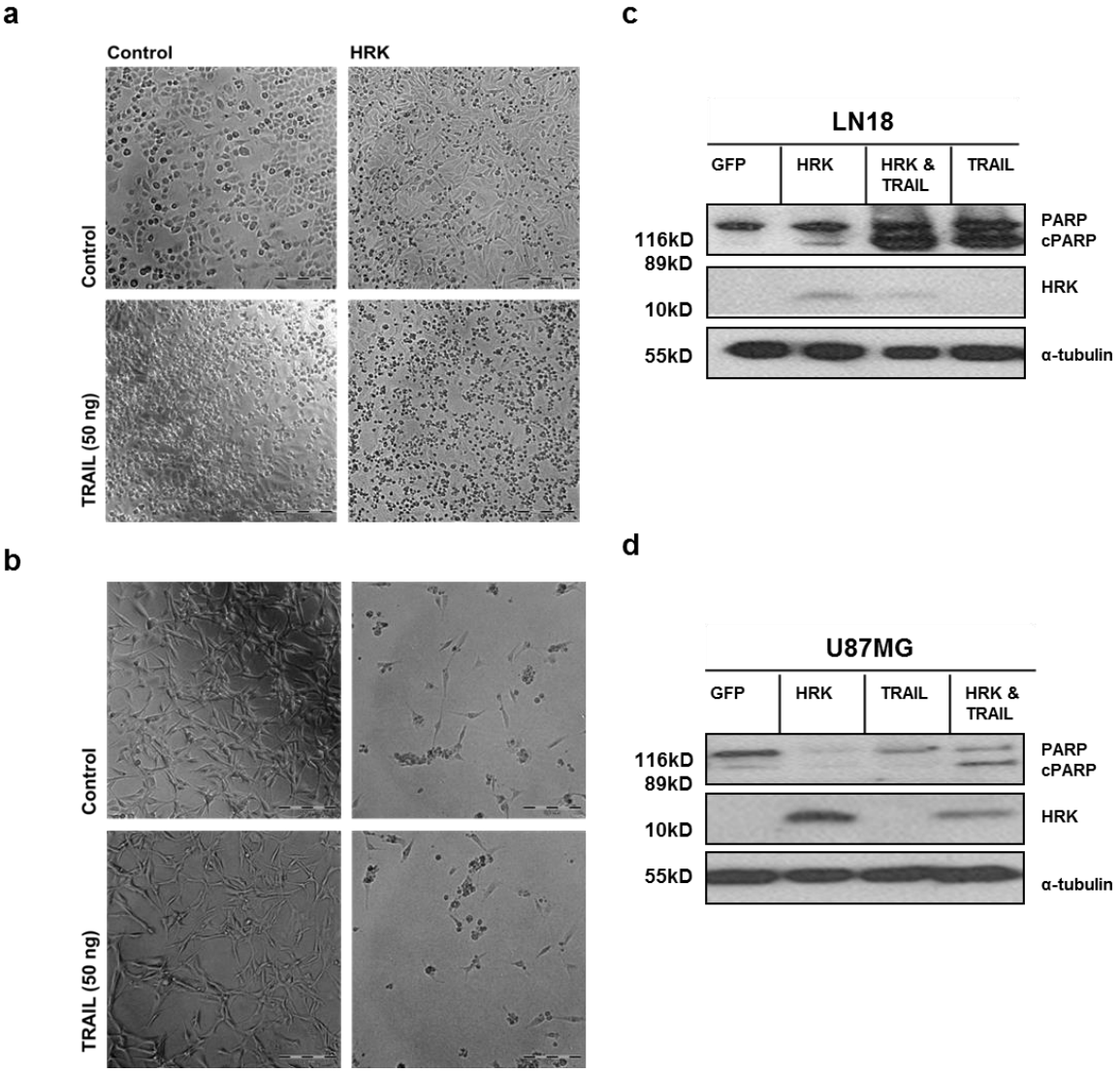


Figure 21: HRK and TRAIL cooperation induces PARP cleavage. Representative images of cell death activity of HRK and TRAIL (50 ng) itself and their combination on LN18 **(a)** and U87MG **(b).** Western blot analysis shows HRK and TRAIL combination induces more PARP cleavage in LN18 **(c)** and U87MG **(d).**

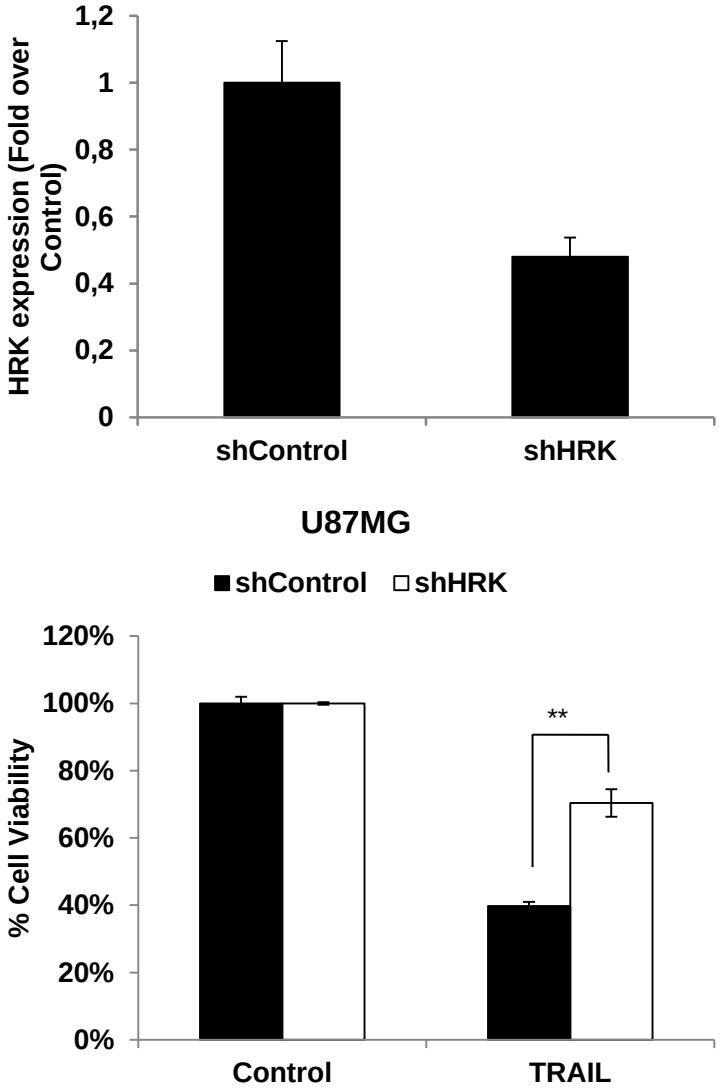
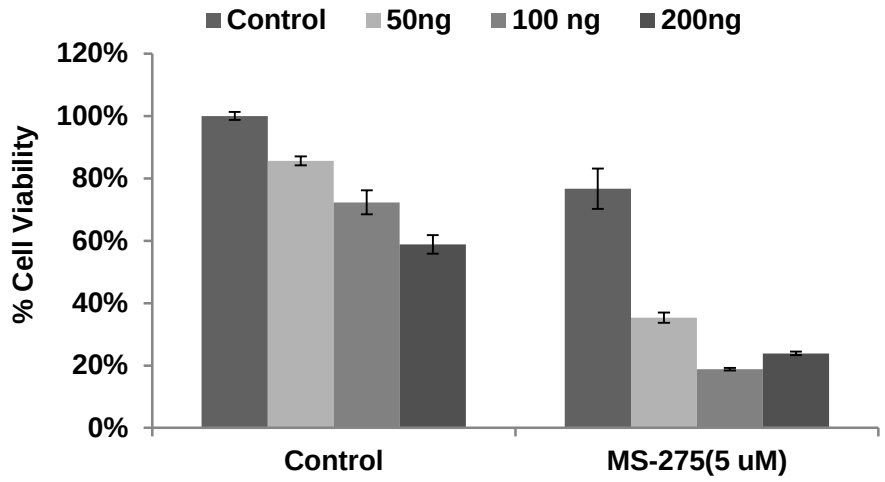


Figure 22: HRK silencing prevents TRAIL induced apoptosis. (a) qRT-PCR analysis of HRK expression in shControl and shHRK transduced U87MG cells. HRK mRNA level relative to GAPDH mRNA level were measured and ΔΔCt method was used to detect relative expression. **(b)** Cell viability analysis of shControl and shHRK transduced U87MG cells upon 75 ng TRAIL treatment. (** indicates p=0,0017)

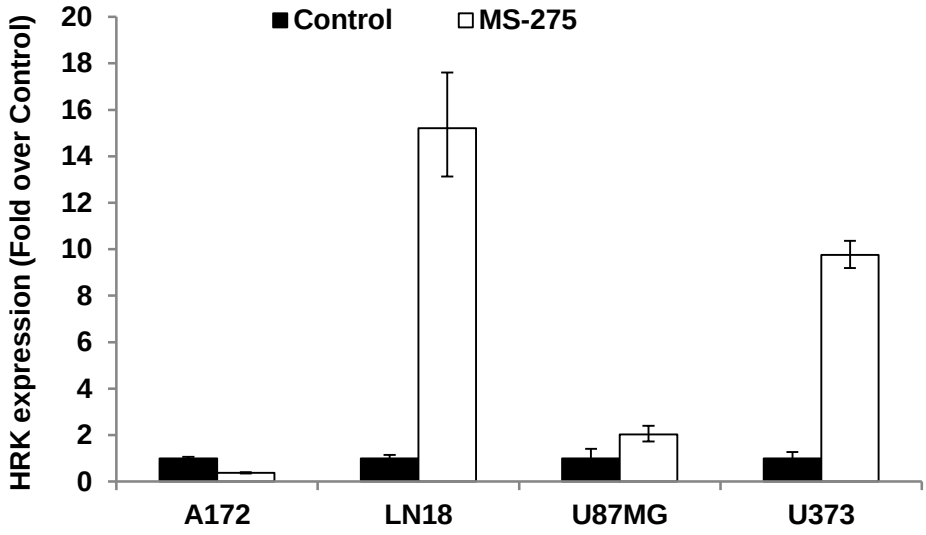
14. HRK expression is responsive to known TRAIL sensitizing agent MS-275

Even though TRAIL response is differential among GBM cell lines, many groups previously showed that TRAIL sensitization is possible (56). A histone deacetylase inhibitor, MS-275 which is known to increase DR4 and DR5 expression, sensitizes TRAIL-resistant GBMs to TRAIL. However, the exact mechanism behind MS-275 mediated sensitization is not known. We first examined the TRAIL sensitization effect of MS-275 in U87MG TRAIL mid-sensitive GBM cell line upon differential doses of TRAIL treatment (0ng, 1ng, 5 ng, 10 ng). Cell viability assay showed that MS-275 sensitized U87MG cells to TRAIL (Figure 23A). Since HRK cooperates with TRAIL and increases this outcome, it may contribute to TRAIL sensitization mechanism of MS-275. To this end, we first examined the HRK expression changes by qRT-PCR upon 24 hour MS-275 treatment (5 uM). Analysis demonstrated that HRK is responsive to MS-275 in GBM cell lines (LN18, U87MG and U373) except A172 (Figure 23B). In order to assess whether HRK upregulation was a result of cell death or not, we assessed the effect of MS-275 on GBM cell viability by treating cells with different doses of MS-275. Cell viability analysis showed that MS-275 does not have any significant effect on cell viability (Figure 23C), suggesting that HRK upregulation was not a consequence of cell death. For further examination, we tested the requirement of HRK for TRAIL sensitization effect of MS-275 by conducting cell viability analysis of shControl and shHRK transduced U87MG cells upon combination of TRAIL (75 ng) and MS-275 (5uM) treatment (Figure 23D). Accordingly, we showed that silencing of HRK in GBM cells prevented MS-275 mediated TRAIL sensitization. This result suggested that HRK can be a key factor for TRAIL sensitization mechanism of MS-275.

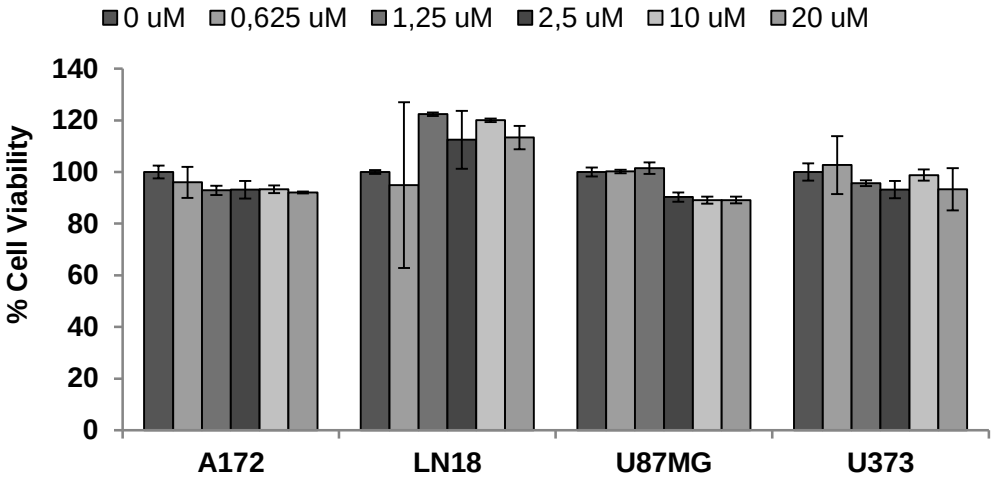
a


Control
50ng
100 ng
200ng
120%
100%
80%
60%
40%
20%
0%
% Cell Viability
Control
MS-275(5 uM)

b


Control
MS-275
20
18
16
14
12
10
8
6
4
2
0
HRK expression (Fold over Control)
A172
LN18
U87MG
U373

c


0 uM
0,625 uM
1,25 uM
2,5 uM
10 uM
20 uM
% Cell Viability
140
120
100
80
60
40
20
0
A172
LN18
U87MG
U373

d

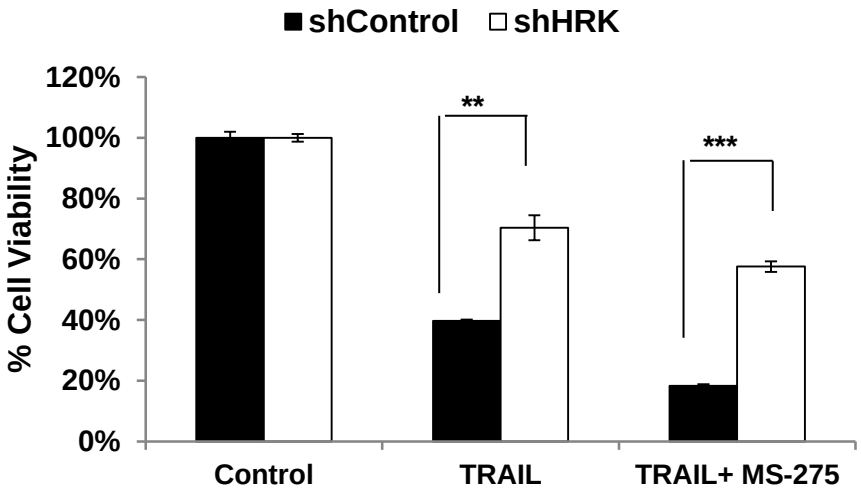
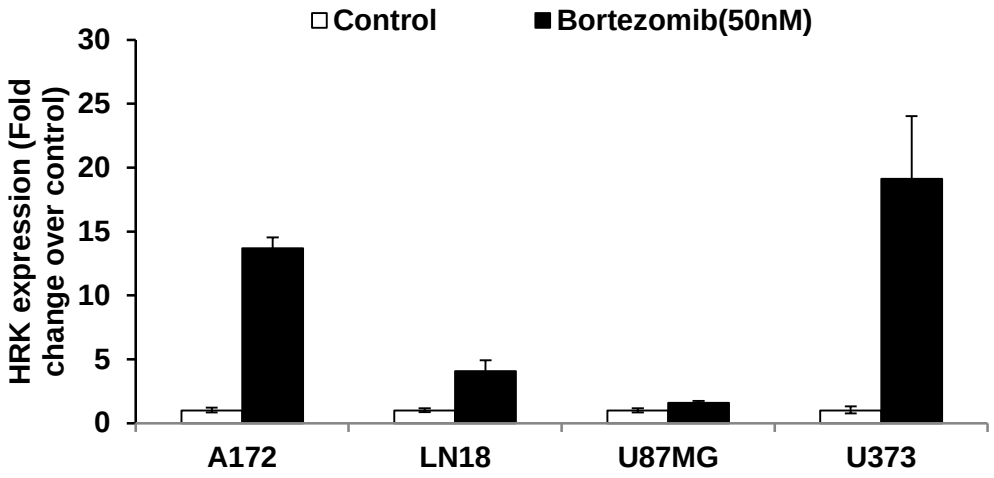

shControl
shHRK
% Cell Viability
120%
100%
80%
60%
40%
20%
0%
**
***
Control
TRAIL
TRAIL+ MS-275

Figure 23: HRK is responsive to MS-275 and is required for its TRAIL sensitization mechanism. (a) Viability analysis of U87MG cells upon differential doses of TRAIL (0ng, 50ng, 100 ng, 200 ng) and 5 uM MS-275 combination. **(b)** qRT-PCR analysis of HRK expression in four established GBM cell lines (A172, LN18, U87MG, U373) upon MS-275 treatment (5 uM). HRK mRNA level relative to GAPDH mRNA level were measured and ΔΔCt method was used to detect relative expression. **(c)** Viability analysis of A172, LN18, U87MG and U373 cells upon differential doses of MS-275 treatment (0 uM, 0,625 uM, 1, 25 uM, 2,5 uM, 10 uM and 20 uM). **(d)** Cell viability analysis of shControl and shHRK transduced U87MG cells upon TRAIL treatment (75 ng) and MS-275 (5 uM) combination. (*, **, *** denotes p< 0, 05, p<0, 01, p<0,001, t-test)

15. HRK is responsive to another TRAIL sensitizing agent Bortezomib

Bortezomib is another known TRAIL sensitizing agent (56). In order to examine the effect of Bortezomib on HRK expression , we treated GBM cells with 50 nM Bortezomib and expression changes were detected by qRT-PCR in A172, LN18 U87MG and U373 cells. Analysis showed that HRK is also responsive to Bortezomib treatment in A172, LN18 and U373 cells **(Figure 24A)**. However, Bortezomib itself does not have any significant effect on GBM cell viability **(Figure 24B).**

a



b

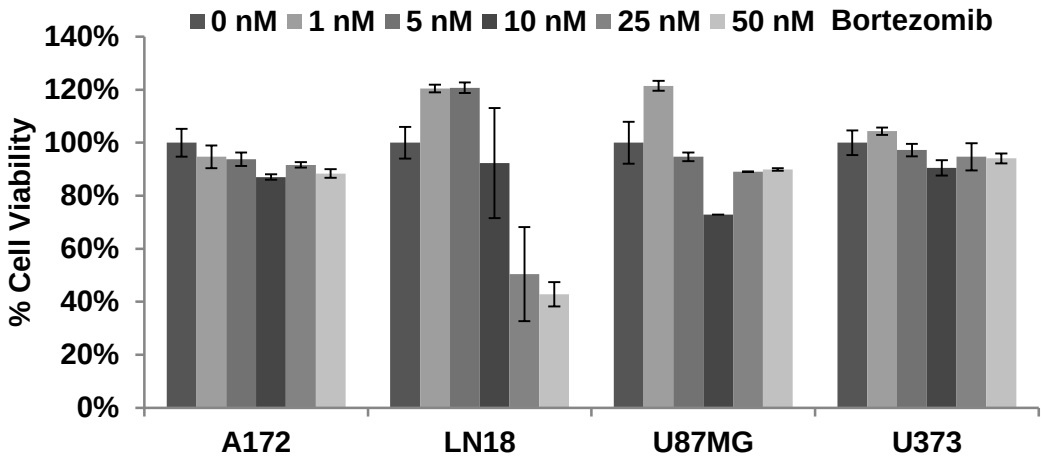
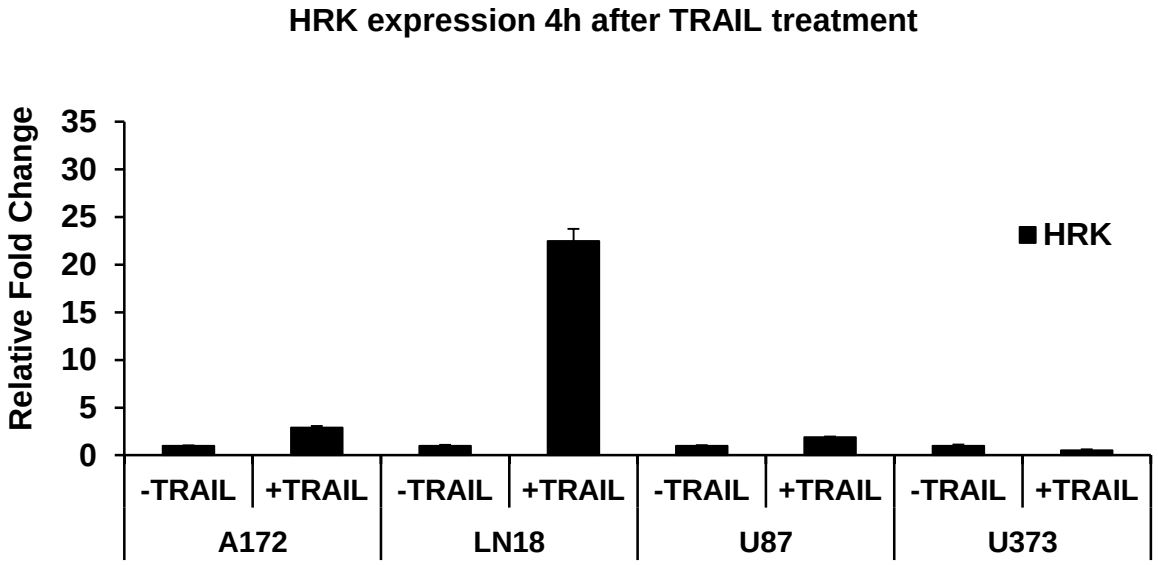


Figure 24: HRK is responsive to Bortezomib. (a) qRT-PCR analysis of HRK expression in four established GBM cell lines (A172, LN18, U87MG, U373) upon Bortezomib treatment (50 nM). HRK mRNA level relative to GAPDH mRNA level were measured and ΔΔCt method was used to detect relative expression. **(b)** Viability analysis of A172, LN18, U87MG and U373 cells upon differential doses Bortezomib treatment (0 nM, 1nM, 5nM, 10nM, 25 nM, 50 nM).

16. HRK is responsive to TRAIL treatment in LN18 and U87MG cells

In order to determine whether HRK expression changes upon TRAIL treatment or not, we collected mRNAs of GBM cell after 4 hour and 12 hour TRAIL treatment and check the HRK expression by qRT-PCR. Analysis showed that HRK expression is upregulated upon TRAIL treatment in LN18 and U87MG cells **(Figure 25).**



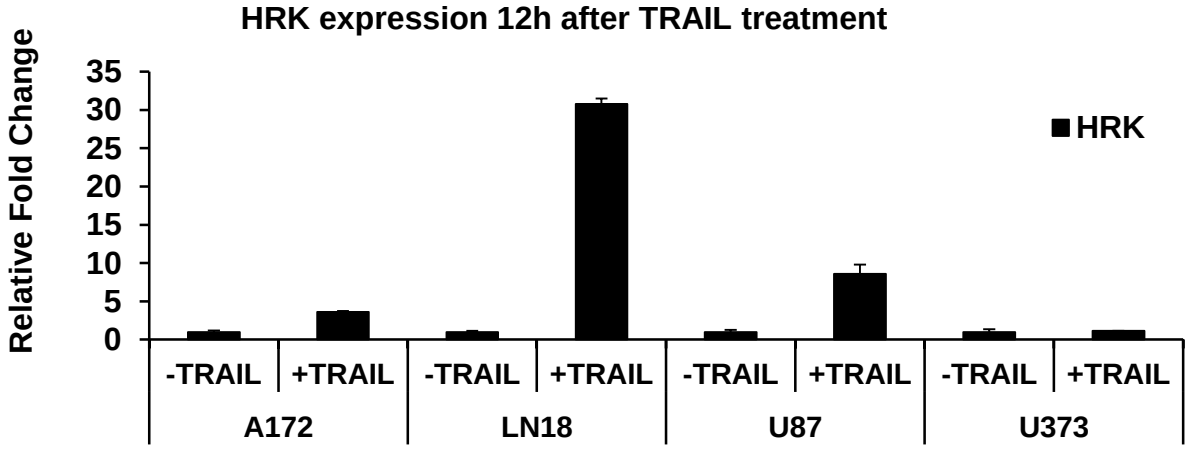


Figure 25: HRK expression is upregulated in LN18 and U87MG cells upon TRAIL treatment. qRT-PCR analysis of HRK expression in A172, LN18,U87MG and U373 cell upon 4 hour and 12 hour TRAIL (50 ng) treatment.

17. Cloning of HRK promoter

As evident in our previous results that showed HRK upregulation in GBM cells, understanding how HRK is regulated would be important. In addition, finding new drugs with an ability induce HRK expression would also be helpful to design promising new therapies in GBMs. Reporter systems are efficient systems to monitor dynamic gene expression changes. Since HRK induced cell death in GBM cells and is responsive to TRAIL sensitization agent, MS-275 and Bortezomib, it can enable us to discover novel TRAIL sensitizing agents by observing the dynamic changes of HRK expression upon drug treatment. To this end, we wished to generate a gene reporter system monitoring HRK expression through combining the putative promoter region of human *harakiri* gene and Firefly Luciferase (Fluc) as a reporter. We first PCR-amplified the 329 bp region of HRK promoter **(Figure 26)** from genomic DNA of human fibroblasts by using primers having KpnI and BglII restriction sites **(Table 5)** and

cloned it into pGL3 reporter vector, which contained the Fluc sequence. After series of cloning and sequencing, we identified one construct (pHRK-pGL3) that had the Fluc expression driven by pHRK. To validate the function of this construct, we first transfected pHRK-pGL3 into 293 T cells and measured Fluc activity. As transfection and positive controls, we used other vectors available in or laboratory, namely, CMV-GFP, CMV-GFP-Fluc, pDR5-Fluc-CMV-GFP in parallel **(Figure 27A).** Fluorescence images showed transfection efficiency **(Figure 27B).** In order to validate the Fluc activity of pHRK-pGL3 vector, we conducted luciferase activity assay **in** CMV-GFP, CMV-GFP-Fluc, pDR5-Fluc-CMV-GFP and pHRK-pGL3 transfected 293t cells. This result showed that pHRK-pGL3 vector gave a measurable Fluc signal **(Figure 27C).** The Fluc activity driven by pHRK was similar to that of another reporter vector (pDR5-Fluc), and lower than constitutively active Fluc vector, as expected.

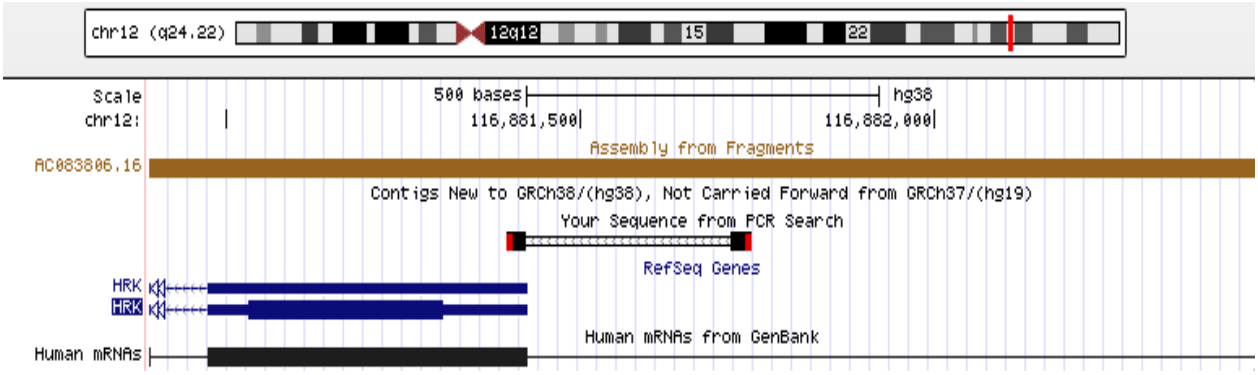
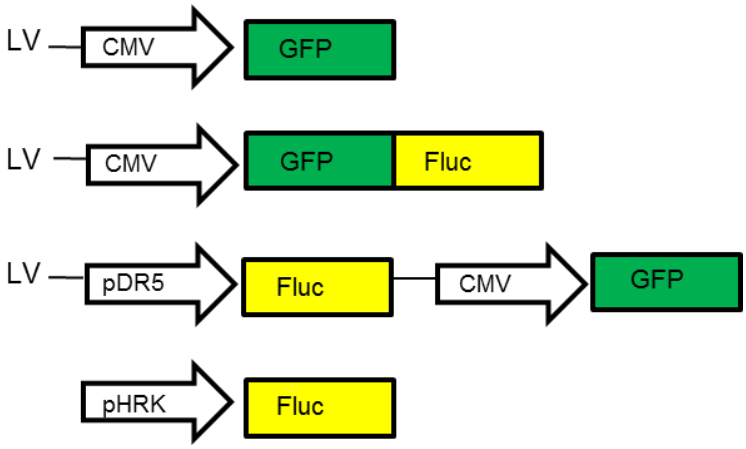
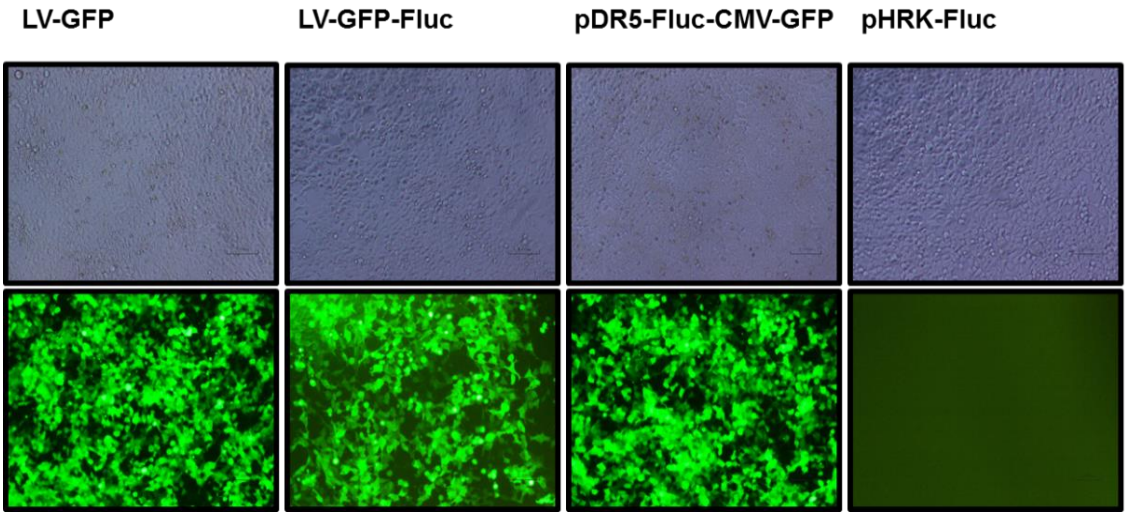


Figure 26: Amplified 329 bp region of putative HRK promoter. The *in silico* PCR result that is taken from UCSC Genome Browser with the primers used for cloning.

a


LV
CMV
GFP
LV
CMV
GFP
Fluc
LV
pDR5
Fluc
CMV
GFP
pHRK
Fluc

b


LV-GFP
LV-GFP-Fluc
pDR5-Fluc-CMV-GFP
pHRK-Fluc

C

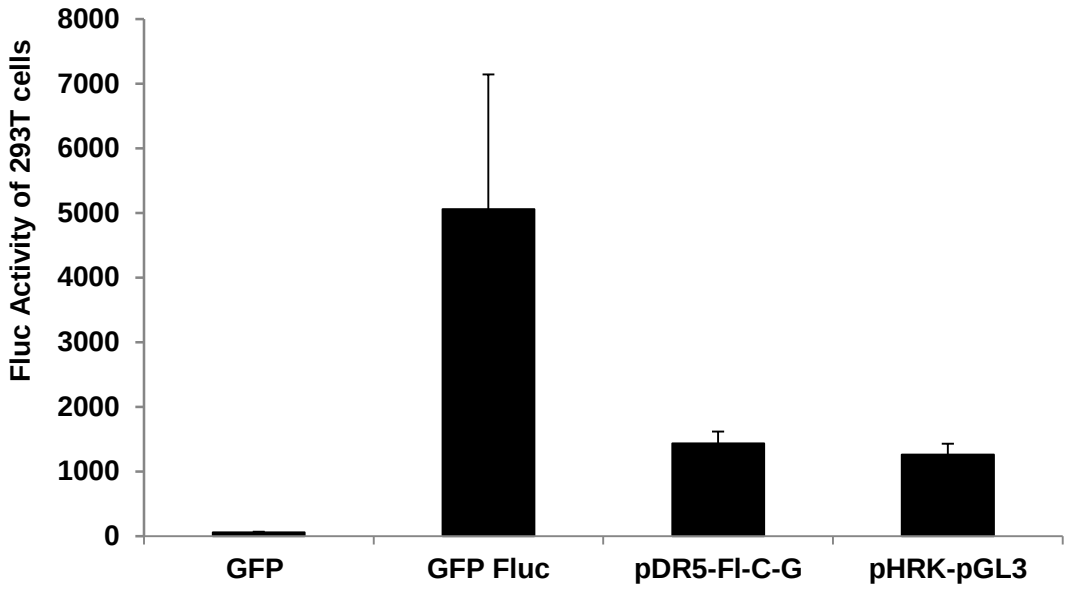


Figure 27: HRK promoter is cloned into pGL3 reporter vector. (a) Cloned pHRK-pGL3 and control vectors are shown **(b)** Fluorescence images of CMV-GFP, CMV-GFP-Fluc, pDR5-Fluc-CMV-GFP and pHRK-pGL3 transfected 293t cells**. (c)** Firefly luciferase activity assay of CMV-GFP, CMV-GFP-Fluc, pDR5-Fluc-CMV-GFP and pHRK-pGL3 transfected 293t cells.10 ug/ml luciferin was used as substrate.

Because GBM cells are not easily transfectable and need viral mediated gene transfer, we cloned pHRK into a lenti viral backbone (please see Materials and Methods). Then, we transfected pHRK-Fluc-CMV-GFP and control vectors, which are CMV-GFP, CMV-GFP-Fluc, pHRK-pGL3 into 293t cells **(Figure 28A)** and performed Fluc activity assay. Result showed that a new clone of HRK promoter in lenti-viral backbone had luciferase activity **(Figure 28B)**.

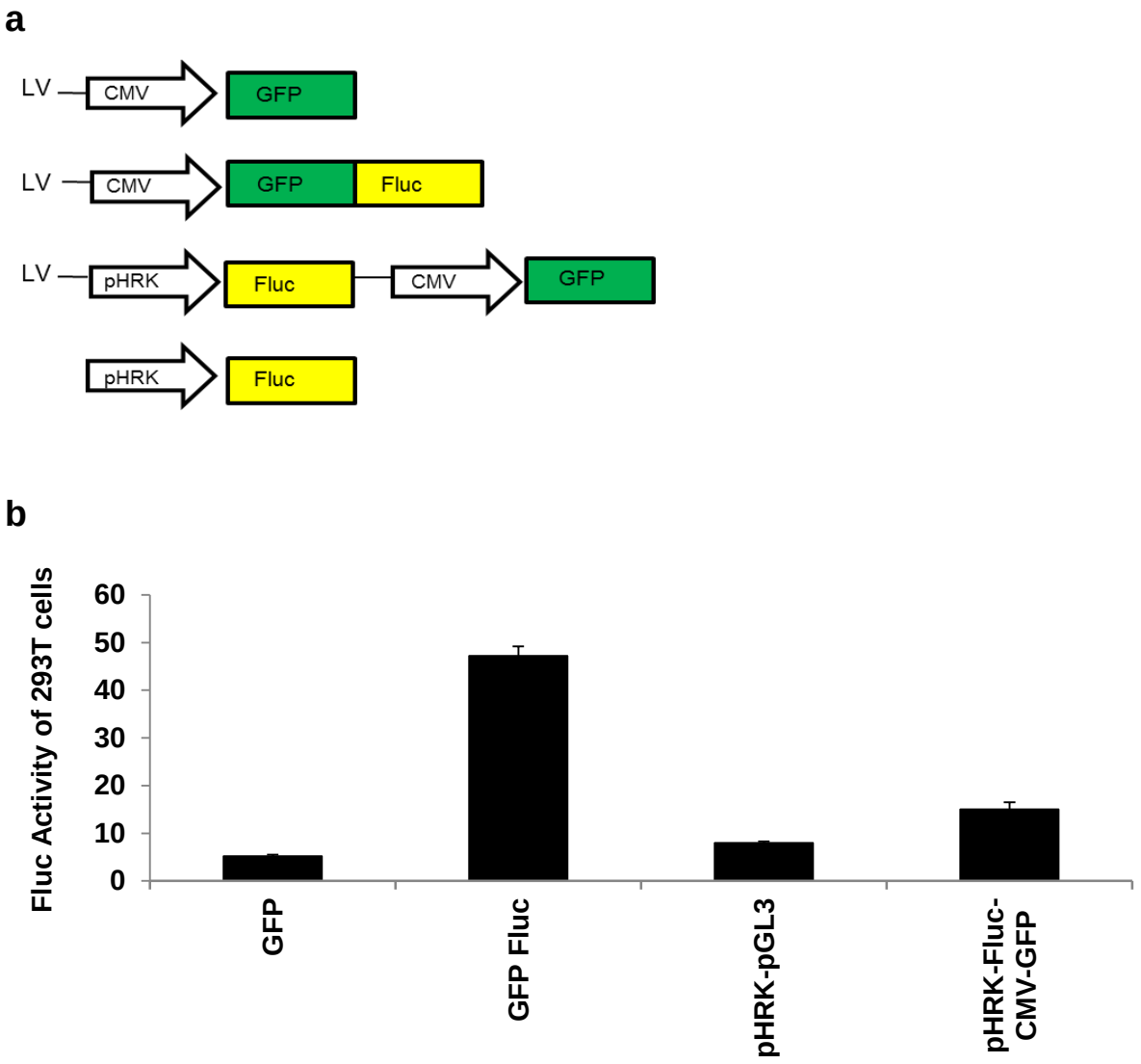


Figure 28: HRK promoter is cloned into lenti-viral vector. **(a)** Cloned pHRK-Fluc-CMV-GFP vector and control vectors are shown **(b)** Firefly luciferase activity assay of CMV-GFP, CMV-GFP-Fluc, pHRK-Fluc-CMV-GFP and pHRK-pGL3 transfected 293t cells.10 ug/ml luciferin was used as substrate. (These experiments were conducted with the assistance of Asli Azizoglu)

Furthermore, we amplified the 797 bp region of HRK promoter to get a longer sequence **(Figure 29)** from genomic DNA of human fibroblast by using primers having KpnI and BglII restriction sites **(Table 5)** and also cloned it into pGL3 reporter vector. Taken together, we have successfully generated reporter constructs to assess HRK regulation in GBM cells in the future.

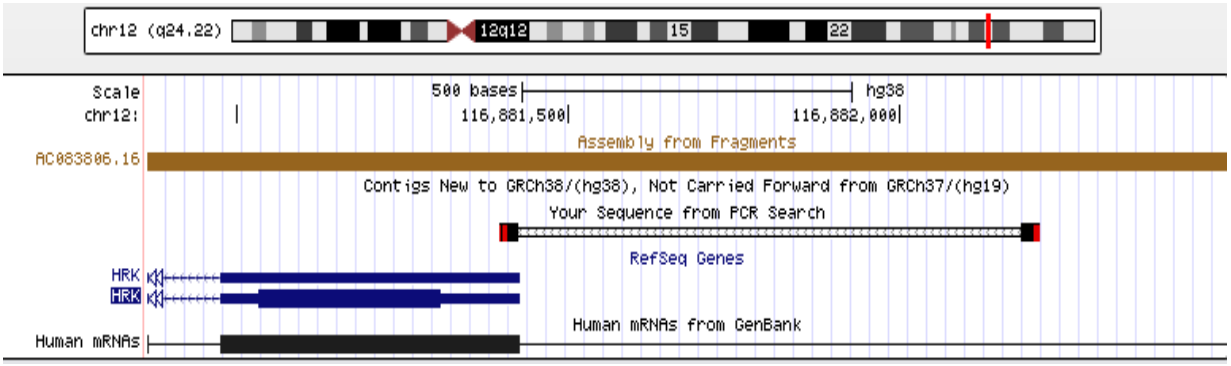
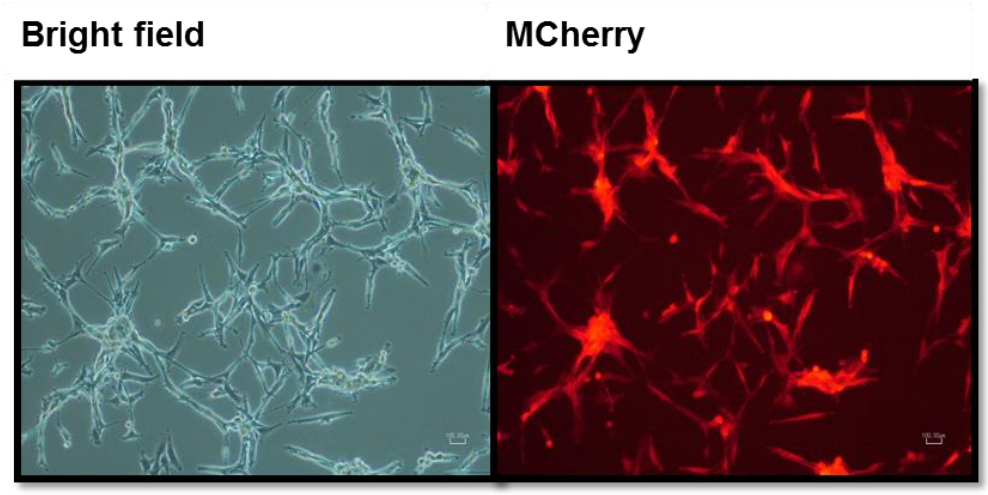


Figure 29: Amplified 797 bp region of putative HRK promoter. *In silico* PCR result that is taken from UCSC Genome Browser (52).

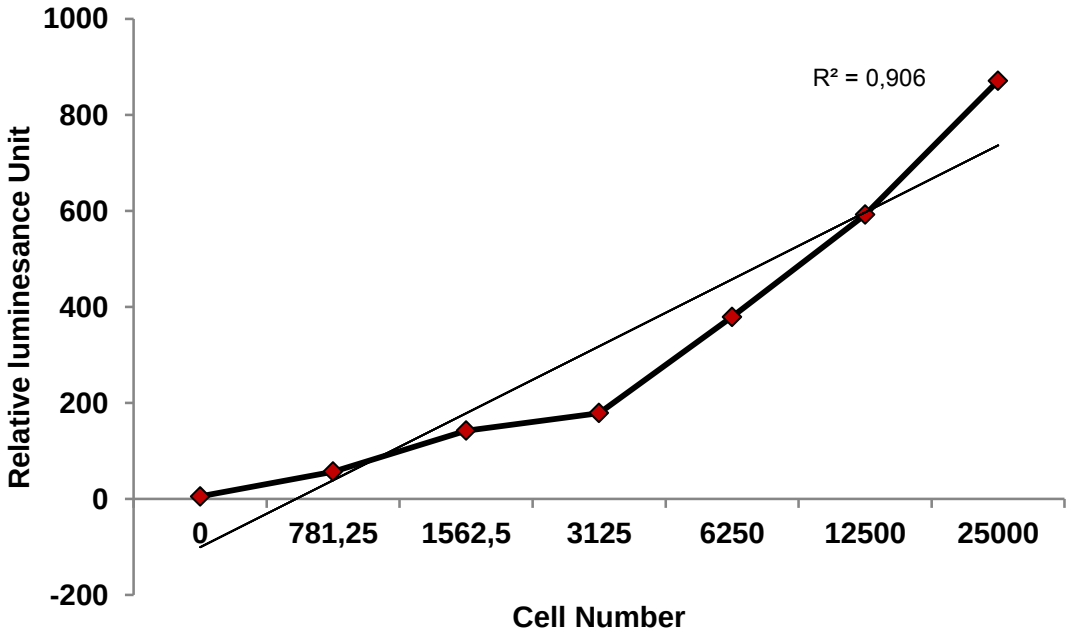
18. HRK overexpression decreases tumor growth *in vivo*

To examine the HRK overexpression effect on tumor growth *in vivo,* we first generated firefly luciferase (Fluc) and mCherry expressing U87MG GBM cells by transducing GBM cells with Fluc-mCherry expressing viral vectors (FMC). FMC expressing U87MG cells showed mCherry protein under fluorescence microscope (**Figure 30A)** and they have Fluc activity (**Figure 30B**). Subcutaneous injection of the cells in immunocompromised mice and long-term analysis of tumor-growth revealed that HRK expression also attenuates tumor growth *in vivo* (**Figure 30C**).

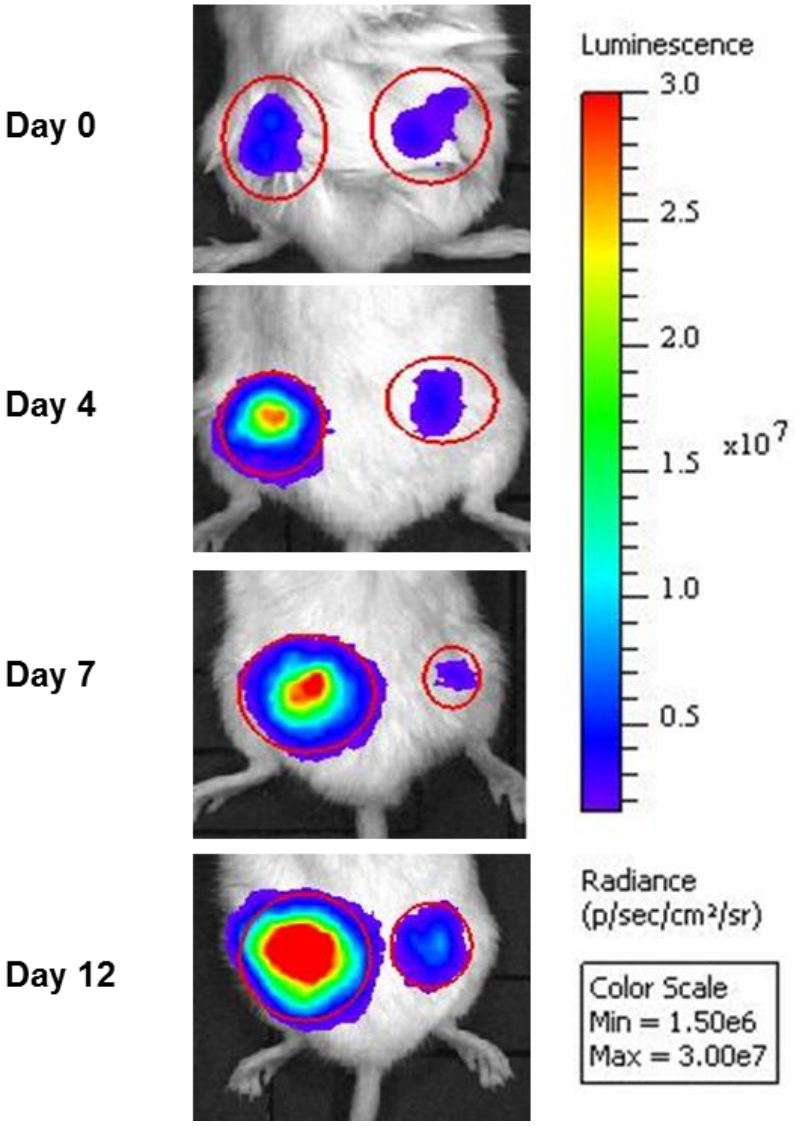
a


Bright field
MCherry

b


1000
800
600
400
200
0
-200
Relative luminesance Unit
R² = 0,906
0
781,25
1562,5
3125
6250
12500
25000
Cell Number

c


Day 0
Day 4
Day 7
Day 12
Luminescence
3.0
2.5
2.0
1.5
1.0
0.5
x10^7
Radiance
(p/sec/cm²/sr)
Color Scale
Min = 1.50e6
Max = 3.00e7

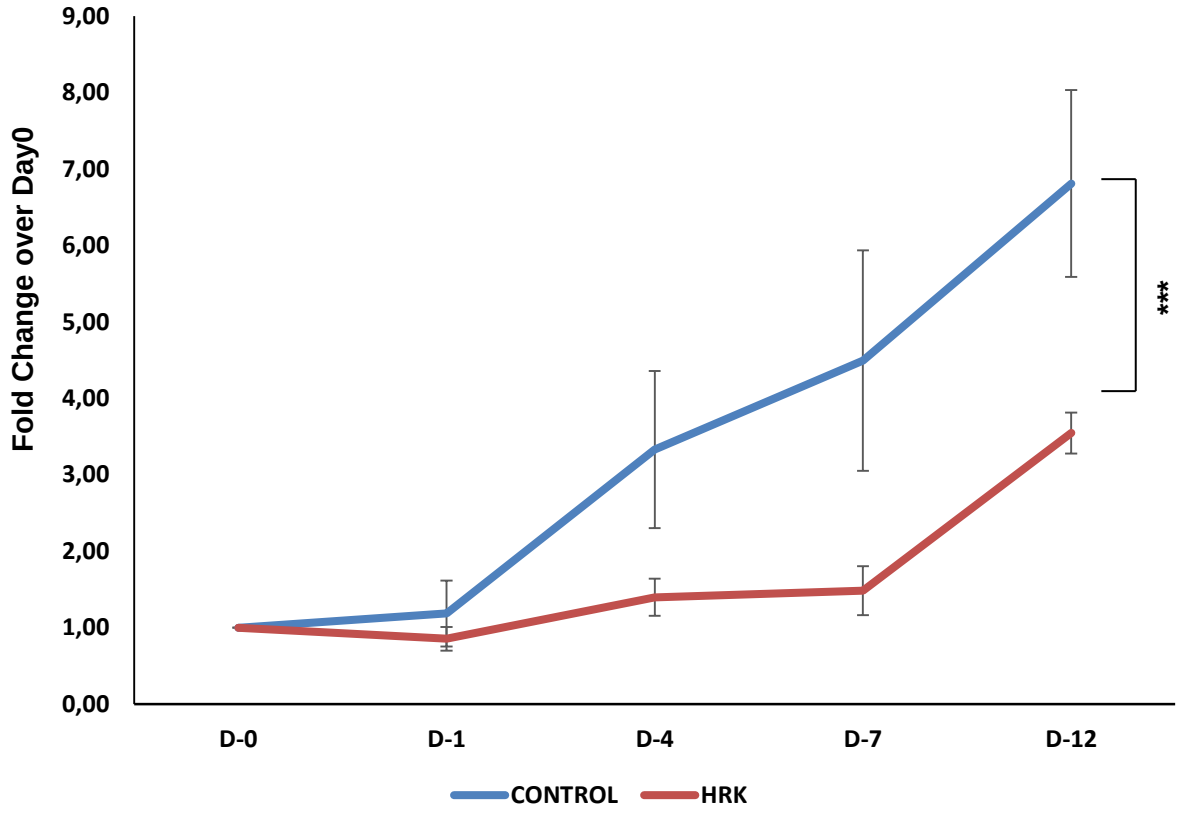


Figure 30: HRK overexpression decreases tumor growth *in vivo*. (a) Representation of Firefly luciferase (Fluc)-mCherry (FMC) expressing U87MG cells fluorescence image. **(b)** Luminescence signal of increasing number (0/25.000) of FMC expressing U87MG cells were measured by luminescence assay upon 100 ng/ml luciferin treatment. (**c**) 2x10e6 cells of Control-FMC and HRK-FMC were injected subcutaneously into SCID mice and assessed for tumor growth dynamics over time. HRK overexpressing tumors grew slower than controls. (These experiments were conducted with Ahmet Cingoz).

CHAPTER 4

Discussion

In this thesis, we examined the role of a pro-apoptotic BH3-only Bcl-2 family member protein, Harakiri (HRK) in GBM cell apoptosis **(Figure 31).** We have shown HRK expression induces cell death in GBM cells, and that its silencing partially prevents extrinsic apoptosis. In the literature, there have been a few studies showing the effects of HRK expression on tumors, and these focused on prostate, breast, ovarian cancers and melanoma (44),(41). However, ours is the first study to show the functional role of HRK in in Glioblastoma Multiforme. Therefore, our findings open up new questions for the potential use of pro-apoptotic therapies in GBMs in the future.

In this study, we also examined the relation between HRK and TRAIL. GBM shows tumor heterogeneity affecting tumor cells' morphologies, growth rates, drug responses and gene transcriptions (3),(12). We observed that GBM cells that are taken from a patient and grown in FBS containing and EF containing media have differential response to TRAIL treatment. Cells growing in EF containing media are TRAIL sensitive whereas cells growing in FBS containing media are TRAIL resistant. The reason behind this difference is not known and might be due to the selection of different subpopulations under different culture conditions. This is also evidence showing tumor heterogeneity in GBMs. Moreover, we observed differential TRAIL response also in established GBM cell lines. A172 cells are sensitive, LN18 and U87MG are mid-sensitive and U373 cells are resistant to TRAIL. Since the balance between anti-apoptotic and pro-apoptotic proteins determine the outcome of the death signal and the expression levels of Bcl-2 family member indicates how close cells are to apoptotic threshold (31), we observed the gene expression differences between TRAIL-

sensitive and resistant subpopulation of GBMs. Interestingly, we found that HRK was the only gene upregulated significantly in TRAIL sensitive subpopulation of GBM cells. This result suggested that HRK can be important regulator in TRAIL induced apoptosis.. We also found that endogenous HRK expression is different among GBM cell lines and correlated with their TRAIL response. HRK expression is very high in A172 cells (sensitive), whereas it is low in LN18, U87MG (mid-sensitive) and very low in U373 (resistant) cells. In the literature, HRK expression is reported to be regulated by loss of heterozygosity and promoter hypermethylation in some cancers including GBMs. (41). Thus, endogenous HRK expression differences among GBM cell lines might be due to the differences in methylation status of their HRK gene. Further studies are needed to test this hypothesis.

In order to examine the functional role of HRK, we overexpressed HRK cDNA in GBM cell lines having differential endogenous expression of HRK. In TRAIL-sensitive A172 GBM cell line, exogenous HRK expression could not affect cell viability of A172. Based on our results, because A172 cells have high endogenous HRK and Bcl-2/Bcl-xL expression **(Appendix III)** exogenous HRK expression was not enough to change the apoptotic threshold in A172 mitochondria. These findings prompt further examination of the expression of all pro- and anti-apoptotic Bcl-2 family members in our cell lines. Alternatively, A172 cells can be Type I cells which use extrinsic pathway for apoptosis and mitochondrial pathway might not be involved the apoptosis mechanism of A172. Further studies are needed to assess these suggestions. In TRAIL resistant U373 GBM cell line, we found that HRK overexpression induced cell death. This result suggests that U373 cells are close to mitochondrial apoptotic threshold and exogenous HRK expression is sufficient to trigger the activation of mitochondrial apoptosis pathway. In addition, this suggests that U373 cells can give better response to chemotherapeutic agents, which usually activate mitochondrial apoptosis pathway. Such agents include Bcl-2/Bcl-XL inhibitors (57), BH3 mimetics (58). However, according to TRAIL response of U373 cells, their exogenous pathway is blocked since TRAIL does not cause cell death in these cells. In parallel, we showed that ectopic HRK expression can trigger cell death also in TRAIL mid-sensitive LN18 and U87MG cells. Since LN18 and

U87MG cells are both responsive to HRK overexpression and TRAIL treatment, they might be categorized as Type II cells, which use both intrinsic and extrinsic arms of apoptosis pathway.

In this thesis, we have determined the TRAIL and HRK combination effect on established GBM cell lines. We have found that HRK cooperates with TRAIL for cell death induction only in TRAIL mid-sensitive LN18 and U87MG cells. In these cells, TRAIL and HRK cooperation induce much more cell death and activate both extrinsic pathway specific caspase-8 and intrinsic pathway specific caspase -9 more compared to TRAIL treatment and HRK overexpression itself. This result supported that HRK and TRAIL cooperation only in U87MG and LN18. Therefore, we have chosen LN18 and U87MG cell lines to check whether HRK expression is required for TRAIL induced apoptosis or not. And, we have showed that knockdown of HRK prevents the TRAIL induced apoptosis in U87MG cells. This result also gives evidence showing U87MG cell is might be a type II cell and needs the mitochondrial pathway activation for TRAIL induced apoptosis. Furthermore, the role of exogenous HRK expression seems to change the balance between pro-apoptotic and anti-apoptotic members in mitochondria and trigger to pass mitochondrial apoptotic threshold. Knockdown of HRK should be performed in also LN18 to draw similar conclusions.

HRK is a sensitizer BH3-only protein and neutralizes Bcl-2 and Bcl-xL to trigger cell death (29),(32). We validated the functional interaction between HRK and Bcl-2/Bcl-xL in GBM cells. When we overexpressed Bcl-2 and Bcl-xL, HRK induced cell death was blocked and we observed phenotype recovery as expected. These findings also support the notion that using apoptosis modulating therapies might offer promising new therapies in GBMs.

MS-275 which is a histone deacetylase inhibitor sensitizes the GBMs to TRAIL (46), but the sensitization mechanism is not well studied. We have shown that HRK is responsive to MS-275 treatment and the knockdown of HRK inhibited the TRAIL sensitizing effect of MS-275. These results suggested that HRK can be key factor for the sensitization mechanism of MS-275. In other words, MS-275 treatment increases the HRK expression, and increased

HRK expression changes the apoptotic balance in mitochondria and enables cell to pass the apoptotic threshold of mitochondrial apoptosis pathway upon TRAIL treatment. Thereby TRAIL activates both extrinsic and mitochondrial pathway and leads to induce apoptosis more.

Bortezomib, a proteasome inhibitor, is also one of the TRAIL sensitizers. We have demonstrated that HRK is also responsive to Bortezomib. Therefore, monitoring gene expression changes of HRK gene upon drug treatment can be great tool to discover novel TRAIL sensitizing agents. To this end, we generated HRK promoter reporter vector to monitor the dynamic changes of HRK expression upon drug treatment. Moreover, because HRK itself has killing activity, drugs increasing HRK expression by itself can be promising treatment options for GBM.

Understanding how HRK is regulated is also very important to dissect the pathways leading to death in GBMs. In the literature, there are few studies demonstrating HRK gene regulation. HRK was shown as a target of JNK-c-Jun pathway (59). Based on this knowledge, to inhibit HRK expression in GBM cells, we used JNK inhibitor (SP600125). However, we could not block the HRK expression contrary to what literature suggested **(Appendix II).**

In recent studies, BH3 only proteins are used to predict the effect of chemotherapy by measuring the early changes in death signaling in tumor cells treated with drugs. This technique is called dynamic BH3 profiling (60). In this technique, tumor cells were treated with different drugs in short time, and then cells were permeabilized and exposed to a specific BH3 only peptides to measure magnitude of MOMP. This measurement determines priming state of cells which shows how close a cell is to the apoptotic threshold. It also identifies cells' dependence on anti-apoptotic proteins. Thus, BH3 profiling provides therapeutic index of certain chemotherapeutics by comparing the MOMP measurements in normal and cancer cells (60). In the light of this information, because HRK can induce cell death in GBMs, it can be used to determine the priming state of GBM cells as well.

In the future, cell growth analysis by xCelligence detecting the effect of TRAIL and HRK combination in LN18 and U87MG cells will be repeated. Because of the late addition of TRAIL, we could not observe their combination effect. Also, for the *in vivo* experiments, tet-inducible system will be used to select the uninfected cells and control the experiment better. We plan to measure the MOMP with the HRK overexpression to gain more insight on HRK induced cell death. Furthermore, in order to detect whether the killing activity of HRK is specific to GBM cells, we will observe the effect of HRK on healthy cells such as fibroblasts and astrocytes. This is important to use HRK as a therapeutic agent for GBM patients. In addition, we will perform a drug screen to discover novel TRAIL sensitizing agents by monitoring the dynamic changes of HRK expression upon drug treatment for GBM treatment. For the clinical application, GBMs can be treated with HRK protein (61) for HRK cDNA can be delivered in nanoparticles(62) to induce cell death in GBMs.

Taken together, the findings of this thesis provided a novel mechanism of apoptosis regulation in GBM cells, that is by HRK and its regulation. Therefore, further understanding of the biology of HRK and finding new drugs based on HRK expression and function might open up new windows in designing effective GBM therapies.

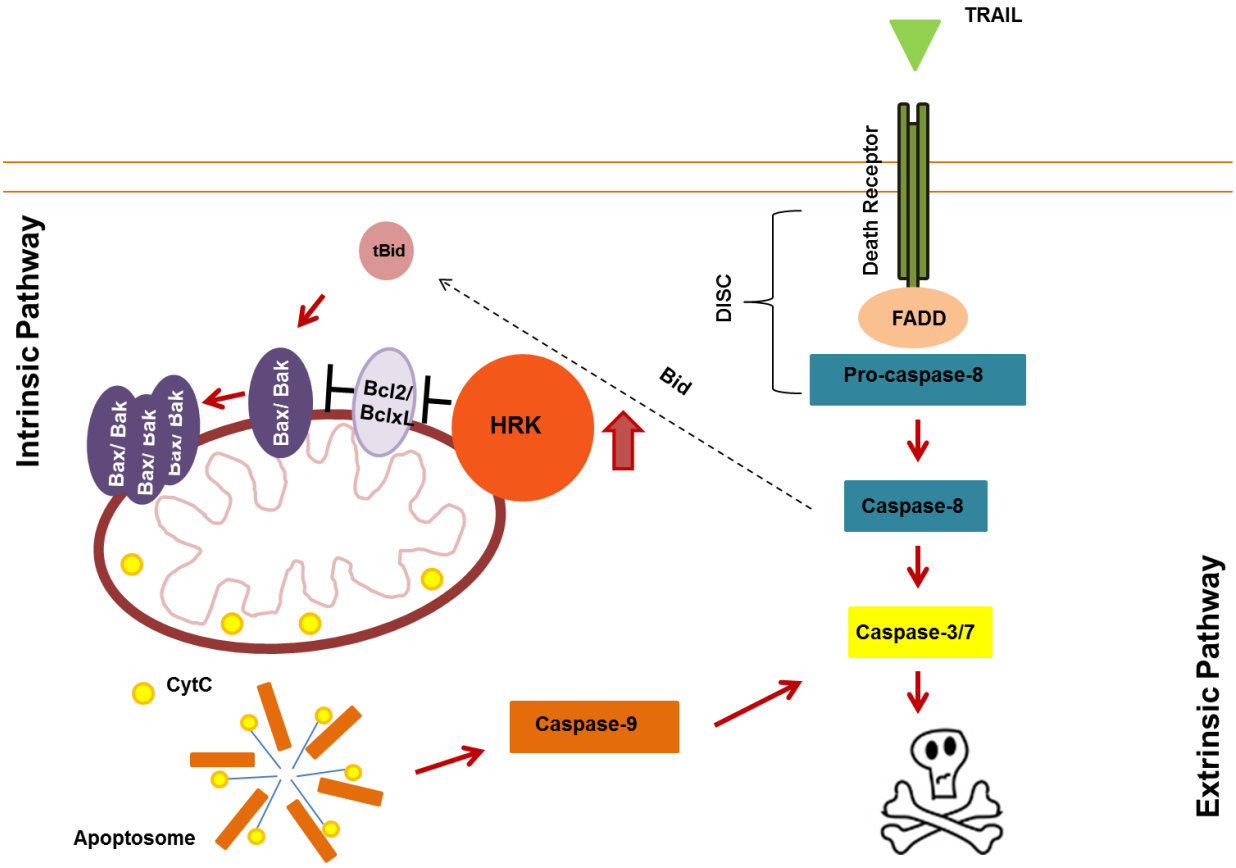


Figure 31: Model for HRK induced cell death. Ectopic HRK expression changes the binding network in mitochondria and induces the activation of mitochondrial apoptotic pathway and leads to cell death in GBMs. On the other hand, TRAIL activates extrinsic pathway and HRK and TRAIL cooperate for apoptosis induction in LN18 and U87MG cells. And, HRK silencing partially blocks the TRAIL-induced apoptosis in U87MG cells.

Appendix I

Complete loss of HRK gene by CRISPR-Cas9 system

To examine the effect of the HRK function and its relation to TRAIL in GBM cells with a secondary approach, we also employed CRISPR-Cas9 system. We designed two gRNA targeting first exon of HRK **(Figure 31), (Table 8).** Since Cas9 enzyme recognizes the gRNA complementary site and cut the DNA nearby the NGG site, gRNAs were designed based on target site sequences following by NGG PAM sequence.

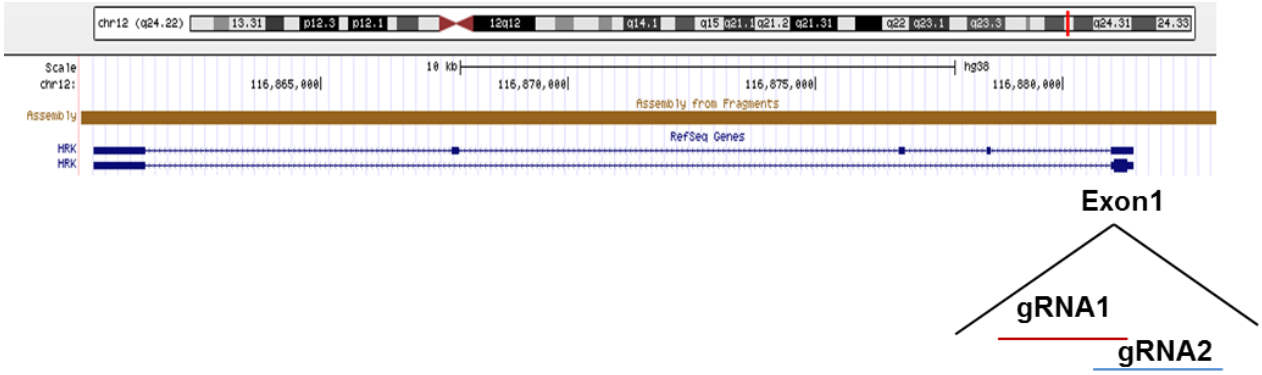


Figure 32: Genomic map of human HRK gene. gRNAs were designed based on the first exon of HRK gene located om chromosome 12.

HRK_G1_1	5’ CACCGCGTCGCCTAGCGCCTTGAGC 3’
HRK_G1_2	5’ AAACGCTCAAGGCGCTAGGCGACGC 3’
HRK_G3_1	5’ CACCG CTGCAGGCGCACACGGCCGG 3’

HRK_G3_2	5’ AAACCCGGCCGTGTGCGCCTGCAGC 3’
HRK_gPCR_For	5’ AACCTCGCGCTCCCTAGC 3’
HRK_gPCR_Rev	5’ CTTACACTTGGAAGGGAAGACTGG 3’
HRK_gSeq_For	5’ AATAGCGCCGTCAGCACAA 3’
HRK_gSeq_Rev	5’ CAAACTTGCCACTCTCCCGC 3’

Table 8: List of designed gRNAs and primers for genomic DNA PCR and sequencing

gRNAs’ both strands have BsmbI restriction sites which are compatible with lentiCRISPR backbone (PxPR_001) **(Figure 32).** Thereby, we cloned gRNAs into lentiCRISPR vector using crispr cloning protocol of Zhang Lab (in collaboration with Ibrahim Cagri Kurt, Koc University). Cloned vectors were sequenced and we identified one gRNA that was cloned properly. Then, we produced viruses of the gRNA vector. We transduced them to U87MG cells and selected them with puromycin (1 ug/mL) treatment.

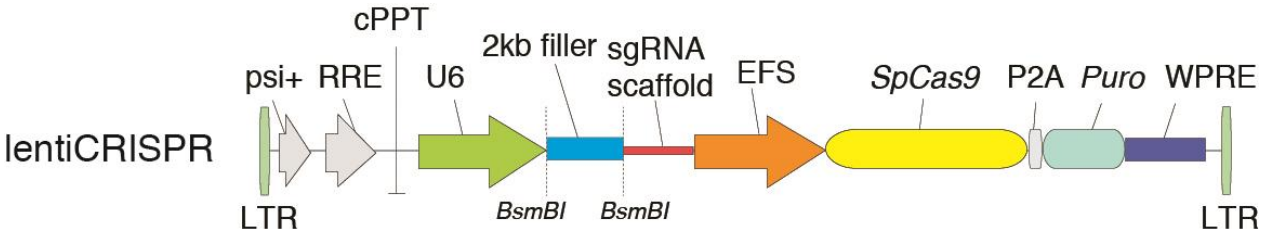


Figure 33: lentiCRISPR vector map (PxPR_001).

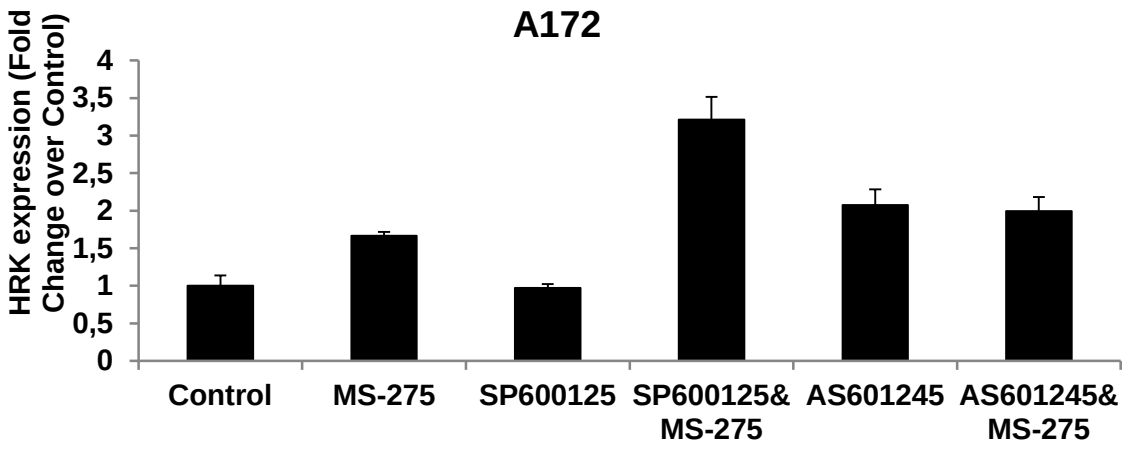
For the functional validation of gRNA, we first isolated the genomic DNA by utilizing MN NucleoSpin Tissue Kit. Then we amplified the target site sequencing for Surveyor assay to detect whether gRNA induced indel mutations or not. However, because of the genomic DNA PCR could not be optimized, CRISPR-induced knock-out in HRK could not be validated, and prompts further experiments

Appendix II

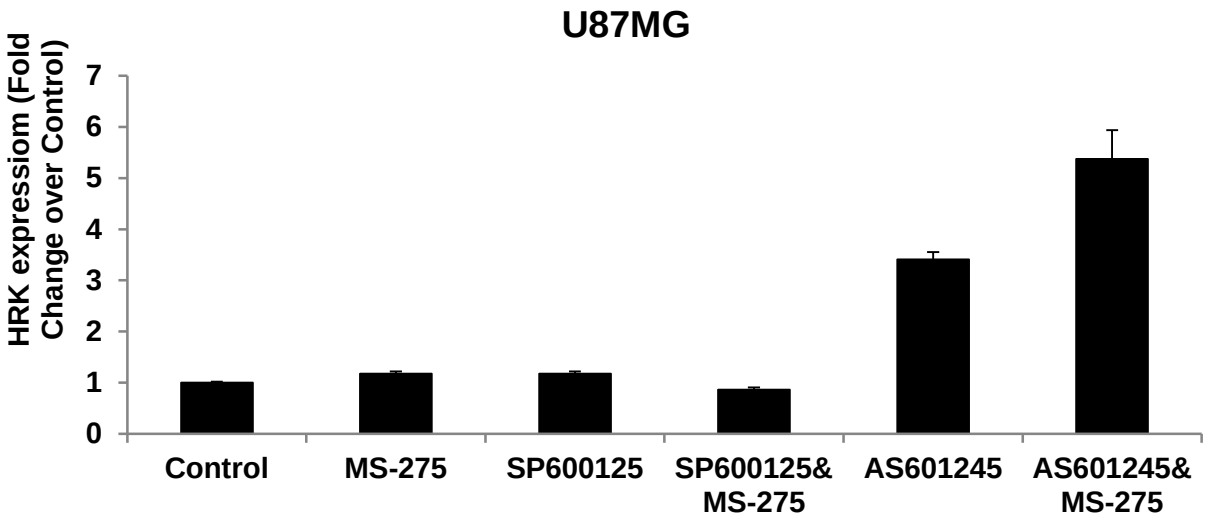
Roles of JNK-c-Jun pathway in HRK expression regulation

In the literature, HRK was found as a target of JNK-c-Jun pathway (38)(59)(44). Therefore, to test HRK regulation by JNK-c-Jun pathway, we treated GBM cells with JNK inhibitors SP600125 targeting JNK1, JNK2 and JNK3 and AS601245 targeting human JNK1, JNK2 and JNK3 (10 mg/ml) and examined the HRK expression changes upon inhibitor treatment and MS-275. We expected that JNK inhibitors reduced the HRK expression and this reduction was recovered by MS-275 treatment. However, RT-PCR analysis showed that SP600125 has not any effect on HRK expression in **(Figure 33A)** A172, **(Figure 33B)** U87MG and **(Figure 33C)** U373 cell. Moreover, AS601245 had toxic effect on GBM cells and led to cell death. Thereby it increased the HRK expression **(Figure 33A, 33B, 33C)**

a


A172
HRK expression (Fold Change over Control)
4
3,5
3
2,5
2
1,5
1
0,5
0
Control
MS-275
SP600125
SP600125& MS-275
AS601245
AS601245& MS-275

b


U87MG
HRK expressiom (Fold Change over Control)
7
6
5
4
3
2
1
0
Control
MS-275
SP600125
SP600125& MS-275
AS601245
AS601245& MS-275

c

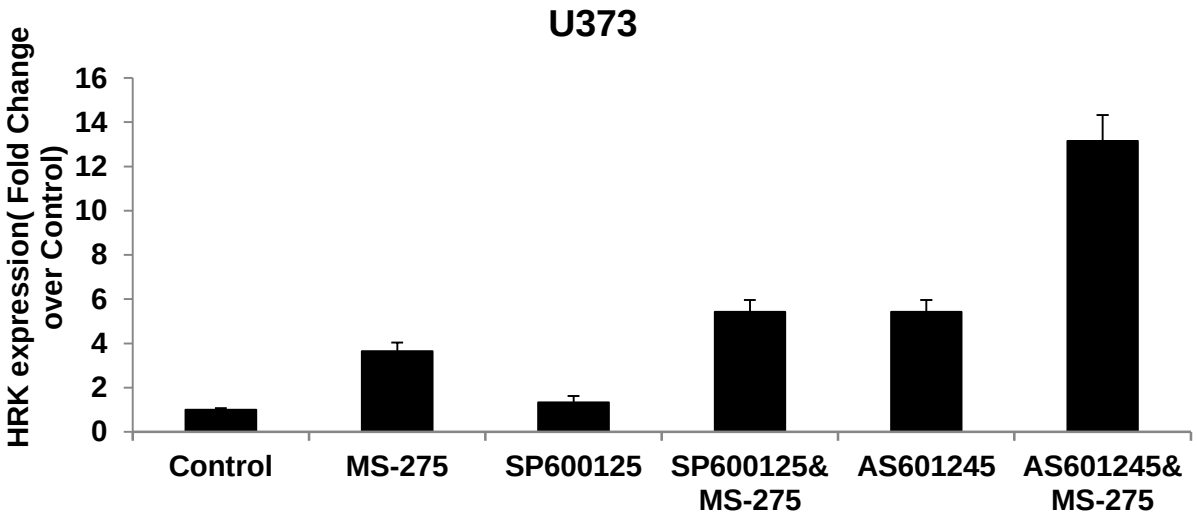


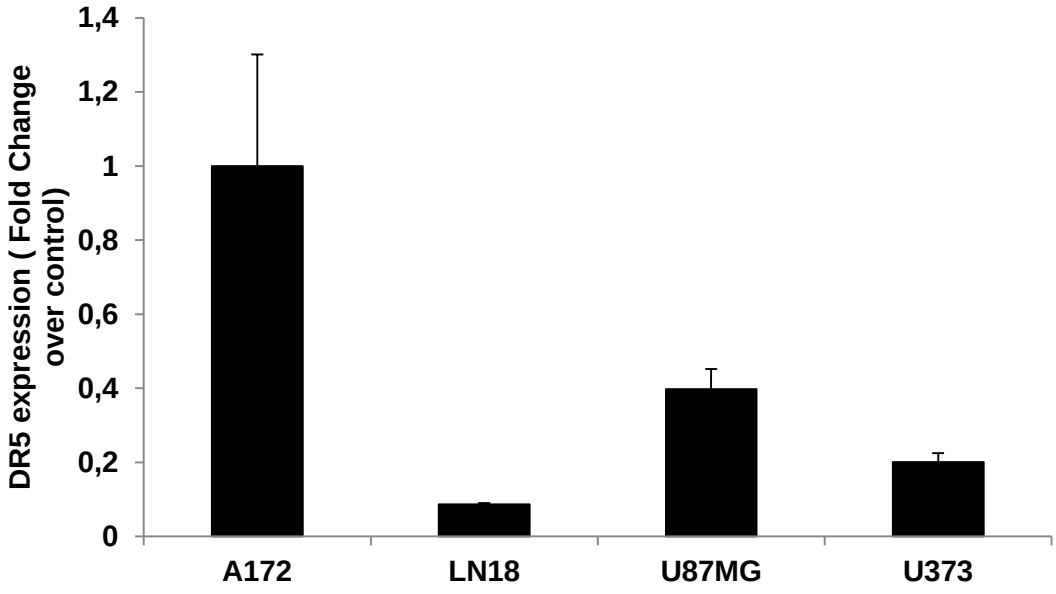
Figure 34: qRT-PCR analysis of HRK expression upon JNK inhibitors SP60012 and AS601245 in A172, U87MG and U373 GBM cell lines.

Appendix III

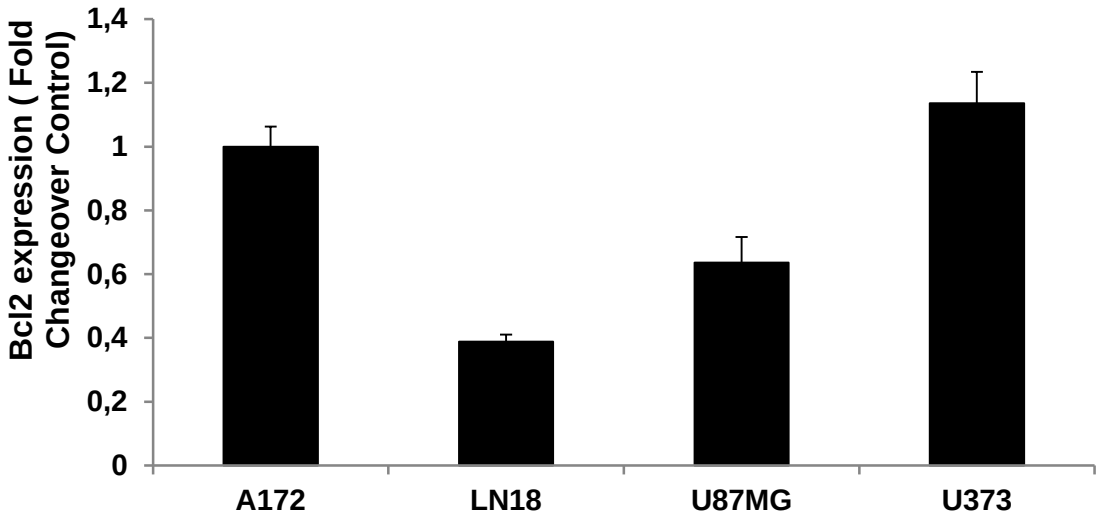
Gene expression profiles of GBM cells

Since four established GBM cell lines (A172, LN18, U87MG and U373) have differential response to HRK overexpression, we wanted to check the endogenous levels of other apoptotic members.

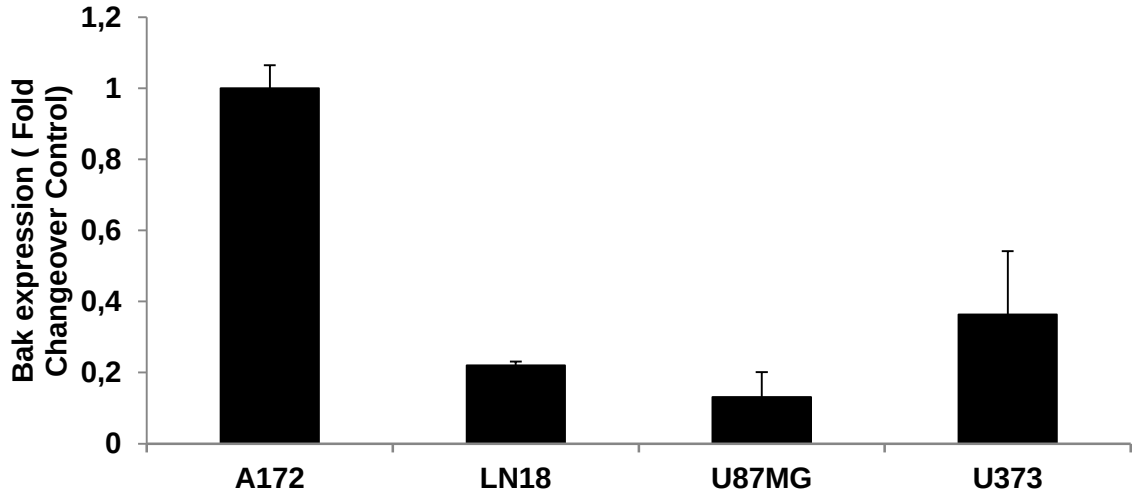
a



b


Bcl2 expression (Fold Changeover Control)
1,4
1,2
1
0,8
0,6
0,4
0,2
0
A172
LN18
U87MG
U373

c


Bak expression (Fold Changeover Control)
1,2
1
0,8
0,6
0,4
0,2
0
A172
LN18
U87MG
U373

d

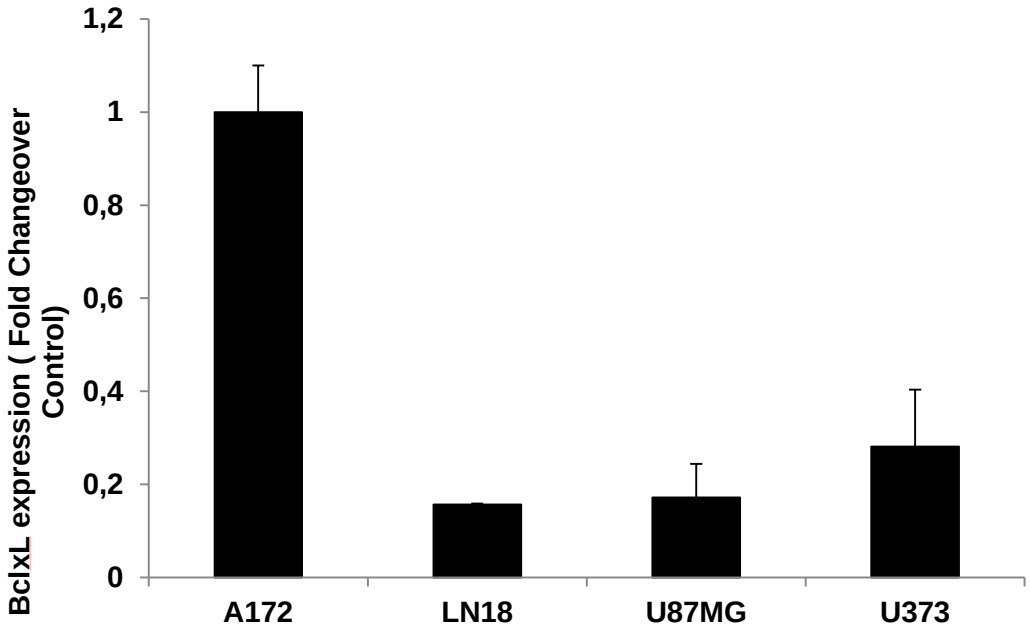


Figure 35: qRT-PCR analysis of DR5, Bcl2, BclxL and Bax in A172, U87MG, LN18 and U373 GBM cell line

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