JOSIP JURAJ STROSSMAYER UNIVERSITY OF OSIJEK
UNIVERSITY OF DUBROVNIK
RUĐER BOŠKOVIĆ INSTITUTE

UNIVERSITY POSTGRADUATE INTERDISCIPLINARY DOCTORAL STUDY OF
MOLECULAR BIOSCIENCES

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EFFECT OF dnMAML PEPTIDE ON GLIOBLASTOMA CELLS

PhD Thesis

Osijek, 2013.

TEMELJNA DOKUMENTACIJSKA KARTICA

Sveučilište Josipa Jurja Strossmayera u Osijeku **Doktorski rad**
Institut Ruđer Bošković
Sveučilište u Dubrovniku
Sveučilišni poslijediplomski interdisciplinarni studij Molekularne bioznanosti

Znanstveno područje: Interdisciplinarno područje znanosti
Znanstvena polja: Temeljne medicinske znanosti, Biologija

UTJECAJ dnMAML PEPTIDA NA RAST STANICA GLIOBLASTOMA

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Rad je izrađen: Zavod za biokemiju, University of Mississippi Medical Center, Jackson, Mississippi; **Mentor**: prof.dr.sc. Dražen Raucher, prof.dr.sc. Vesna Babić-Ivančić

Kratki sažetak disertacije (oko 400 znakova)
Glioblastoma multiforme (GBM) najzastupljeniji među tumorima mozga. Konvencionalna terapija daje slabe rezultate. Na staničnom modelu GBM pokazali smo da inhibicija Notch puta pomoću SynB1-ELP-dnMAML daje dobre rezultate. Sporiji rast zbog inhibicije samog Notch puta i njegovih utjecaja na ukupni stanični metabolizam predstavlja dobru osnovu za efikasno alternativno liječenje GBM.

Broj stranica: 93
Broj slika: 19
Broj tablica: 2
Broj literaturnih navoda: 127
Broj priloga: -
Jezik izvornika: engleski

Ključne riječi: glioblastom, dnMAML, Notch, ciljana terapija, proteinski inhibitor

Datum obrane: 22. travnja 2013.

Stručno povjerenstvo za obranu:

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6. **dr.sc. Mira Grdiša,** znanstvena savjetnica, redoviti profesor (zamjena člana)

Rad je pohranjen u:
Gradskoj i sveučilišnoj knjižnici Osijek, Europska avenija 24, Osijek; Sveučilištu Josipa Jurja Strossmayera u Osijeku, Trg sv. Trojstva 3, Osijek

BASIC DOCUMENTATION CARD

Josip Juraj Strossmayer University of Osijek **PhD thesis**
Ruđer Bošković Institute
University of Dubrovnik
University Postgraduate Interdisciplinary Doctoral Study of Molecular biosciences

Scientific Area: Interdisciplinary Area of Science
Scientific Fields: Basic medical science, Biology

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Thesis performed at Department of Biochemistry, University of Mississippi Medical Centre
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Short abstract
Glioblastoma multiforme (GBM) is the most aggressive form of cancer in humans. Effectiveness of therapy is limited. Aberrations of Notch signaling were found in many types of cancer including GBM. Inhibition of Notch with SynB1-ELP-dnMAML is effective in vitro and effects both canonical and non canonical targets. That is why it represents a sound foundation for alternative therapy of GBM.

Number of pages: 93
Number of figures: 19
Number of tables: 2
Number of references: 127
Original in: English

Key words: glioblastoma, Notch, dnMAML, targeted therapy, protein inhibitor

Date of the thesis defense: 22. April 2013.

Reviewers:

1. **prof.dr.sc. Ljubica Glavaš-Obrovac,** full professor
2. **prof.dr.sc. Dražen Raucher,** full professor
3. **prof.dr.sc. Vesna Babić-Ivančić,** associate professor
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Thesis deposited in:
City and University Library of Osijek, Europska avenija 24, Osijek; Josip Juraj Strossmayer University of Osijek, Trg sv. Trojstva 3, Osijek

This thesis was completed at the Department of Biochemistry, University of Mississippi Medical Center, Jackson, under co-supervision of dr.sc. Dražen Raucher and prof.dr.sc. Vesna Babić-Ivančić as part of University Postgraduate Interdisciplinary Doctoral Study of Molecular biosciences.

ACKNOWLEDGEMENTS

First and foremost my thanks go to my mentors prof.dr.sc. Vesna Babić-Ivančić and dr.sc. Dražen Raucher. I thank the former for pushing me out the door and am gratefull to the letter for welcoming me.

To prof.dr.sc. Antun Tucak, prof.emmeritus, my eternal gratitude for letting me choose my own path.

All this would not be possible without the help, advices and friendship of dr.sc. Shama Moktan, dr.sc. Leslie Walker, dr.sc. Gene Bidwell and especially dr.sc. Matea Mikecin. They say a person's worth is determined by her friends and I'm honored to count you as mine.

Last but not least to the rest of the Raucher lab: Leigh Whitney, Emily, Maria, Rebecca and Rowshan. Thank y'all for making my Mississippi experience that much enjoyable.

Special thanks to dr. sc. Lakshman Varanasi for all the help keeping me company in the lab during many long nights and to dr. sc. Jung Su Ryu for tying up loose ends

Thank you to my friends and colleagues at MEFOS for welcoming me back and making it seem I never left.

A triple dedication goes out to…

My safety net on all my attempts…	*…my family*
My rock and partner in crime…	*…my husband*
My sunshine and silver lining in every cloud …	*…my daughter*

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1. INTRODUCTION

Today cancer is one of the most investigated diseases in the world. Advances in modern medicine have brought us to a point where deadly diseases of the past can be dealt with in simple and efficient ways. Modern way of life and its comforts result in the longevity of the population and for that reason an increased appearance of autoimmune, neurodegenerative diseases as well as tumors. Although a reason for the appearance of cancer cannot be precisely pinpointed the above mentioned factors are confirmed to contribute to cancers growing incidence in the world population. Advances in therapy have been great but remain insufficient. Same is true for research efforts that seem to grow constantly but provide very few new therapies. Results presented in this work will involve one type of cancer - glioblastoma multiforme (GBM) with a high malignant potential and a high degree of morbidity and mortality and a new approach to treatment that holds great promise if taken through to clinical applications.

1.1. GLIOBLASTOMA (GBM)

When people talk about commonly known types of cancers talk rarely touches the subject of brain cancer. That maybe because the brain is as mysterious and full of inconsistencies in what we know about its workings as cancer itself. The other reason is purely statistical: in the vast span of cancer types brain tumors are not as frequent as some other types including breast cancer, leukemias, prostate cancer and alike.

The data from Central Brain Tumor Registry of the US (CBTRUS) and the International Agency for Research on Cancer predict that there will be around 70 000 new cases of brain tumor diagnosed in 2013. Approximately 25 000 of those will be malignant tumors. Incidence rates worldwide for primary malignant brain tumors using a sample population were set around 5 per 100 000 for developed countries. Rates for malignant tumors are higher in males than in females and also between developed and less developed countries. CBTRUS data shows that these numbers in the US are around 6.5 per 100 000 (CBTRUS 2012). Compared to breast cancer that will occur in 1 out of every 8 women or at the rate of between 90 and 120 per 100 000 women these numbers appear small and unfortunately the amount of research diminishes almost proportionally.

In that rare group glioblastomas (GBMs) are a small subgroup but one that makes all the difference if you ever have the misfortune to have to face it. GBMs account for about 70% of the newly diagnosed malignant brain tumors. According to the World Health Organization classification (Louis et al. 2007) glioblastomas fall into the astrocytoma group and are grade IV malignancy. What does that mean? Grade IV are malignant, mitotically active tumors associated with rapid disease progression, frequent recurrences and a fatal outcome. Although this is true for grade IV tumors of all origins, there are additional problems in glioblastoma therapy. Those issues come from the nature of the environment where they develop in - the brain. Natural protection of the brain in form of the blood-brain barrier (BBB) significantly decreases the number of available therapeutic options. Current standard treatment includes "the cancer triad": maximal surgical resection (if possible), radiation therapy with concomitant chemotherapy. Today, in the treatment of GBMs, temozolomide is the chemotherapeutic of choice. Unfortunately, this therapeutic approach doesn't seem to be effective since the median survival of glioblastoma patients who undergo therapy is between 12 and 15 months, just a few months more than patients who receive no treatment. There is no underlying cause for GBMs that can be identified in majority of cases and only about 5% of diagnosed patients have a family history of GBMs. Primary GBMs typically occur in older population while in younger population it is more often a case of secondary GBM developing from low grade astrocytoma over a longer time period (Porter et al. 2010). These two types differ in their molecular patterns but cannot be otherwise distinguished from each other and have similar response to therapy (Wen and Kesari 2008). In spite of all progress and changes in therapy recurrence rate for GBMs is extremely high with about 90% of the tumors recurring in the original site (Hochberg and Pruitt 1980).

All of the above mentioned makes glioblastoma and the people inflicted with this disease are prefect candidates for alternative therapeutic approaches. Fortunately, there is no want for specific possible targets while tumors of this type carry a wide number of altered pathways, receptors and genes. Bartek et al. in their 2012 paper explore some of these options and from the data collected concluded that an effective new GBM therapeutic agent must block several various pathways. Special attention should be given to blocking cell to cell interactions between tumor cells and their

endothelial neighbors that, at least in GBM, seem to be maintaining tumor stem cells and are responsible for high frequency of recurrence in GBM (Zhu et al. 2011).

Currently several alternative GBM therapies are in clinical trials with an even greater number in preclinical stage. In this thesis I will present the work that addresses one potential target for advanced drug design. By using peptide based approach to treat parts of the signaling network normally inaccessible to conventional pharmaceutics there is a possibility to provide higher specificity and efficiency. Targets that cannot be treated systemically because of overwhelming side effects can be reached this way. Although still at preclinical stage, the work presented in this thesis shows that Notch pathway inhibition in GBM is a valid target.

1.2. NOTCH PATHWAY

Notch pathway is a highly conserved in all multi cellular organisms. It was discovered for the first time almost a century ago in *Drosophilla melanogaster* mutants. Flies with a partial loss of function had particular wing morphology with characteristic notches on wing margins which gave rise to the name of the pathway. Initially there was not much interest in the newly discovered characteristic but it grew more and more interesting in the 1980s and continues to intrigue scientists today. Notch gene was sequenced and it turned out that its protein product is a transmembrane receptor. Further research discovered that it is a highly conserved receptor and its role in development and maintenance slowly started to emerge. Notch pathway is a mediator of short range cell-to-cell communications during development influencing the final outcome depending on cellular context. Mammals have four different Notch receptors (Notch 1-4) that have both redundant and unique functions (Kopan and Ilagan 2009).

1.2.1. CANONICAL NOTCH SIGNALING

Soon after the discovery of Notch receptor, based on the knowledge available at the time, search for ligands started. Today we know that human Notch receptors have five possible ligands (Delta 1,3 and 4; Jagged 1 and 2), being transmembrane proteins as well, and that they act in overlapping manner but also have their distinct roles (Krebs et

al. 2003; Cheng et al. 2007). The steps, from signal generation to the final effectors, of the Notch signaling pathway are known. Pathway modifications and regulation, however, are still being investigated. Notch pathway is generally a short distance signaling system that enables communication between adjacent cells. Signal generation starts when a ligand expressed on one cell interacts with the receptor on one of its neighboring cells. This interaction can be *trans* or *cis* meaning that it can result in an activating (*trans*) or inhibitory (*cis*) signal. Upon ligand binding, receptor activation proceeds with a sequence of proteolytic cleavages by a series of membrane embedded proteases, most importantly γ-secretase. This process yields an intracellular domain of the receptor (NICD, Notch intracellular domain) that translocates to the nucleus where it binds to a transcription activation complex and activates transcription of Notch target genes (Artavanis-Tsakonas et al. 1999; Kopan and Ilagan 2009). Main targets are genes from Hes and Hey families. Hes (Hairy enhancer of split) and Hey (Hairy enchancer of split with YRPW motif) are transcription factors belonging to the basic loop-helix-loop family. Their key role is regulation of embryonic development and differentiation (Fischer and Gessler 2007). Transcription activation complex is composed of several elements and regulates Notch gene transcription through cooperative binding. Main components of the complex are: CSL (CBF1, Suppressor of hairless and Lag1), transcription factor, NICD and Mastermind-like (MAML), a co-activator. When NICD gets into the nucleus it binds CSL and recruits MAML into the complex. Binding MAML and NICD switches CSL from a repressor to an activator of transcription. These three components make a scaffold on which a larger transcription assembly can be built and transcription can proceed (Nam et al. 2003; McElhinny et al. 2008). This type of signal translation is termed canonical and is involved in differentiation and tissue homeostasis. Schematic of activation is shown in Figure 1.

This multistep process is regulated by various mechanisms on each level so that the final effect can be very different and provide much more diversity than four receptors and five ligands can offer. Since the receptor itself is cleaved to produce signal, it can signal only once. Maintaining the level of signal is achieved by changes in endosomal trafficking and posttranslational modification of the resulting receptor segments, directing them to recycling or degradation.

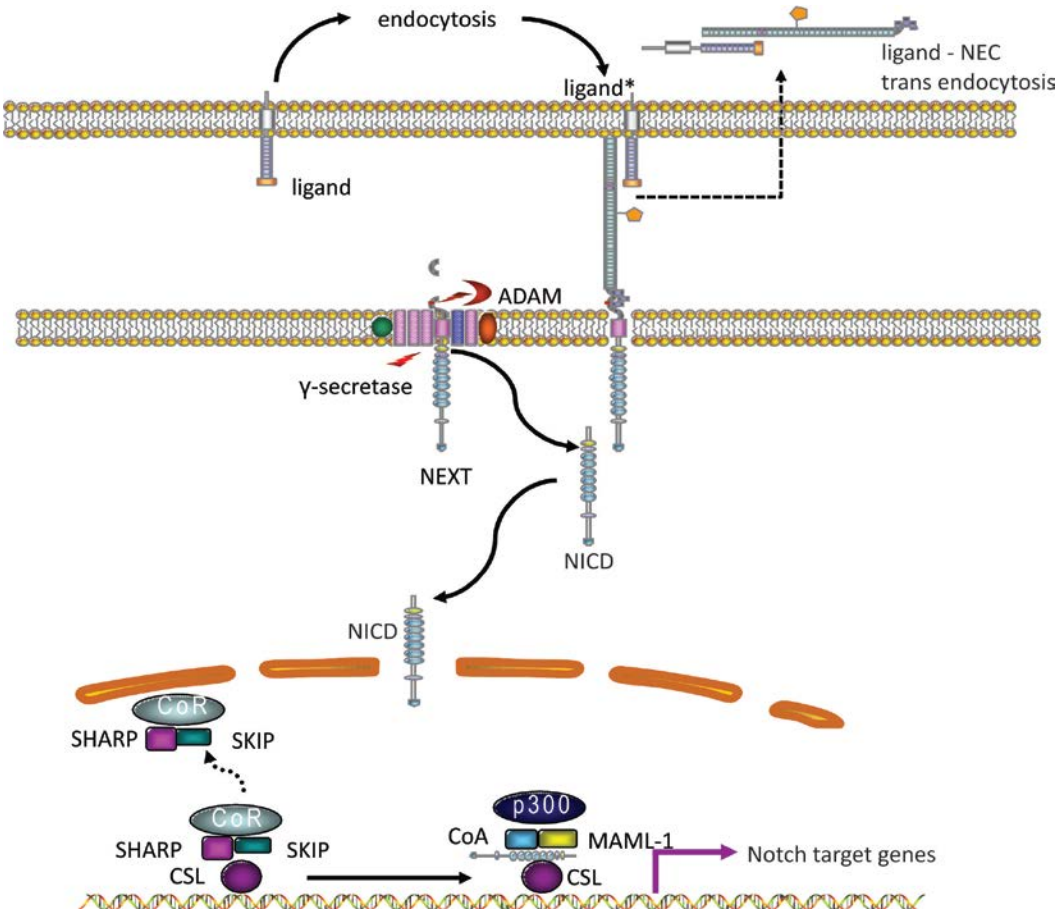

Figure 1. Notch canonical signaling. Simplified schematic of Notch canonical signaling showing stepwise process that leads to Notch genes transcription. Upon activation receptor is cleaved and NICD translocates into the nucleus. In the nucleus NICD displaces co-repressor complex bound to DNA and binds to transactivation complex counterparts. After this, Notch target genes are activated and NICD and the receptor are subsequently degraded.

These mechanisms are not completely clear but some of the main participants are known. Ubiquitin ligases like Numb regulate degradation of the receptor, changing its half life and availability for biding. Receptor glycosylation by Fringe glycosyltransferases (Lunatic, Manic and Radical in mammals) governs receptor-ligand specificity and the strength of their binding (Miele 2006). Since Notch ligands are also soluble and do not have to be integrated in the membrane, this is a way to avoid activation of the pathway. The key activation step, cleavage by γ-secretase, is controlled by four enzyme complexes that show different specific activities. Their role in overall signaling control is still not completely elucidated but there are hypothesis of location and membrane composition effects on cleavage and the resulting signal strength and duration. This

regulation is enabled by cleaving NICD in slightly different position within the same region yielding NICD with various N-terminus amino acids. These ends impact the half life of NICD and control the level of activation. Final gene activation takes place in the nucleus after NICD forms a complex with CSL and MAML cofactor and depends on the number and combination of enhancers and their respective affinity for NICD/MAML/CSL complex (Kopan and Ilagan 2009).

The more is known about Notch and the way it can be regulated and controlled the more the image of the pathway is replaced with an image of a network with complex internal control mechanisms (Fiúza and Arias 2007).

1.2.2. NON-CANONICAL NOTCH SIGNALING

The canonical signaling was the first to be discovered and studied, but more complex experiments discovered certain discrepancies. Levels of receptor and ligands present and the expression of the known Notch target genes from the Hes family contradicted canonical activation mechanism. Upon closer look it was discovered that Notch genes can be activated in alternative ways. Further research showed that NICD can interact with other co-activators besides CSL and can independently activate both canonical Notch targets as well as other tissue-specific genes. In these cases NICD can act mainly in the cytoplasm and does not need to be cleaved. Similar additional interactions were found for MAML proteins leading to the conclusion that both activators and repressor of Notch are shared with other pathways. Today, after extensive research, much is known about non-canonical signaling. There seems to be two basic types. Type I signaling (Figure 2. a) requires the activation of the receptor and release of the intracellular domain, but activates genes independently of CSL, main target of NICD in canonical signaling. This way NICD can interact with other signaling pathways and activate target genes through alternative means. Main target for this type of signal transduction are other development pathways like Wnt and Hedgehog. Type II signaling is completely independent and does not need the receptor to be cleaved. Examples of type II signaling are high levels of Hes family genes without the corresponding high levels of Notch activity. It covers effects that MAML and NICD have on components of other signaling cascades (p53, Akt, mTOR), along with some additional activators of

NICD shown in Figure 2. b (Sanalkumar et al. 2010; Kopan and Ilagan 2009; Andersen et al. 2012; Zhao et al. 2010).

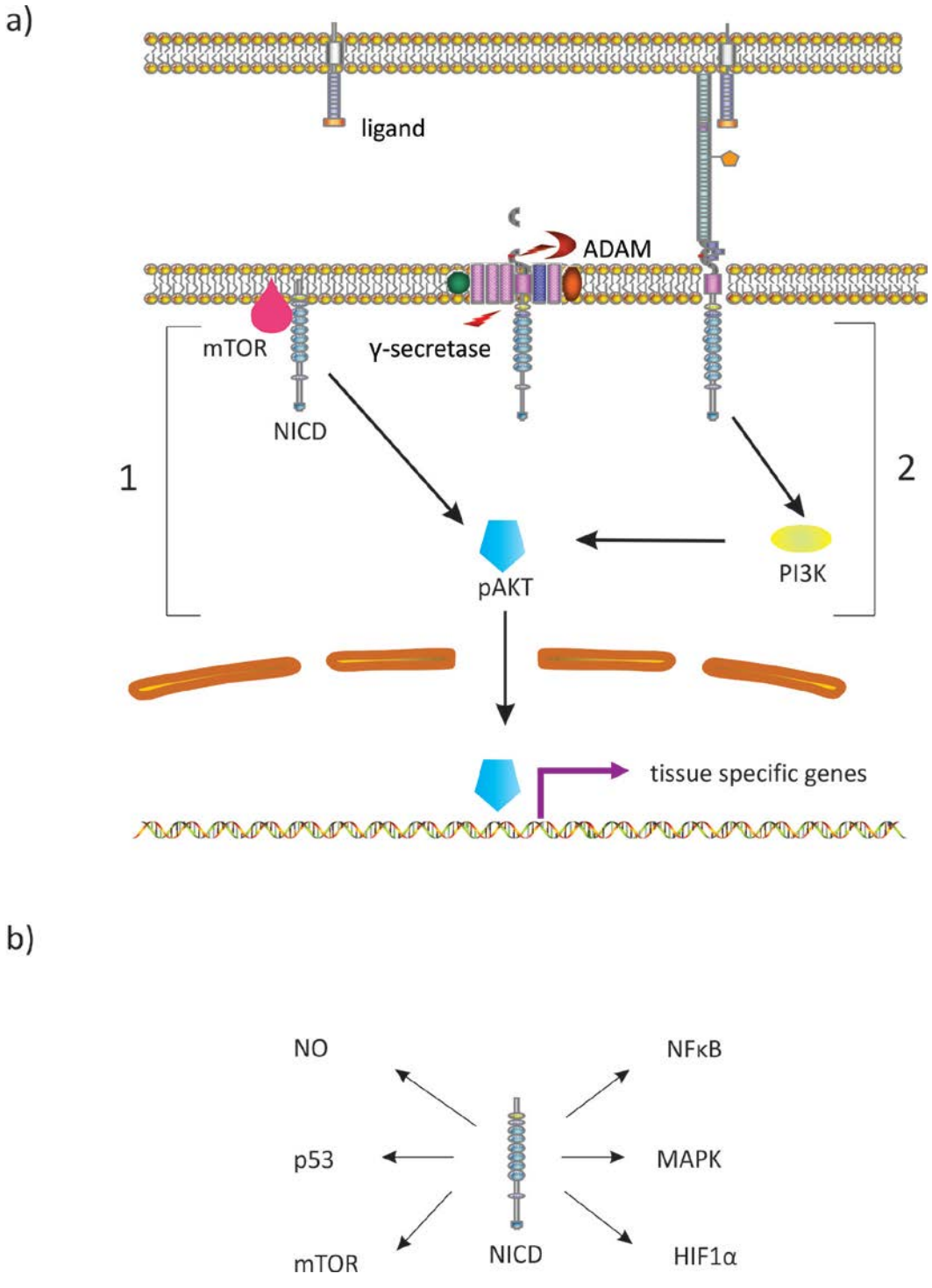

Figure 2. Non canonical Notch signaling.
a) Mode of activating genes outside the Notch pathway and tissue specific factors without binding CSL (1) or without the need to cleave the receptor (2)
b) NICD activates pathway crosstalk and this is an illustration of some of the factors that interact with or are regulated through NICD

1.2.3. NOTCH IN CANCER

Cancer presents a group of cells that have undergone a number of changes in their cellular processes and are growing uncontrollably or at best with little consideration for the safety mechanisms that govern normal cell proliferation. Notch signaling network is crucial in important points during development, cell differentiation, cellular homeostasis and renewal of organs in adult life, thus having an enormous impact on tumorigenesis.

This hypothesis was first confirmed when patients with T-ALL were screened and activating mutations of Notch were found in about 60% of all cases (Roy et al. 2007; Lobry et al. 2011). These findings made Notch mutations a main oncogenic lesion in T-ALL. With the link between Notch and T-ALL well established this cancer is the starting point for all Notch related research.

After this discovery, Notch signaling aberrations were implicated in a number of other solid tumors including breast cancer, melanoma, non-small lung carcinoma (NSCLC) and colorectal cancer (Ranganathan et al. 2011). All of the above showed increased activation of Notch related genes, but a mutation similar to the one found in T-ALL was never identified in more than a few random cases. Genetic sequencing, done in these carcinomas, as well as some others, showed that Notch ligands are rarely mutated and that Notch1 receptor gene is the most mutation-prone among them, but only on rare occasions, mutations affect a functional domain. In GBMs similar infrequent mutations were found outside the functional domains but still indicating that Notch1 is the main oncogene. Additionally, more than 80% of primary GBMs over-express activated cleaved form of NICD (Egloff and Grandis 2012; Kanamori et al. 2007), pointing to the activation of Notch in ligand-mediated manner. Excessive expression of ligands such as Jagged was linked to more progressive forms of breast cancer and poor outcome (Reedijk et al. 2005; Dickson et al. 2007). Different types of cancer show different activation mechanisms often linked to non-canonical Notch activation by hypoxia or through cross-talk with related pathways like Wnt and AKT/mTOR (Roy et al. 2007; Qiang et al. 2012). In glioblastoma one of the factors that activates Notch outside of the both pathways and represents another group of activators is nitric oxide (NO) that can readily diffuse between cells (Charles et al. 2010).

However, as one would expect from diverse effects that Notch has in non-tumor tissues, its oncogenic role is everything but straightforward. In certain cellular environments and types of cancer activation of Notch can lead to exactly opposite results and act as a very powerful tumor suppressor. In hepatocellular carcinoma and in basal cell skin cancers the activation of Notch leads to cell cycle arrest and apoptosis (Viatour et al. 2011; Rangarajan et al. 2001). Its tumor inhibitory role in this case is thought to result from the interaction of Notch with Sonic Hedgehog pathway. Loss of function mutations affecting Notch receptors are identified in more than 20% of head and neck squamous cell carcinoma patients, indicating its important tumor suppressor activity (Agrawal et al. 2011).

This highlights the dual role of this single pathway and puts a huge question on how to target Notch. Although the interest in γ-secretase inhibitors (GSI) has been growing, their systemic use needs to be put on hold, till more information on potential systemic consequences of Notch inhibition is gathered (Lobry et al. 2011). Alternative approaches can compensate for the shortcomings of GSIs and should overcome the resistance towards GSIs that develops in some cases. Synergistic action on Notch, Wnt and hypoxic pathways, as well as targeting signals coming from the surrounding epithelial tissue is one of the possibilities (Lino et al. 2010).

1.2.4. NOTCH AND GROWTH REGULATION

With the development of long-living multicellular organisms it was imperative to find a way to allow cell proliferation when needed and at the same time suppressing excessive cell division. With the molecular basis of cancer being discovered daily, a promise of more refined and effective therapies is made. In spite of continuous proof that cancer is a highly heterogeneous and diverse disease there are common changes that can be found in every cancer investigated. In almost all cases deregulated cell proliferation and suppressed cell death are found to be critical events in cancer development. With its high hierarchical position in the development, changes in Notch signaling influence both cell proliferation and cell death.

1.2.4.1. NOTCH AND CELL CYCLE

Cell cycle is creation of cell clones to allow growth or replace dying cells. Standard eukaryotic cell cycle is divided into four distinct phases that do not overlap. G_1 and G_2 phases are gaps that insure accumulation of protein with main events occurring in the S and M phases. It is a highly organized and tightly regulated process (Maddika et al. 2007). Cell cycle is mainly regulated through cyclin dependent kinases (CDKs) protein kinases that activated at specific points during the cell cycle push the cell through the phases. G_1 and G_2 are characteristic for somatic cells and are not necessary for proper functioning of the cell cycle machinery as can be seen in embryonic cells that have rapidly alternating S and M phases without gaps. This gives an insight on how cell cycle can proceed in cancer cells without these restriction points (Sherr 2000). Notch influences the cell cycle through the control of CDKs and related proteins expression rather than through direct interaction (Ronchini and Capobianco 2001). In T-cell acute lymphoblastic leukemia/lymphoma (T-ALL) cells Notch inhibition is associated with reduced CDKs and cyclin D1 expression along with increased p21 levels. In affected cells this is accomplished by inhibition of AKT pathway and cells show increase of the number of cells in G_0/G_1 phases(Guo et al. 2009). This was confirmed in small cell lung cancer (SCLC) where it was additionally shown that Notch induction of cell cycle arrest is independent of the levels of Hes-1, thus establishing the importance of non-canonical Notch signaling (Sriuranpong et al. 2001). Duality of Notch is evident in this, as well as other aspects of cellular control. In the fore-mentioned SCLC model, Notch activation induces cell cycle arrest, whereas in tissues like myocardium, activation of Notch leads to re-entry of quiescent cardiomyocytes into active division. CSL does this through cyclin D1 and not by binding to NICD (Campa et al. 2008). In GBMs Notch has a similar effect as in the myocardium. Activating Notch leads to increased cell division through both increased Hey-1 and AKT pathways (Hulleman et al. 2009).

There are plenty of additional ways to sustain cell cycle progression, if Notch is blocked. The option to go into G_0 and re-enter cell cycle when conditions change always exists. Insuring permanent block of uncontrolled growth can only be done by killing the cells in question.

1.2.4.2. NOTCH AND APOPTOSIS

Apoptosis, also called programmed cell death, is the mechanism by which excess cells are removed. This evolutionary conserved process was first described on the model of *Caenorhabditis elegans* and it has been the most investigated cell death process. Most of the mechanistic facts about apoptosis can be investigated on lower organisms and translated into higher with high level of reliability (Tamm et al. 2001).

There are two principal ways of activating apoptosis. First, the death receptor mediated apoptosis, which is a result of signals outside the cells and is therefore called extrinsic. Second, internally apoptosis can be initiated by the release of cytochrome c from the mitochondria activating the intrinsic pathway. These two pathways converge on the executioner caspases. Activation of both branches is strictly regulated by a series of inhibitors that regulate receptor binding as well as activation of caspases and permeability of the mitochondrial membrane.

In cancer apoptosis is evaded in several ways, mainly by over expressing anti apoptotic components, and at the same time by lowering the expression of pro-apoptotic molecules. Modulating p53 signals and survival signals by AKT are also important steps to avoiding proper execution of apoptosis (Igney and Krammer 2002; Evan and Vousden 2001).

Due to the importance that Notch has during development, it has often been identified as the master switch, deciding about the cells' fate. For example, in non-transformed cellular environment Notch activation and concomitant apoptosis is the part of selection of cell clones during differentiation as shown in the development of mature T cells. Notch activation has anti-apoptotic effect in T cells and can regulate negative selection through death by neglect (regulating the level of cytokines necessary for growth). In transformed cells Notch aberrations are found in many lineages. In most cases, over expression of ligand or receptor results in permanent activation of Notch signaling. If such cells are treated with any type of Notch inhibitor, apoptosis is initiated (Miele and Osborne 1999). Exact mechanism of apoptosis induction through Notch may vary due to numerous interactions with important regulators such as p53 and AKT (Zhao et al. 2007; Guo et al. 2009).

Overall, evidence of Notch involvement in cell cycle and apoptosis regulation are present in both normal and transformed cells. It can be concluded that the final result

of altering Notch activity depends largely on the intermediates and the levels of signal transmitted.

1.2.5. MAML AND DNMAML

Mastermind-like (MAML) family of proteins has 3 members in mammalian organisms and has gotten its name because of the similarities between them and the *Drosophilla* Mastermind protein both in structure and in function. MAML proteins function as transcriptional co-activators in Notch signaling and in several other pathways. MAML is essential for the assembly of transcription activation complex of Notch target genes. MAML proteins are structurally simple (Figure 3.) and can be the base for binding more complex proteins and getting them in close contact necessary for proper function (Nam et al. 2003). Gene sequence discovered that N-terminal domain of all 3 MAML proteins is highly conserved and its function involves interaction with NICD and other members of the transcription activation complex. C-terminal region contains transcription activation domain and the sequences differ much more in that part between different MAML proteins. All three proteins are capable with interacting with each of the four NICD but with different affinities determined by slight variations in their N-terminal domains (Wu et al. 2002).

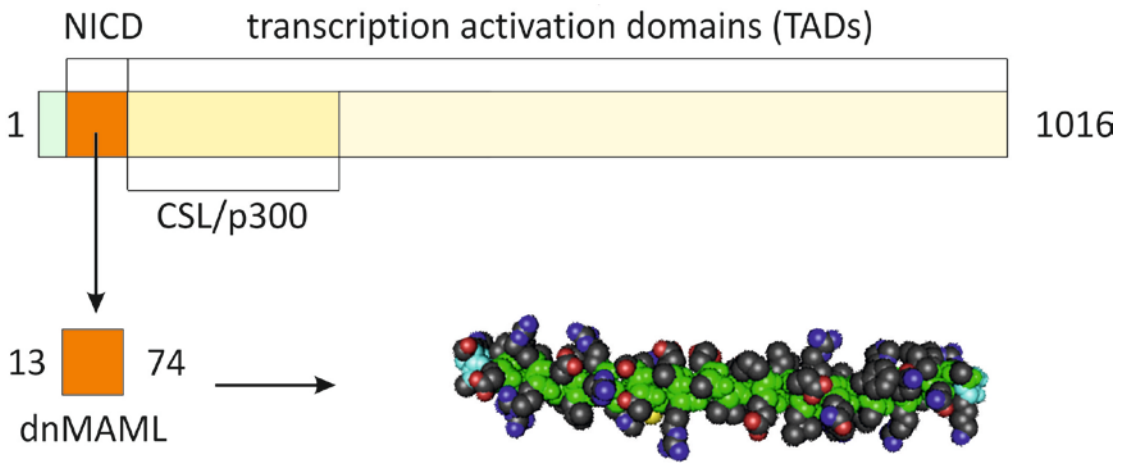

Figure 3. Schematic structure of MAML1 and dnMAML Schematic of MAML1 shows all domains and their function. dnMAML originates from NICD binding domain of MAML1 and its structure is shown in the space fill model

MAML proteins have Notch-independent functions as well. These functions resemble non-canonical signaling roles of NICD. MAML proteins enable cross-talk between several pathways such as Wnt, catenin and p53 (Saint Just Ribeiro and Wallberg 2009).

MAML mutants can be divided in two general groups, one being unable to activate transcription and the other unable to bind its respective cofactors and NICD. All these mutants have a dominant negative effect on Notch signaling (Wu et al. 2002).

dnMAML1 mutant is a truncated version of MAML1 protein, consisting of 62 amino acids (13-74) from the N-terminal basic domain of MAML1 (Figure 3). It lacks the transactivation domain and cannot activate transcription. It interferes with the endogenous function of MAML proteins and inhibits transcriptional activation from all four Notch receptors. The N-terminal portion that makes up dnMAML1 is completely functional and is presumed to be able to mimic MAML1 in Notch-independent functions. That is true for p53 where binding is accomplished between N-terminal part of MAML and the DNA binding domain of p53. The family of MAML co-activators make excellent candidates for targeting since they modulate a wide number of signaling pathways (McElhinny et al. 2008).

1.3. DRUG TARGETING, ELP AND HYPERTHERMIA

The phrase “magic bullet” was coined over a century ago, describing drugs that would have high specificity and act only on their intended target to completely eradicate cancer. The man who used it first was Paul Erlich. His work has made possible many advances in cancer treatment at the time, so he is also considered as the founder of chemotherapy (Strebhardt and Ullrich 2008). The search for the illusive single target drug has recorded only failures. The rapidly proliferating cancer cells have many aberrant signaling pathways, many redundant ways to evade blocks and continue on their way. At the same time, all these alterations offer themselves as valid targets for another strategy of treatment for cancer cells at a molecular level.

Drug targeting at the molecular level was made possible by the discovery of DNA structures in the early 1950s, followed by the discovery of oncogenes and tumor suppressor genes in the 70s and 80s. Today field of molecular therapeutics has several

well known successes, for example 5-flurouracil as an analogue of DNA base that inhibits DNA replication, the humanized antibody trastuzumab that targets ERBB2 receptor, and the ATP analogue imatinib that inhibits the BCR-ABL fusion protein for the treatment of chronic myeloid leukemia (Strebhardt and Ullrich 2008). The expanding knowledge of molecular interactions leads to continuous discovery of novel ways to target specific functions in the cells. However, specificity of the drug itself is not enough to evade side-effects. Clinical applications of all above mentioned therapies have shown some limitations mainly due to off-target toxicity. For example, the drug imatinib is also associated with low frequency of congestive heart failures, and is ineffective against the mutated form of ABL that is frequently found in CML patients (Strebhardt and Ullrich 2008). The problem is that no matter how specific molecular therapeutics are, until we deliver them with the same level of selectiveness, they will have the same undesired side-effects on normal cells, albeit in a somewhat lower degree. To be able to fully use the potential of molecular targeting we need an effective delivery system in order to reduce toxicity to normal cells.

Due to the fast growth of tumors and their great need for nutrients, they have certain characteristics that can be successfully used for targeting. For example, macromolecules (greater than 40 kDa in size) preferentially accumulate in solid tumors due to the hyper permeability of the tumor vasculature and the poor lymphatic drainage system. This phenomenon of an abnormal tumor vasculature leads to an effective retention of macromolecules and is known as the enhanced permeability and retention (EPR) effect (Matsumura and Maeda 1986). Thus, the EPR effect leads to passive targeting of macromolecules to the tumor region. Macromolecular delivery systems can and have been used in a variety of forms. They can be based on liposomes, nanoparticles, or synthetic and natural polymers (Kopecek 2003; Haider et al. 2004).

Poly(ethylene)-glycol (PEG) based liposomes of Doxorubicin (Doxil) are used in the treatment of ovarian cancer (Rakowski et al. 2011). Similarly, albumin-based formulation of paclitaxel (Abraxane) is used for the treatment of advance breast cancer (Guarneri et al. 2012). Several natural and synthetic water-soluble polymers, such as poly(ethylene glycol), dextrans and N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers, are in various phases of human clinical trials (Hu and Jing 2009; Khare et al. 2009; Maeda 2010).

A particular interest within the macromolecular delivery systems has been focused on stimulus-responsive polymers. These polymers change their characteristics in specific circumstances or after a specific stimulus, such as heat, light or magnetic force was applied and thus can be further actively targeted to the desired area. This change is a non linear event that takes place in a narrow window. These stimulus-responsive polymers are preferred over direct binding strategies for delivery that depend on the receptor binding to deliver the load (Schmaljohann 2006). Receptors and their levels of expression vary within the tumor so delivery cannot be uniform and can give rise to resistant subpopulation of cells within the tumor. Stimulus-responsive polymers have another great advantage when compared to receptor mediated direct binding. They are widely applicable. Since the stimulus is in most cases applied externally (heat or light, for instance) there is no need for additional modification after the drugs are incorporated or bound. The same carrier-drug complex can be used on various tumors regardless of their individual differences which makes them very easily applicable in clinical settings (De Las Heras Alarcon et al. 2005).

1.3.1. ELASTIN LIKE POLYPEPTIDES

Derived from the hydrophobic domain of tropoelastin, elastin-like polypeptide (ELP) is composed of pentapeptide repeats of VPGXG, where X is any amino acid except proline. ELPs undergo a phase transition at a specified temperature known as the inverse transition temperature (T_t). ELPs are soluble in aqueous solution below their T_t and aggregate form above their T_t (Urry 1988; Urry 1992; Li et al. 2001). The T_t of ELP is inversely related to the polarity of the ELP molecule. It is also dependent on chain length of the pentapeptide repeat. ELP is genetically engineered therefore; its T_t can be adjusted to any desired temperature by varying the chain length of the pentapeptide repeat, and the composition and mole fraction of X, which influences the hydrophobicity of the molecule (Urry et al. 1991; Meyer & Chilkoti 2004). Changing the composition of the pentapeptide influences only T_t but it does not interfere with structure so various composition ELP can be used for the same purpose under different temperatures (Arkin and Bilsel 2010). For the thermal targeting of ELP for therapeutic purposes, a T_t slightly higher than the physiological temperature (39 – 41 °C) is desirable

to avoid the incidence of edema and necrosis in healthy tissue surrounding a heated tumor (Liu et al. 2006).

ELPs are genetically engineered using a process called recursive directional ligation (Meyer and Chilkoti 2002). Basically, an oligonucleotide cassette containing 10-16 pentapeptide repeats is introduced into a plasmid vector (pUC19). The cassette is excised and re-ligated back into the vector so that the two cassettes are now fused directionally and in frame. This process is repeated until ELP of desired molecular weight is produced. The protein is then expressed in *E. coli* using a hyper-expression protocol (Meyer and Chilkoti 1999). This way a library of ELPs with different repeats and molecular weights can be built. An ELP –based polypeptide (MW 59.2 kDa) that has 150 pentapeptide repeats with valine, glycine and alanine in a 5:3:2 ratio in position X was constructed so that its T_t is around 40 °C (Liu et al. 2006). This ELP, termed ELP1, is an ideal carrier for thermal targeting and has been used for thermally targeted delivery of small molecule drugs such as paclitaxel and doxorubicin, as well as other anti-cancer therapeutic peptides (Bidwell and Raucher 2005; Bidwell et al. 2007; Massodi et al. 2009; Bidwell et al. 2010; Massodi et al. 2010; Moktan et al. 2010). ELPs can also be used for gene delivery under the same conditions (Chen et al. 2008). Since hyperthermia is expected to enhance the vascular permeability of the tumor, the application of heat could also augment tumor vasculature penetration by the ELP macromolecule. Additionally, with a terminal half-life of 8.7 h and a two-fold accumulation in heated versus unheated sites (Liu et al. 2006), ELP1 has the potential to increase the therapeutic index of the drug cargo. From a synthesis stand-point, ELPs are easy to work with because they can be purified in large quantities by simple inverse transition cycling (Bidwell and Raucher 2005). Attention should be paid to the fact that adding peptides or drugs to ELP lowers the T_t so it has to be confirmed after all modifications have been completed (Meyer et al. 2001). As a thermo-sensitive macromolecule the utilities of ELP are two-fold – it can increase the stability of the cargo drug or peptide, and it can increase the specificity of the drug to the tumor site through passive targeting by EPR and active targeting by hyperthermia as described above. ELP-based therapies can potentially accumulate the chemotherapeutics to the tumor site and therefore, reduce chemotherapeutics associated side-effects, provide a better treatment outcome and improve patient's quality of life in general.

1.3.2. CELL PENETRATING PEPTIDES (CPPS)

ELP by itself can enter the cell, but levels of internalized polypeptide increase significantly if a cell penetrating peptide (CPP) is used (Bidwell and Raucher 2010). CPPs are a very diverse group of small peptides that have replaced vector molecules such as antibodies and sugars used for targeted transport and greatly improved delivery strategies for various drugs and other agents.

Over the past decades, from the discovery of CPP capabilities of Tat (peptide derived from HIV virus), more than 200 various peptides have been reported to be able to internalize their cargo successfully into cells. CPPs are not type- or tissue- specific and rely only on their positive charge to perform the role. The transduction properties of a CPP are determined by their origin, as well as by the sequence characteristics, according to which they can be divided to subgroups (Sebbage 2009). They all rapidly cross the cell membrane without disrupting it but the exact mechanism varies depending on the CPP and proposed explanations are still a matter of a heated debate. The best explanation is that the same CPP can use various methods of entry (direct penetration, pore formation, endocytosis) depending on the cargo it carries and that it can use more than one method at the same time. Major drawback of CPPs is their susceptibility to proteolytic cleavage and lack of specificity but delivery systems have been devised to overcome that. In the end benefits of using CPPs are far greater than the problems that arise in their use (Koren and Torchilin 2012). One of the aforementioned benefits is the possibility to target specific cellular compartments with different CPPs (Bidwell et al. 2009). This gives additional opportunity for increased specificity and delivery optimization.

SynB peptides are a group of CPPs derived from antimicrobial protein protegrin (PG-1) isolated originally from porcine leukocytes. SynB1 is an 18 amino acids long peptide that can successfully cross cellular membranes. Blood brain barrier (BBB) poses a different challenge all together. This multilayer structure with efficient system of tight junctions is the main problem in treating any brain disorder including GBMs. SynB1 has been shown to successfully cross the BBB without compromising its protective role. Other CPPs showed a more extensive disruption of the BBB resulting in possible problems if used in actual treatment (Rousselle et al. 2001; Drin et al. 2002).

For all of the above-mentioned reasons SynB1 was the chosen CPP in this work that used with ELP can help protect it from proteolysis and provide specificity.

1.3.3. HYPERTHERMIA IN CANCER THERAPY

Mild hyperthermia is a non-invasive method to increase tumor temperature in the range of 40 – 44 °C and is used as an adjuvant in chemotherapy and or radiation therapy (Schildkopf et al. 2010). Although hyperthermia as a therapy was introduced over 25 years ago, due to the advancement in our understanding of tumor biology, and significant improvement in hyperthermia application and imagining technologies, it is only now gaining rapid clinical acceptance (Hurwitz 2010). Application of mild hyperthermia to a tumor site transiently improves blood flow and oxygenation, and sensitizes cancer cells to chemotherapy and radiation (Hokland et al. 2010). Hyperthermia has been introduced in the treatment of glioblastoma, head and neck cancer, breast cancer, cancer of the gastrointestinal or urogenital tract, and sarcoma (Dewhirst et al. 1997; Falk and Issels 2001; Takahashi et al. 2002). Hyperthermia is accomplished using microwave, radio-frequency, and high-intensity focused ultrasound (HIFU) that allows precise heating of deep-seated tissues. These heating devices are coupled with imaging tools to guide as well as to better monitor the response to and efficacy of heating. For example, HIFU technology uses a high-intensity convergent ultrasound beam generated by high power transducers to produce heat. As an acoustic wave propagates through the tissue, part of it is absorbed and converted to heat. With focused beams, a very small area of interest can be precisely heated deep in tissues (Cohen et al. 2007; Ram et al. 2006). In a magnetic resonance imaging (MRI) guided HIFU, the entire process is monitored by MRI, which facilitates precise monitoring and control of temperature fluctuation to maximize heat response. Consequently, the methods and techniques necessary to employ thermal targeting of thermally responsive polymers are already available in the clinical setting.

1.3.4. ADVANTAGES OF USING RESPONSIVE POLYMERS AND HYPERTHERMIA

There are several advantages of using thermally responsive polymers in combination with hyperthermia. First, hyperthermia preferentially increases the permeability of tumor vasculature compared to normal vasculature, which can further augment the

delivery of drugs by thermo responsive carriers to tumors (Issels 1995; Feyerabend et al. 1997; van Vulpen et al. 2002). In addition, due to abnormalities of tumor vasculature, aberrant vascular architecture, and lack of lymphatic drainage tumors cannot perfuse heat adequately. Consequently, because the heat dissipation is slower in tumor than that in normal tissues, as the tumor is heated temperature of the tumor continues to rise. The tumor targeting and retention of thermo responsive polymer drug carriers are significantly enhanced. Retention can be additionally increased with application of heat in cycles rather than continuously (Dreher et al. 2007). Second, by applying selective local heating technologies like MRI-guided HIFU, thermo responsive polymers can target solid tumors in any organ or tissue in the body. Lastly, the significant advantage of ELPs over other thermally sensitive carriers, such as temperature sensitive liposomes (Kong and Dewhirst 1999), is that accumulation of the drug on the target tissue occurs through the phase transition of the carrier rather than through heat-triggered release of the drug. Unlike other delivery systems, a concentration gradient is therefore not required to drive thermally responsive polymers into the heated tumor. Even when their blood concentration is less than the total concentration in the tumor, thermally responsive polypeptides continue to accumulate because of aggregation in the heated tumor, and alteration of its parent form (Kratz et al. 2011). Therefore, the polypeptide-drug conjugate may be injected at a low concentration systemically, while still achieving a higher concentration in the tumor. For these reasons the strategy of using thermally responsive polymers like ELP in conjunction with hyperthermia is very promising for delivery of anti-cancer drugs to solid tumors.

In conclusion, as science and medicine work together to continuously improve treatment options and quality of life for those suffering of cancer, protein-based therapeutics show more and more promise. Ease of production and enormous potential to reach those targets that so far were classified as undruggable by the pharmaceutical industry make protein/peptide based therapies closer to personalized medicine than any other therapeutic approach. Potential drawbacks, like bad pharmacokinetics and short half life due to proteolytic enzymes present in cells and circulation, have been addressed by various types of delivery systems and protective methods. In the variety of systems that have been proven successful in those areas ELP-based delivery offers an

additional advantage in the possibility of active targeting by the application of heat. In clinical surroundings the use of controlled hyperthermia concurrently with chemotherapy is already in practice, so the transition from bench to clinic should be easily achieved. Combination of efficient and easily manufactured delivery systems combined with the most recent discoveries in cellular biology and drug design will hopefully bring us, in not so distant future, to a point when being treated for cancer will not represent an obstacle to leading a normal life and in case of cancer like glioblastoma will give the patients a fighting chance of beating their disease.

2. HYPOTHESIS AND AIMS

It has been shown repeatedly in literature that Notch pathway is over expressed in a very large portion of glioblastomas. Since the current method of choice for blocking Notch activity by inhibiting γ-secretase cleavage of the Notch receptor can have many potential off target effects and questionable efficacy in the brain due to the presence of the blood brain barrier exploring new approaches is desirable.

dnMAML is efficient in blocking the same pathway without affecting other γ-secretase regulated processes. It could be a replacement for GSIs in glioblastoma.

Hypothesis was that by attaching ELP and an appropriate CPP to dnMAML, it can be efficiently delivered to the brain and into the cell and inhibit growth through blocking over-expressed Notch targets.

To competently test this hypothesis several steps need to be completed. These steps include following specific aims:

- cloning of the N-terminal fragment of MAML1 protein - further named dnMAML into a vector carrying ELP and SynB1 cell penetrating peptide.
- expression of the pure protein products in a bacterial based system and their purification by thermal cycling, taking advantage of the ELPs temperature sensitive transition properties
- testing of the inhibition potential of SynB1-ELP-dnMAML in selected glioblastoma cell lines and its confirmation by appropriate controls
- exploration of the precise mechanism of inhibition by testing levels of apoptosis induction and cell cycle distribution
- Notch inhibition monitoring through measurement of expression of main target genes for the canonical pathway
- Notch inhibition monitoring through measurement of protein levels for non-canonical targets, as well as Notch independent dnMAML targets

Main objective is to show that SynB1-ELP-dnMAML can act as a potent inhibitor of the GBM derived cells' growth and that it does so by affecting both canonical and non-canonical Notch targets in addition to dnMAML targets independent of Notch. This work should therefore present SynB1-ELP-dnMAML, as not only adequate replacement for GSI, but a more specific and safer alternative to GSIs.

3. MATERIALS AND METHODS

3.1. SYNTHESIS OF SYNB1-ELP-DNMAML

In order to synthesize the required peptide a step wise process is used where necessary sequences are added in a block like manner to the pET25b(+) vector (Novagen, Madison, WI). To mediate the intracellular uptake of ELP, the amino-terminus of ELP was modified by the addition of the SynB1 peptide.

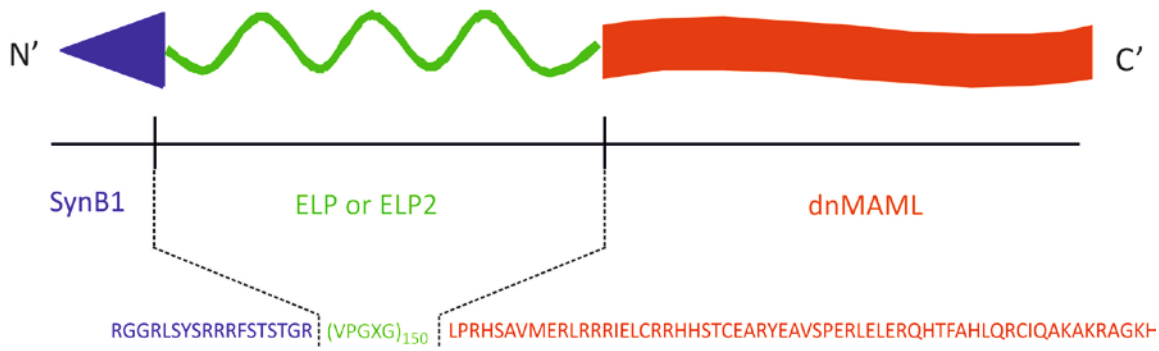

Figure 4. SynB1-ELP-dnMAML structure shematic with respective amino acid sequences by section

A double stranded 5'phosphorylated oligonucleotide cassette encoding the sequence of amino acids for SynB1 (RGGRLSYSRRRFSTSTGR) was cloned within the *NdeI* and *SfiI* sites of a pET25b(+) vector. DNA sequence of dnMAML was obtained from pMIGR1 containing the full *MAML1* sequence (generous gift from dr. Antonio Pannuti, University of Mississippi Medical Center Cancer Institute) by touchdown PCR reaction focused on the 13-74 amino acid portion using specific primers (IDT, Coralville, IA). Primers were designed according to amino acids sequence of the human *MAML1* sequence with added recognition sites for *SfiI* and *BamHI* (5' - GGCC GGCC GGGCC - 3' and 5' - TAT GGA TCC GCC - 3', respectively). Touchdown PCR was done with 1 °C decrease in annealing temperature starting from 65 °C until T_m of the primers was reached at 54 °C. Resulting PCR product was then cloned within *SfiI* and *BamH1* sites in pET25b(+). In the final step, the ELP sequences from pUC19-ELP1 and pUC19-ELP2 (synthesized as previously described (Meyer and Chilkoti 1999)) were cut out with *Pf1MI* and *BglI* and introduced into the *SfiI* site of pSynB1-*SfiI*-dnMAML to generate pSynB1-ELP1-dnMAML and its thermally unresponsive control pSynB1-ELP2-dnMAML. Ligation products were transfected into DH5α *E. Coli* competent cells. DNA from the bacteria carrying the plasmid was purified and tested for ELP. Samples with successfully inserted ELP showed

a characteristic 2kb band after digestion with *NdeI* and *BamHI*. Samples positive for this band were sent for sequencing. Bacterial stock with confirmed correct DNA sequence was stored and used for further work.

Table 1. List of all polypeptides used

Name	Thermally responsive	Functional domain	molecular weight / kDa
SynB1-ELP	YES	NO	61.8
SynB1-ELP-dnMAML	YES	YES	74.1
SynB1-ELP2-dnMAML	NO	YES	75.7

3.2. PURIFICATION OF ELP-BASED POLYPEPTIDES

Plasmids with the correct sequences were transformed into *E. coli* BLR(DE3) competent cells (Novagen, Madison WI). Proteins were expressed using a hyper-expression protocol (Daniell et al. 1997). Transformed DE3 culture was inoculated into 500 mL of TB Dry growth media (MoBio, Carlsbad, CA) supplemented with 50 µg/mL ampicillin and 2 mL glycerol (Sigma, St. Louis, MO) and incubated at 37 °C, 220 rpm for 18-20 h. Cells were harvested from the media by centrifugation (3,000 x g, 10 min, 10 °C) and frozen for a minimum of 1h at -80 °C to facilitate subsequent lysis. The cells were then sonicated in PBS to lyse the cells (Fisher Scientific 550 Sonic Dismembrator, Fisher Scientific, Pittsburg, PA). A centrifugation step (13,000 x g, 45 min, 10 °C) was carried out to remove cell debris. 0.5A% w/v polyethylene imine (PEI - Sigma, St. Louis, MO) was added to the resulting supernatant fraction to precipitate nucleic acids which were then removed by centrifugation (13,000 x g, 30 min, 10 °C). Finally, the phase transition of ELP was induced at room temperature by adding NaCl up to 2M concentration and a visual change in turbidity of the supernatant collected from the last spin. The sample was warmed briefly in a water bath set to 40 - 42 °C to further induce transition, centrifuged (11,000 g, 10 min, 30 °C), and the protein pellet was collected. Protein was suspended in cold phosphate buffered saline and divided into smaller aliquots for further cycling. Samples we cooled to 4°C then centrifuged for 60s to remove insoluble debris. Sodium chloride was added to the supernatant and samples warmed in the water bath again to induce phase transition. Protein aggregates were the spun down for

60s and soluble impurities removed in the supernatant. Protein pellets were re-suspended in cold PBS (shown in Figure 5.). These steps were repeated for minimum 3 for up to 5 times to give pure ELP samples. This process is known as inverse thermal cycling (Meyer and Chilkoti 1999).

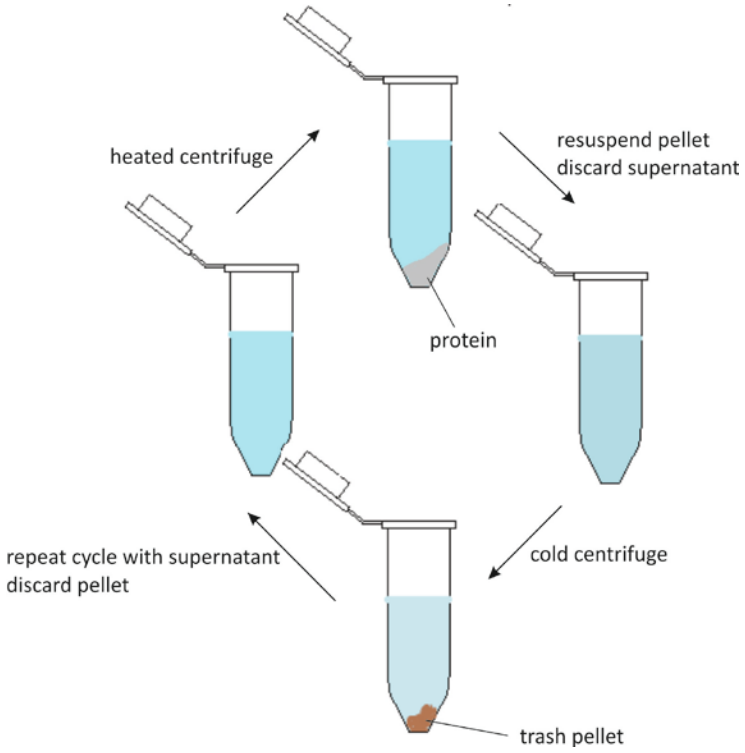

Figure 5. Schematic illustration of thermal cycling used for protein purification. Heating the protein solution above T_t allows the protein to aggregate and can be spun down. Centrifugation in a cooled centrifuge after cooling the resuspended protein (preferably to 4°C) spins down insoluble trash. Entire procedure is repeated until no visible trash pellet is formed after cold centrifuge (3-5 times approximately).

3.3. CHARACTERIZATION OF THE TRANSITION TEMPERATURE

The effect of the addition of peptides on ELP phase transition temperature was determined by monitoring the change in optical density of the protein solution with respect to temperature. Solutions of SynB1-ELP-dnMAML at different concentrations in complete media were heated at a constant rate of 1 °C/min using the thermal feature of a multi-cell holder UV-vis spectrophotometer (Cary 100, Varian Instruments, Palo

Alto, CA). Absorbance data was converted to percent of maximum for each curve and plotted against temperature. The T_t from this graph is defined as the temperature that results in 50% of the maximum absorbance. Concentration dependence of T_t was determined by fitting the data to a logarithmic equation. The resulting graph gave the range of concentrations that can be used with effective phase transition of ELP within the 37 – 42 °C mild hyperthermia region.

3.4. LABELING POLYPEPTIDES WITH FLUORESCENT PROBES

For the labeling with fluorescent probes, protein was diluted to 100 µM in 50 mM Na_2HPO_4 buffer, pH 7.0, and incubated with 10-fold molar excess of tris-(2-carboxyethyl)phosphine (TCEP – Invitrogen, Eugene, OR) at 4°C for 20 min. Either the thiol reactive 5-iodoacetaminefluorescein or tetramethylrhodamine-5-iodoacetamide dihydroioide (Invitrogen, Eugene, OR) was added in 2-fold molar excess to the protein. Since the dyes are not directly soluble in the conjugation buffer, they were dissolved in 10 µL of DMSO before adding to the conjugation mixture. The conjugation was carried out with continuous stirring at 4 °C overnight. The free stain was removed by inverse thermal cycling the protein 3 to 5 times. The labeling efficiency was assessed by UV-visible spectrophotometry (UV-1600 Shimadzu, Shimadzu, Columbia, MD) at 541 nm for rhodamine, at 495 for fluorescein, and at 280 nm for protein. The final protein concentration was calculated by using Beer-Lambert formula ($A = c * \varepsilon * L$; ε – extinction coefficient, L – path length) and subtracting the percentage of absorbance contributed by the dye. Labeling efficiency varied from 10% to 15%.

3.5. CELL CULTURE

The D54 and U251 cells stably transfected with luciferase were obtained from dr. Lacey McNally (University of Alabama, Birmingham). Cells were cultured in DMEM/F12 1:1 media (Cellgrow, Manassas, VA) supplemented with 10% fetal bovine serum (FBS – Altlanta Biologicals, Lawrenceville, GA), 100 units/mL penicillin, 100 µg/mL streptomycin, and 25 µg/mL amphotericin B (Invitrogen, Carlsbad, CA). All cell cultures

were maintained at 37 °C atmosphere with 5% CO_2. Hyperthermic treatment was performed in a 42 °C incubator with 5% CO_2.

3.6. CELLULAR UPTAKE OF SYNB1-ELP-DNMAML

D54 and U251 cells were plated ($2x10^5$ per well) in 6-well tissue culture plates. Cells were allowed to grow for 24h at 37 °C. The next day cells were treated with 30 µM fluorescein-labeled SynB1-ELP1-dnMAML or SynB1-ELP2-dnMAML for 1h at 37 or 42 °C. After treatment, cells were washed with PBS and collected using non-enzymatic cell dissociation buffer (Invitrogen, Carlsbad, CA). Total uptake of the fluorescein labeled polypeptide was measured by measuring fluorescence intensity in FL1 channel (10 000 cells per sample) using flow cytometry (Gallios, Beckman Coulter, Indianapolis, IN). Cell debris was excluded from the forward vs. side scatter plot. The fluorescence raw data was corrected for labeling efficiency of each polyeptide, and compared to background values. In order to distinguish total cell binding from true cellular internalization, 10-15 µL of Trypan Blue (Cellgrow, Manassas, VA) was added to the cell samples to quench the extracellular fluorescent signal (Hed et al. 1987; Raucher and Chilkoti 2001).

3.7. LASER SCANNING CONFOCAL MICROSCOPY

To confirm sub cellular localization of SynB1-ELP-dnMAML D54 cells were plated at ~50% confluence on cover slips. Cells incubated at 37 °C for 24h were exposed to 30 µM of rhodamine-labeled SynB1-ELP1-dnMAML at 37 or 42 °C for 1h. After treatment, cells were allowed to grow at 37 °C for 1h before fixing in 4% paraformaldehyde (PFA - Fisher Scientific, Pittsburg, PA). Nuclei were stained with 25 nM Sytox Green (Invitrogen, Carlsbad, CA) and coverslips mounted on slides. Cells were then visualized using Nikon Eclipse C1 scanning confocal microscope (Nikon Instruments, Melville, NY) and images analyzed using Nikon EZOne software.

3.8. THERMALL PULL-DOWN ASSAY

Cells were plated to ~80% confluence in 75cm^2 flasks. Cells were lysed with T-PER lysis buffer (Fisher Scientific, St. Louis, MI) supplemented with protease inhibitors (Complete Mini, Roche, Indianapolis, IN). Aliquots of the resulting lysate (200 µL) were incubated with 100 µM SynB1-ELP-dnMAML or SynB1-ELP for 2h at 4 °C with gentle agitation to maximize interaction. Upon completion phase transition of ELP polypetides was induced by warming the samples to 42 °C to allow polypeptide aggregation and centrifuged to collect aggregated polypeptide. Resulting pellets were resuspended in 100 µL of the fresh lysis buffer. Solutions were then incubated over night at 4 °C under constant agitation to remove non-specifically bound proteins. Procedure is schematically shown in Figure 6. Aggregation step was repeated the following day and final samples dissolved in sample buffer with reducing agent added before being subjected to SDS-PAGE and transferred to 0,2 µm PVDF membrane (BioRad, Hercules, CA, USA). Western blot analysis was carried out with anti-Notch1 (D1E11, Cell Signaling, Danvers, MA) and visualized with HRP-labeled secondary antibodies (Sigma, St. Louis, MI) using Super Signal West Pico Chemiluminescent Substrate (Fisher Scientific, Pittsburg, PA).

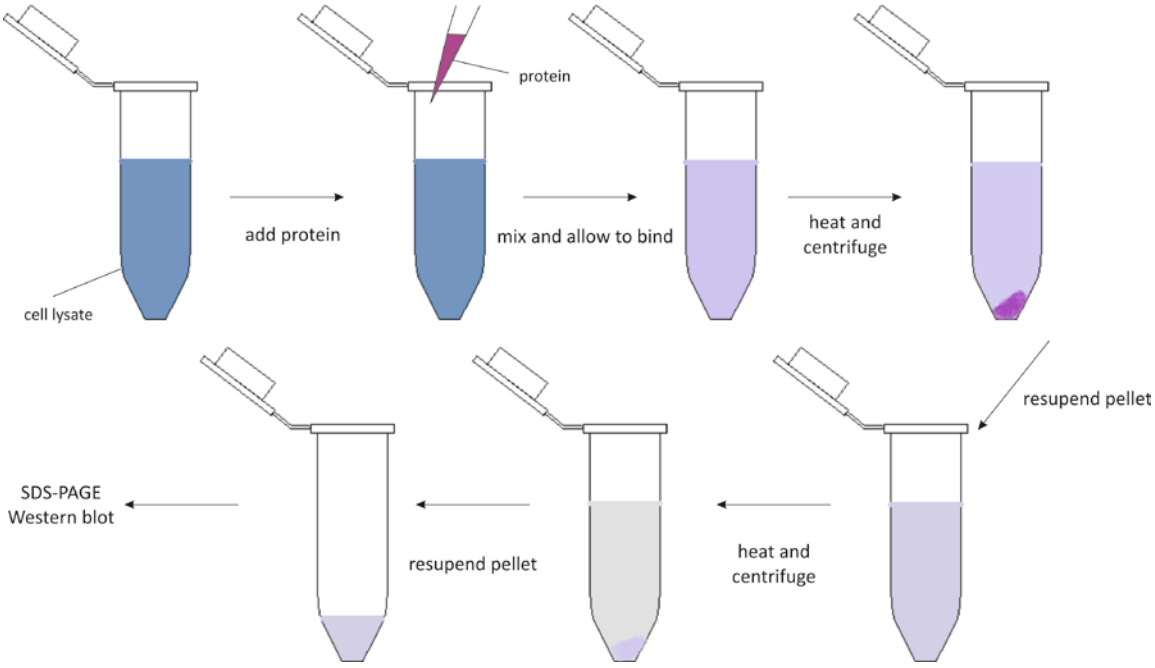

Figure 6. Illustration of the pull-down procedure. Using the thermal aggregation of ELP all the protein that bind to the dnMAML are pulled from the lysate. All non-specificly bound protein is washed by gently resuspending the protein pellet in lysis buffer. After SDS-PAGE and transfer to the membrane samples are probed for presence of Notch 1.

3.9. CELL PROLIFERATION

Both D54 and U251 cells were plated in 6-well plates (5000 cells/mL) and incubated for 24h at 37 °C. On day 0 cells were either left untreated or treated with various concentrations of SynB1-ELP1-dnMAML and SynB1-ELP2-dnMAML for 1h at 37 or 42 °C. Treatments were removed and replaced with fresh media. Cells were incubated at 37 °C for 72h, and the treatment was repeated again on day 3. On day 6, 72 h after the second treatment, cells were collected by trypsinization, washed in 1 mL PBS, and samples divided. Half of the sample was counted using Coulter Counter (Beckman Coulter, Indianapolis, IN) and the other half stained by Trypan Blue (Cellgrow, Manassas, VA) and counted in hemacytometer (Hausser Scientific, Horsham, PA) to confirm the count obtained by the Coulter Counter. Since both gave the same results Coulter Counter was used for all other experiments.

3.10. APOPTOSIS ASSAYS

Both D54 and U251 cells were plated 20 000 cells/mL in 25cm^2 flasks. Cells were left untreated or treated with 30 µM SynB1-ELP1-dnMAML or SynB1-ELP at 37 or 42 °C for 1 h on day 0 and day 3. Treatment was removed and cells were allowed to grow at 37 °C in fresh media. On day 4 both floating and adherent cells were harvested and washed in Annexin binding buffer containing 10 mM HEPES (Sigma, St. Louis, MO, USA) pH 7.4, 140 mM NaCl and 2.5 mM CaCl2 (Sigma, St. Louis, MO, USA). After washing cells were counted and equal number of cells was taken for each sample. Samples were then stained with 2 µg/ml of propidium iodide (PI) and Alexa-488-Annexin V reagent (Invitrogen, Carlsbad, CA) from Dead Cell Apoptosis Kit according to manufacturer's recommendations to distinguish the necrotic and the apoptotic cells. Cells treated overnight with 50 mM etoposide were used as apoptosis positive control. FITC and PI signals were measured using FL1 and FL3, respectively, by the Gallios flow cytometer and analyzed by Kaluza software (Beckman Coulter, Indianapolis, IN). Cell debris was excluded from the analysis using forward vs. side scatter plot. Samples were gated according to PI only and Alexa-488-Annexin V only stained controls.

3.11. CELL CYCLE DISTRIBUTION

Cell cycle analysis was done by BrdU incorporation assay. For this experiment 40 000 cells/mL of both cell lines were plated in 25cm^2 flasks. Cells were then treated in the same way as for the apoptosis induction assay. On day 4 cells were pulsed for 1h with 10 µM BrdU (Sigma, St. Louis, MO) in the dark, before being harvested. Collected cells were fixed in cold 70% ethanol on ice overnight. Fixed cells were washed in cold PBS and incubated for 15 min in 2 N HCl with 2% Triton-X (Sigma, St. Louis, MO, USA) to denature DNA. After incubation cells were centrifuged to collect and washed in 0.1 M $Na_2B_4O_7$ (Sigma, St. Louis, MO, USA) to neutralize the acid. After neutralization cells were washed in cold PBS and counted. The samples ($5x10^5$ cell per sample) were incubated with Alexa 488 labeled anti-BrdU antibody (clone MoBU-1, Invitrogen, Eugene, OR, USA), at 4 °C overnight, to determine levels of incorporated BrdU. Total DNA content was assessed by PI staining (Sigma, St. Louis, MO). Alexa 488 fluorescence

and PI signal were measured in FL1 and FL3 channel, respectively, using a fluorescence-activated cell scanner (Gallios, Beckman Coulter, Indianapolis, IN). Cell aggregates and debris were excluded from analysis with the help of forward scatter vs. FL3 intensity plot. The plots of PI and Alexa 488 fluorescence intensity were gated into respective regions representing cell cycle phases to determine the percentage of cells in each phase using Kaluza software (Beckman Coulter, Indianapolis, IN).

3.12. WESTERN BLOT ANALYSIS

To evaluate the effect on treatment with SynB1-ELP-dnMAML on targets outside the Notch pathway and known pathway cross-talk targets cells were plated in 6-well plates to approximately 80% confluence before treatment with SynB1-ELP-dnMAML or SynB1-ELP at 37 or 42 °C for 1h. Treatment media was then replaced with fresh media and cell allowed to grow for 24h at 37 °C. After that they were harvested according to the protocols described by Martinez (Martinez et al. 2010). Cells were washed in cold PBS and lysed in T-PER lysis buffer before being frozen at -80 °C for one hour. Samples were then thawed on ice, collected and sonicated. Total protein concentration was determined by BCA protein assay (Fisher Scientific, St. Louis, MI). Amount of protein of 15 µg per sample were denatured in loading buffer with reducing agent at 95 °C for 5 minutes. Samples were loaded onto 4% - 12% precast gels (NuPage, Invitrogen, Carlsbad, CA) and electrophoresis run for 1h at 200V. After electrophoresis samples were transferred on PVDF membranes by wet blotting for 1h at 35V. Completed transfer was confirmed by staining the membrane with Ponceau. Membrane was then incubated for 1h in 5% non-fat milk for blocking. Western blot analysis was carried out using antibodies listed in Table 2. Each antibody was diluted to working concentration in 5% milk and incubated with the membrane overnight. Membrane was subsequently washed before incubating it with secondary antibody for 1h. Secondary antibodies labeled with horseradish peroxidase (HRP) were used for visualization with Super Signal West Pico Chemiluminescent Substrate (Fisher Scientific, Pittsburg, PA). Blot films were developed using Kodak X-OMAT 2000A X-ray film processor (Kodak, Rochester, NY).

Table 2. List of antibodies used for Western blot and pull-down assays (* denotes antibodies used in the pull-down assay)

Antibody	Raised in	Clone	Manufacturer	Dilution
anti-MAPK (Erk1/2)	rabbit	D13.14.E	Cell signaling, Danvers, MA	1:2000
anti-pAkt	rabbit	D9E	Cell signaling, Danvers, MA	1:2000
anti-p53	mouse	DO-1	Santa Cruz Biotechnology, Dallas, TX	1:500
anti-actin	mouse	AC-15	Sigma, St. Louis, MI	1:10000
anti mouse	goat	polyclonal	Sigma, St. Louis, MI	1:10000
anti-rabbit*	goat	polyclonal	Sigma, St. Louis, MI	1:15000
anti-Notch1*	rabbit	D1E11	Cell signaling, Danvers, MA	1:1000

3.13. qPCR ASSAY

qPCR assay was done to determine direct inhibition of known Notch downstream genes *Hes-1* and *Hey-L*. Cells were plated to approximately 80% confluence in 75cm^2 before treating them with SynB1-ELP-dnMAML or SynB1-ELP for 1h at 37 or 42 °C for 1 h. Cells were the allowed to grow for 24h before total RNA extraction and clean up with Rneasy kit (Qiagen, Germantown, MD) according to manufacturer's protocols. RNA amount of 600 ng per sample was used to generate cDNA using Superscript III First Strand System (Invitrogen, Carlsbad, CA). qPCR samples were prepared using SYBR Green PCR Mix (Invitrogen, Carlsbad, CA). Final reaction volume of 25 µL was prepared with 2µL of cDNA, 1.5µL of water, 12.5 µL of SYBR Green and 10 µL of 0.5 µM Hes-1, Hey-L or 18S primers. qPCR was run using pre determined optimized protocols (i.e. for Hes-1: 13min at 95 °C, 41 cycles (95 °C for 10s; 57 °C for 15s; 72 °C for 20s), melting curve 65 °C-95 °C - 0.05s). qPCR was performed on CFX 384 Thermal Cycler (Biorad, Hercules, CA). Results were analyzed using provided CFX Manager Software. Expression levels of the Hes-1 and Hey-L genes were normalized to internal control gene expression, which was in this case 18S ribosomal RNA.

3.14. STATISTICAL ANALYSIS

All described experiments were done in triplicate. Where indicated, statistical difference between groups was calculated using Analyze It add-in software for Microsoft Excel. All data groups in an experiment were compared using one-way analysis of variance (ANOVA) pair wise comparison with Bonferroni correction to reduce the possibility of false positive results. All analyses were done with 95% confidence interval (α=0,05).

4. RESULTS

4.1. PROTEIN PURIFICATION

When the final construct has been confirmed and successfully transformed into *E. coli* BLR(D3), bacterial proteins are purified by the process described in detail in Materials and Methods section. To show how effectively proteins can be purified each step was sampled and all the samples were submitted to SDS-PAGE. A representative gel is shown in Figure 7.

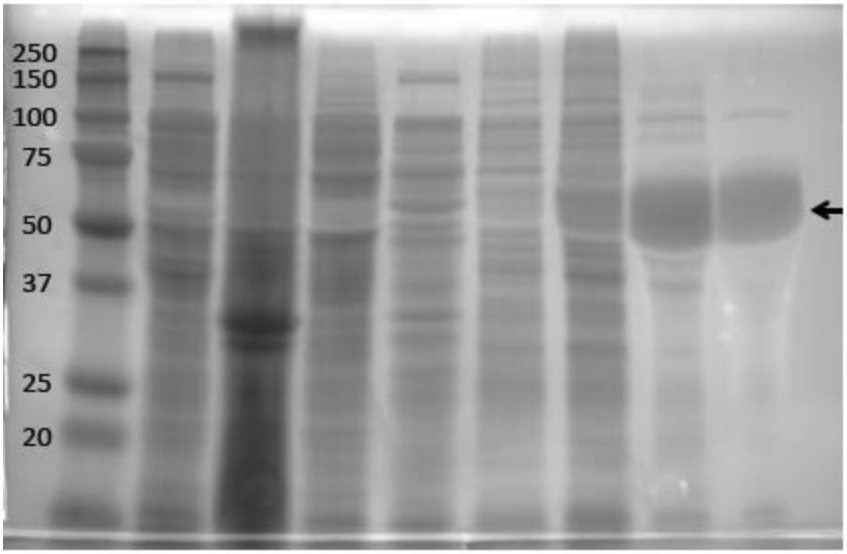

Figure 7. ELP purification by thermal cycling Representative SDS-PAGE gel showing each step of the purification process. Lane 1 contains the size marker, lanes 2-5 supernatant and pellet from initial centrifugation steps and lanes 6-9 samples from consecutive thermal cycles. Arrow indicates ELP band

4.2. CHARACTERIZATION OF SYNB1-ELP-DNMAML

The key to a functional ELP is having the protein undergo transition within the desired range of temperature, in this case between 37°C and 42°C. It is necessary to confirm transition temperature after every addition because any change in sequence and length of the peptide lowers transition temperature (Urry et al. 1991). Temperature transition experiments were performed in complete cell culture media in order to get the most accurate prediction on how the protein will behave in cell culture conditions. As can be seen from Figure 8., addition of SynB1 and dnMAML did not prevent ELP from aggregating and it decreased the transition temperature slightly. From the data collected, a range of concentration of SynB1-ELP-dnMAML which show transition in the desired range, can then be determined (Figure 8. b).

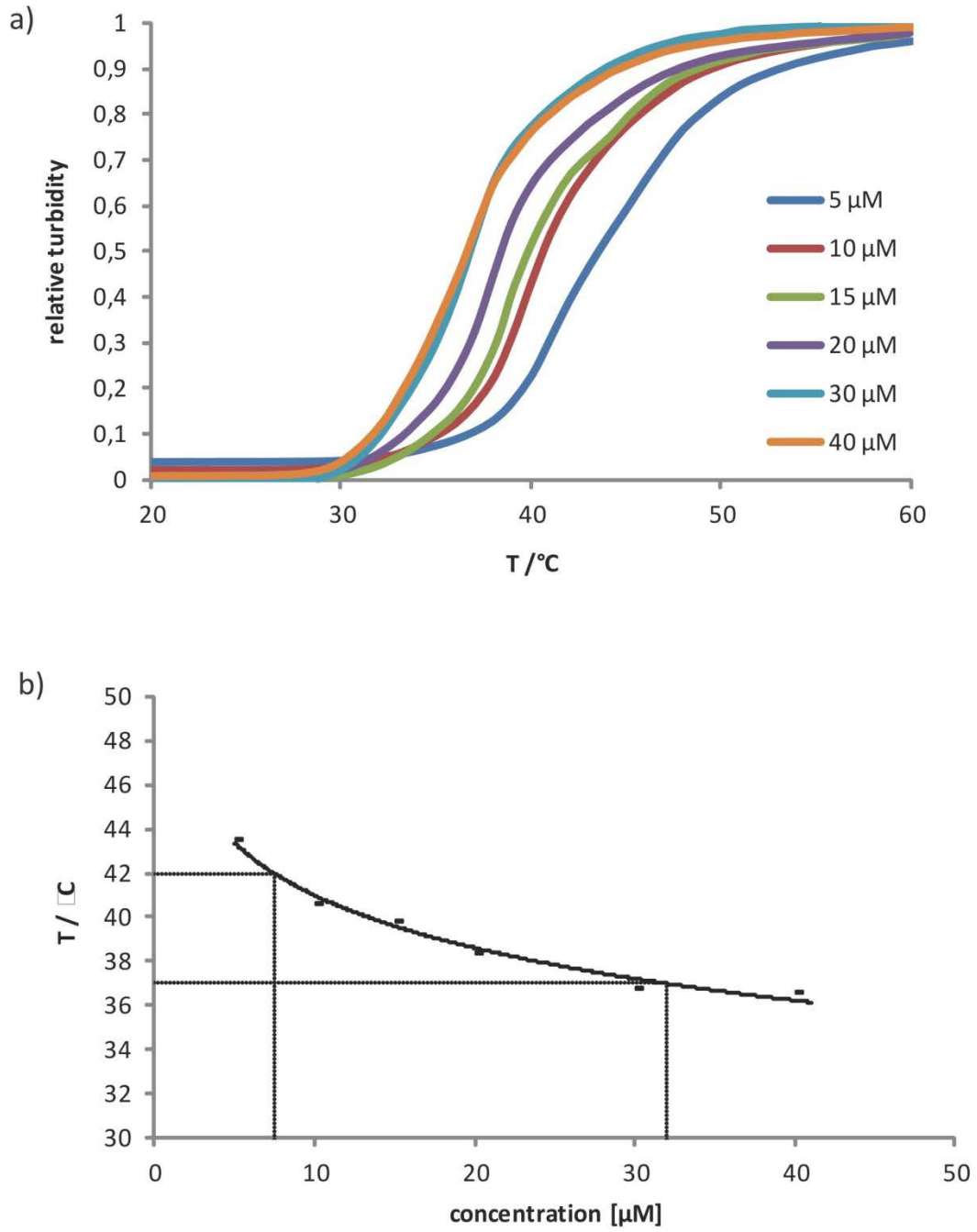

Figure 8. Thermal transition of SynB1-ELP-dnMAML

a) Turbidity of various concentrations of SynB1-ELP-dnMAML as a function of temperature was measured in complete cell culture medium to determine transition temperature (T_t) for each concentration. T_t is defined as the temperature value at which turbidity reaches half of the measured maximum.

b) T_t values from the previous graph plotted against their respective concentrations and fitted to a logarithmic curve. This way a range of concentrations in the desired temperature range is determined.

By determining the range of concentrations that undergo transition in the chosen temperature range, optimum heat effect can be achieved. For SynB1-ELP-dnMAML concentration range was 7.5µM and 32µM. With that in mind all the following experiments were done with protein concentrations of 10, 20 and 30µM. A thermally unresponsive control peptide SynB1-ELP2-dnMAML was constructed and transition temperature determined and compared to that of SynB1-ELP-dnMAML (Figure 9.) Transition temperature of ELP2 is around 57°C and therefore does not display any heat effect under experimental conditions. As another functional control SynB1-ELP vehicle construct was used.

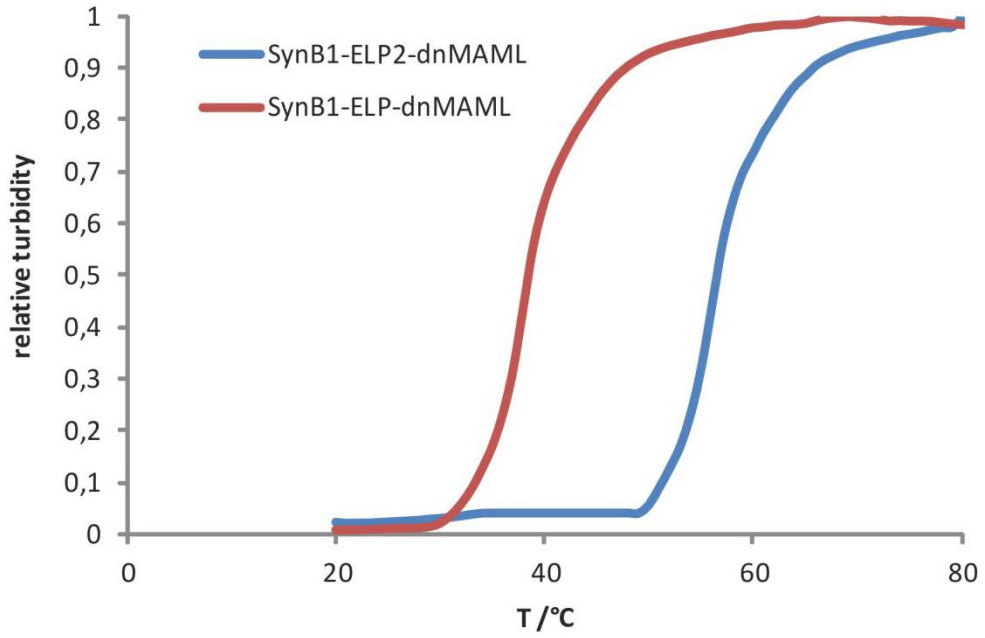

Figure 9. Thermal transition of SynB1-ELP-dnMAML and SynB1-ELP2-dnMAML. Turbidity of 20 µM solution of SynB1-ELP-dnMAML and its thermally unresponsive control SynB1-ELP2-dnMAML is plotted as function of temperature to compare transition temperatures of the 2 proteins. T_t of SynB1-ELP2-dnMAML is above the experimental conditions and can therefore be used as a thermally unresponsive control.

4.3. UPTAKE AND SUB-CELLULAR LOCALIZATION

The uptake and sub-cellular localization of the peptide constructs are directly linked to the CPP used. SynB1 was previously shown to have a strong cytoplasmic localization (Moktan and Raucher 2012). Scanning confocal microscopy results of D54 cells treated

with 30 µM rhodamine labeled SynB1-ELP-dnMAML show cytoplasmic accumulation of protein. Fluorescence is more pronounced on slides treated under hyperthermic conditions. Protein fluorescence is present in punctuated dots in the cytoplasm, characteristic of entry via endocytosis. Membrane shows no fluorescence indicating that the protein has entered the cells completely. As can be seen in Figure 10., in the far right column, there is no visible overlay of fluorescent signal between Sytox (green) and rhodamine (red).

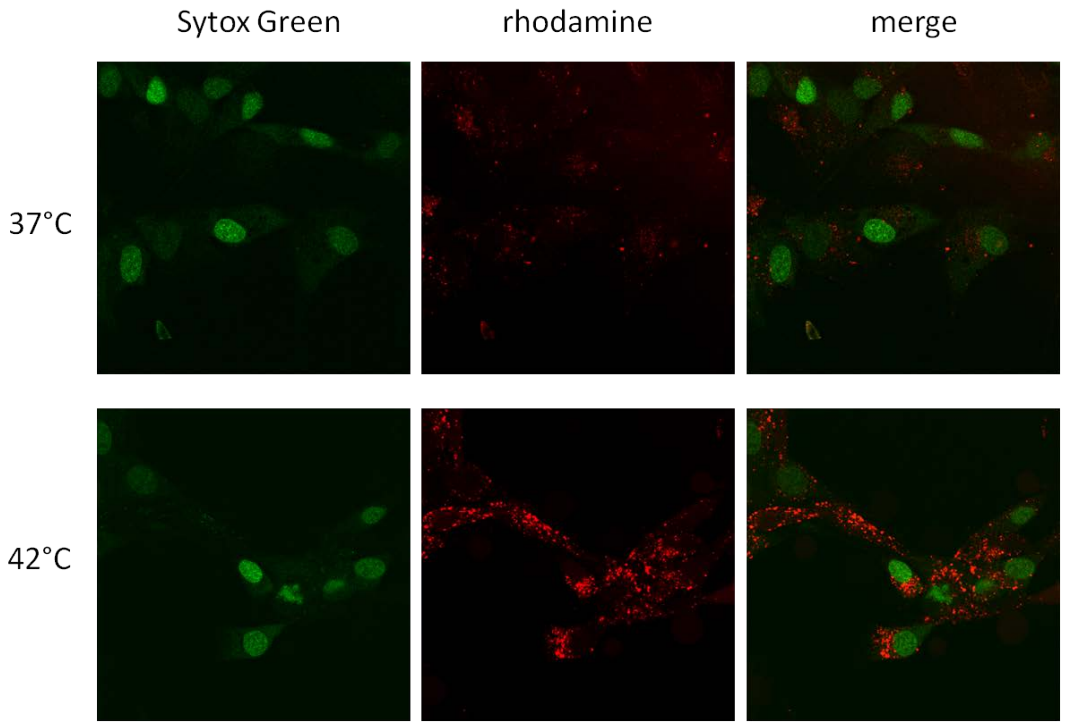

Figure 10. Scaning confocal microscope images of D54 cells treated with rhodamine labeled SynB1-ELP-dnMAML Green fluorescing Sytox Green dye dyes the nuclei. Rhodamine flurescence (red) shows presence of the protein. Two channel overlay helps determine subcellular localization of the protein. Protein is confined in the cytoplasm in punctuated pattern characteristic for endosomal entry.

The level of protein in the cell was quantified by flow cytometry. In this case cells were treated with equal amounts (20 µM) of fluorescein labeled SynB1-ELP-dnMAML and SynB1-ELP2-dnMAML. Experiment was done on both cell lines. Results show that heated SynB1-ELP-dnMAML samples take up more protein than the unheated samples and more than SynB1-ELP2-dnMAML heated samples (Figure 11.). D54 cells take up the protein more effectively. The uptake of heated SynB1-ELP-dnMAML was approximately

4-fold compared to the unheated sample and SynB1-ELP2-dnMAML (Figure 11. a.) Under the same conditions, the uptake in U251 cells is lower, only doubling when heat is applied (Figure 11. b.). The difference observed between SynB1-ELP2-dnMAML heated and unheated samples can be attributed to increased membrane leakage due to increased temperature and the efficiency of the CPP itself to internalize everything that does come in contact with the cell membrane.

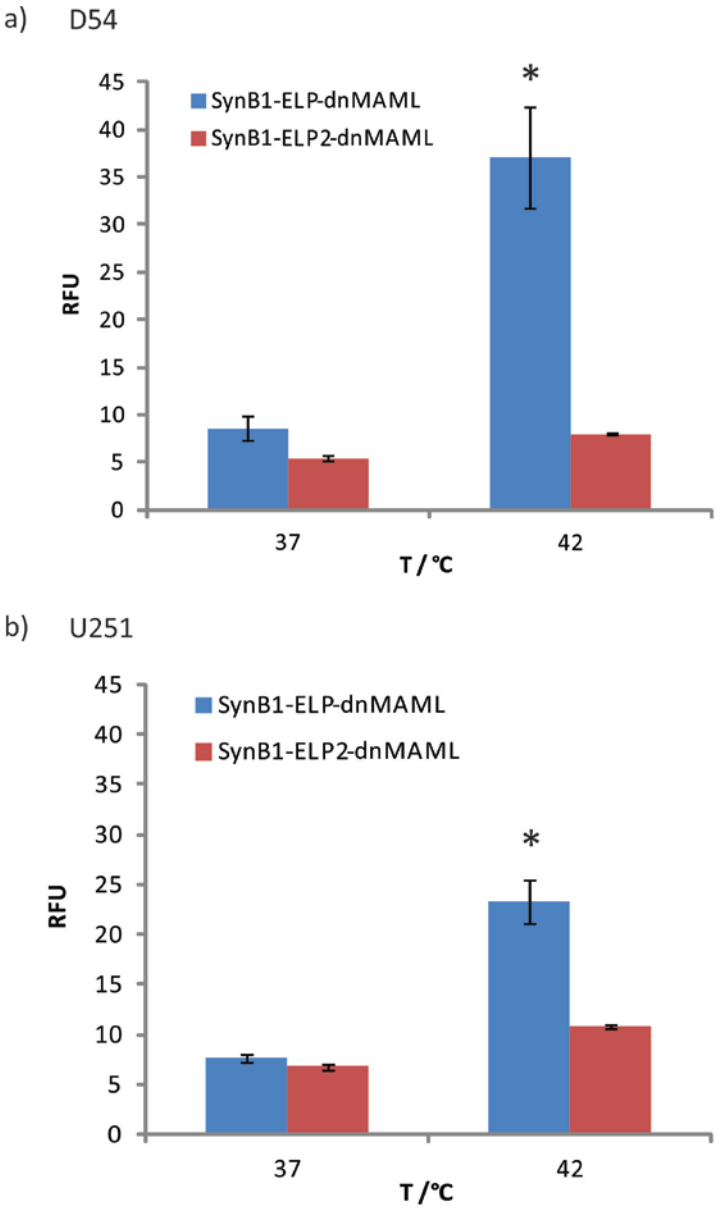

Figure 11. Uptake of fluorescein labeled polypeptides. D54 (a) and U251 (b) cells were treated with equal amounts of SynB1-ELP-dnMAML and SynB1-ELP2-dnMAML for 1h with or without hyperthermia. Fluorescence was measured by flow cytometry. Samples that showed significantly higher uptake than control and unheated sample ($p<0.001$) are denoted by asterisk (*). Relative fluorescence units (RFU) represent measured fluorescence corrected for labeling efficiency

4.4. THERMAL PULL-DOWN OF NOTCH

Inhibition of Notch pathway with dnMAML can be both canonical and non canonical. Canonical inhibition requires direct interaction between Notch 1 and SynB1-ELP-dnMAML. Thermal properties of SynB1-ELP-dnMAML make it possible to pull down all proteins bound to it. This time Notch 1 was identified in D54 cell line lysate demonstrating that SynB1-ELP-dnMAML does come into direct contact with Notch 1. Under the same experimental conditions no detectable Notch 1 is pulled down by SynB1-ELP.

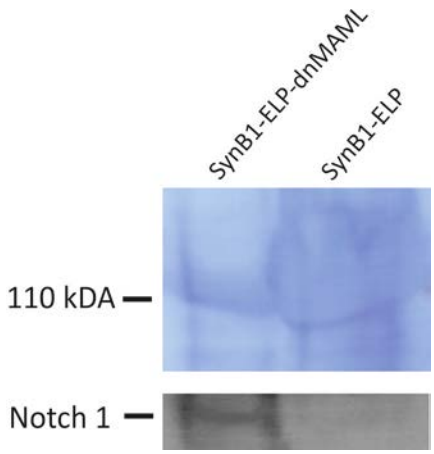

Figure 12. Western blot of Notch1 pull-down by SynB1-ELP-dnMAML. Whole cell lysates of D54 cells were incubated with equal amounts of SynB1-ELP-dnMAML and SynB1-ELP to confirm that dnMAML specifically binds to Notch1. Membrane was stained with Naphtol Blue Black after transfer and probed with Notch1 antibody

4.5. PROLIFERATION INHIBITION

D54 and U251 cells were chosen because of their metabolic and genetic differences. Both cell lines were treated with growing concentrations of SynB1-ELP-dnMAML for 1h at different temperatures in two 72h cycles. Same was done with SynB1-ELP to show that the vehicle protein itself is not cytotoxic, and with SynB1-ELP2-dnMAML to determine the effect of temperature on the efficiency of treatment. Resulting growth inhibition in both cell lines are shown in Figure 13. and Figure 14.

Both cell lines responded well to treatment with SynB1-ELP-dnMAML with inhibition effect ranging from 20% to 75% depending on the cell line and the application of heat.

In D54 cells significant inhibition was shown for all protein concentrations when compared to untreated controls. Level of inhibition is concentration dependent and is raised significantly by application of heat (48% and 18% of cells survived 30 μM protein treatment at 37 °C and 42 °C, respectively, $p < 0.0001$). For U251 cells overall growth rates are higher and effect of protein treatment is lower at all concentrations but still significant when compared to untreated controls. Observed heat effect was in this case not significant (50% to 44% of cells survived 30 μM protein treatment at 37 °C and 42 °C, respectively, $p < 0.0001$). As expected, SynB1-ELP2-dnMAML did not show similar inhibition confirming that phase transition and aggregation is important to achieve therapeutic levels of the protein in the cell. SynB1-ELP had no cytotoxic effect at any of the tested concentrations (Figure 13. b and 14. b).

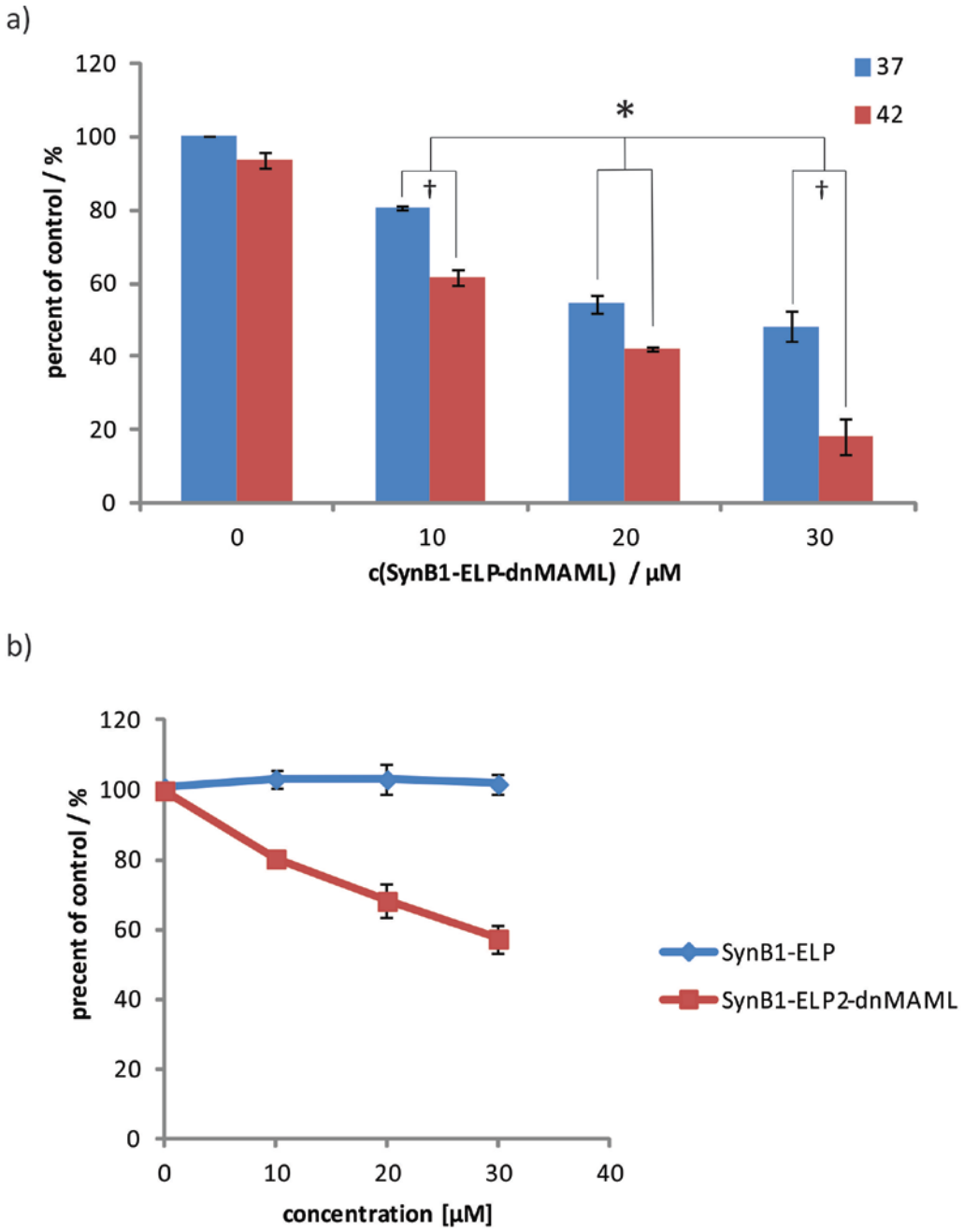

Figure 13. D54 proliferation assay. Graph **(a)** shows growth of D54 cells after 6-day treatment with SynB1-ELP-dnMAML at indicated concentrations and temperatures. Asterisk (*) indicates groups of treatments that significantly differ from controls ($p<0.0001$). Dagger (†) indicates groups with significant difference between heated and unheated samples at equal protein concentration ($p<0.0001$). Graph **(b)** shows growth pattern of cells treated with the vehicle polypeptide (SynB1-ELP) and the thermally unresponsive control (SynB1-ELP2-dnMAML) at 42°C. All results are presented by average of three independent experiments ± SD.

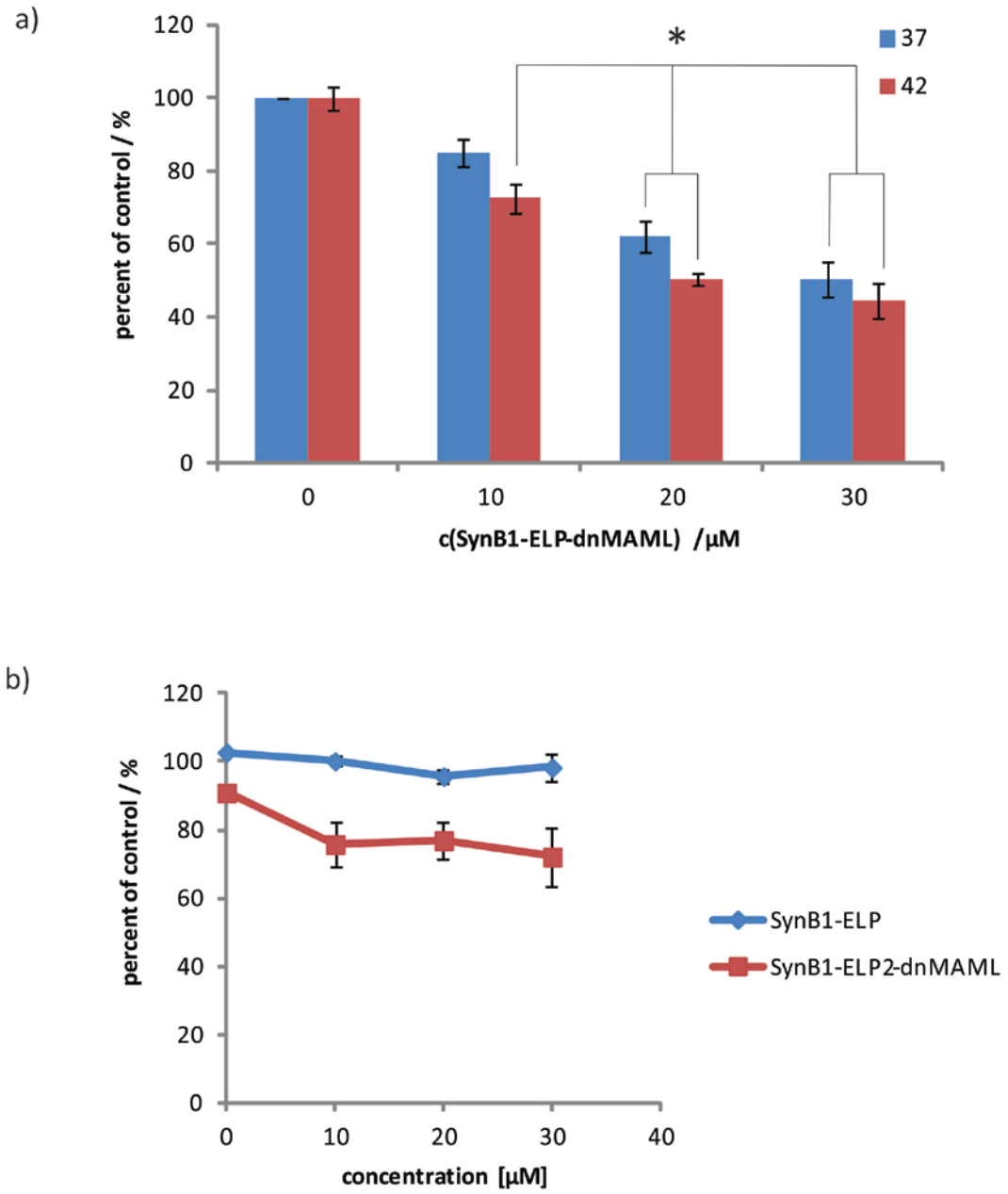

Figure 14. U251 proliferation assay. Graph (a) shows growth of U251 cells after 6-day treatment with SynB1-ELP-dnMAML at indicated concentrations and temperatures. Asterisk (*) indicates groups of treatments that significantly differ from controls ($p<0.0001$). Graph (b) shows growth pattern of cells treated with the vehicle polypeptide (SynB1-ELP) and the thermally unresponsive control (SynB1-ELP2-dnMAML) at 42°C. All results are presented by average of three independent experiments ± SD.

4.6. APOPTOSIS

Apoptosis is the way any organism deals with cells that are not needed any more or which have acquired too much damage. In cancer treatment apoptosis is targeted more than other methods of cell death and the goal is to activate apoptosis pathways in all treated cancer cells (De Bruin and Medema 2008). This way there is a lower risk of inflammation and other disease-related processes that could thwart cancer eradication. In D54 cells treatment with SynB1-ELP-dnMAML induces apoptosis in 20% cells more than the vehicle protein SynB1-ELP. Hyperthermia increases the percentage apoptotic cells to 40% in SynB1-ELP-dnMAML samples while it does not affect cells treated with SynB1-ELP (Figure 15. a). Treatment of D54 cells with SynB1-ELP-dnMAML in concurrence with hypethermia increases apoptosis 8-fold with respect to untreated cells. This makes for significant apoptosis induction for both treatment alternatives when compared to either control (p levels of $p<0.0001$ for all comparisons). Apoptosis in U251 cells are not induced this much. In heated SynB1-ELP-dnMAML samples, apoptotic cells make up for 20% of the total population. Treatment with SynB1-ELP causes apoptosis in 10% of the cells (Figure 15. b). Combination of hyperthermia and SynB1-ELP-dnMAML results in significant apoptosis induction ($p<0.0001$). In U251 cells effects of SynB1-ELP-dnMAML without hyperthermia are similar to those achieved with heat alone.

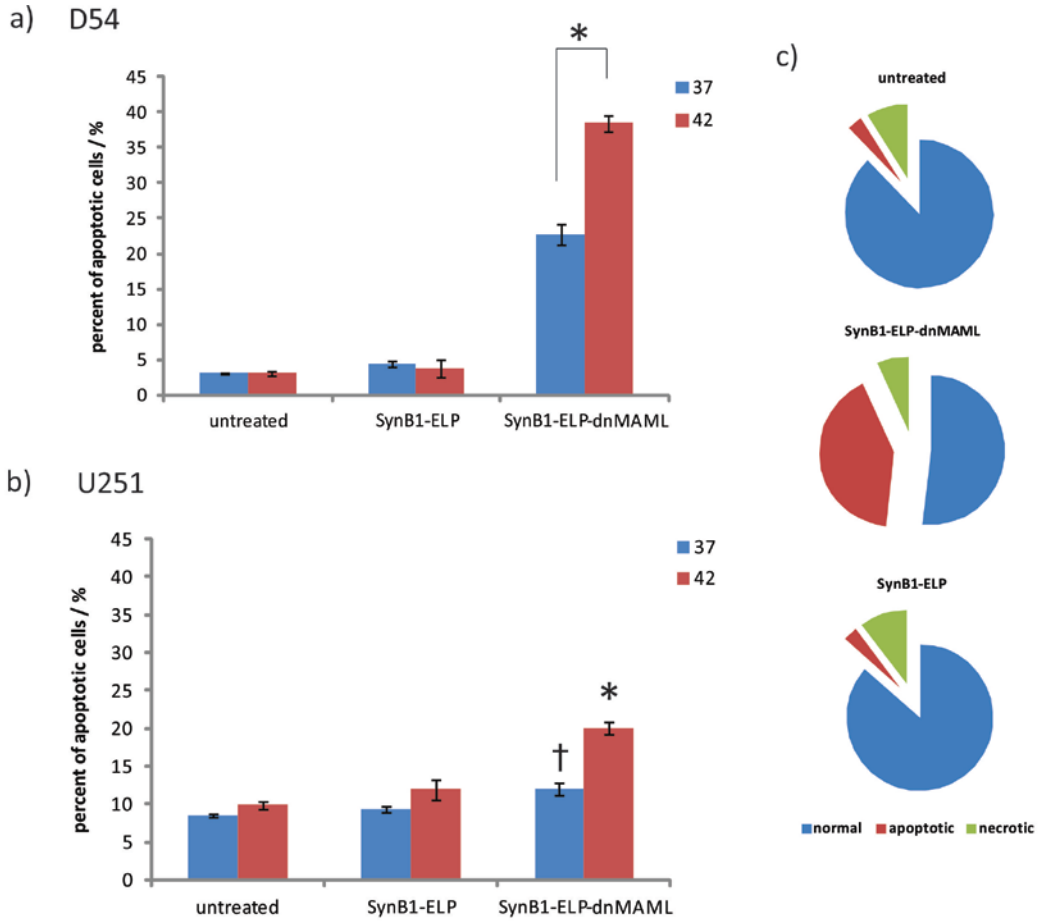

Figure 15. Apoptosis induction. D54 **(a)** and U251 **(b)** cells were treated with 30 µM of SynB1-ELP or SynB1-ELP-dnMAML on days 1 and 4. Percentage of apoptotic cells was determined by Annexin assay using flow cytometry after collecting both floating and attached cells on day 5. Percentage of apoptotic cells is plotted against protein treatment for each temperature. Asterisk (*) indicates groups with significant induction of apoptosis when compared to all samples and their normothermic counterparts ($p < 0.0001$). Dagger (†) represents sample that showed no significant increase when compared to heated control samples, both untreated and SynB1-ELP. Plot **(c)** shows representative samples to illustrate how all analyzed cell populations change depending on the treatment applied.

4.7. CELL-CYCLE DISTRIBUTION

Mechanism of growth inhibition also can mean stopping the cell cycle at crucial check points. Cancer cells are known to have low cell cycle control that allows many damaged cells to continue dividing. Cell cycle block is a less desirable way to stop cancer because cells can regain their full growth potential but it can also give the cell enough time to activate control mechanisms. Both cell lines treated with SynB1-ELP-dnMAML

demonstrate fewer cells in S phase and there is a visible lag in cell cycle, with more cells in both G0/G1 and G2/M phase (Figure 16. and 17.). SynB1-ELP-dnMAML treated samples show significant lag when compared to SynB1-ELP (significant difference in the number of cells in S phase p=0.004 for D54 cells and p=0.003 for U251 cells).

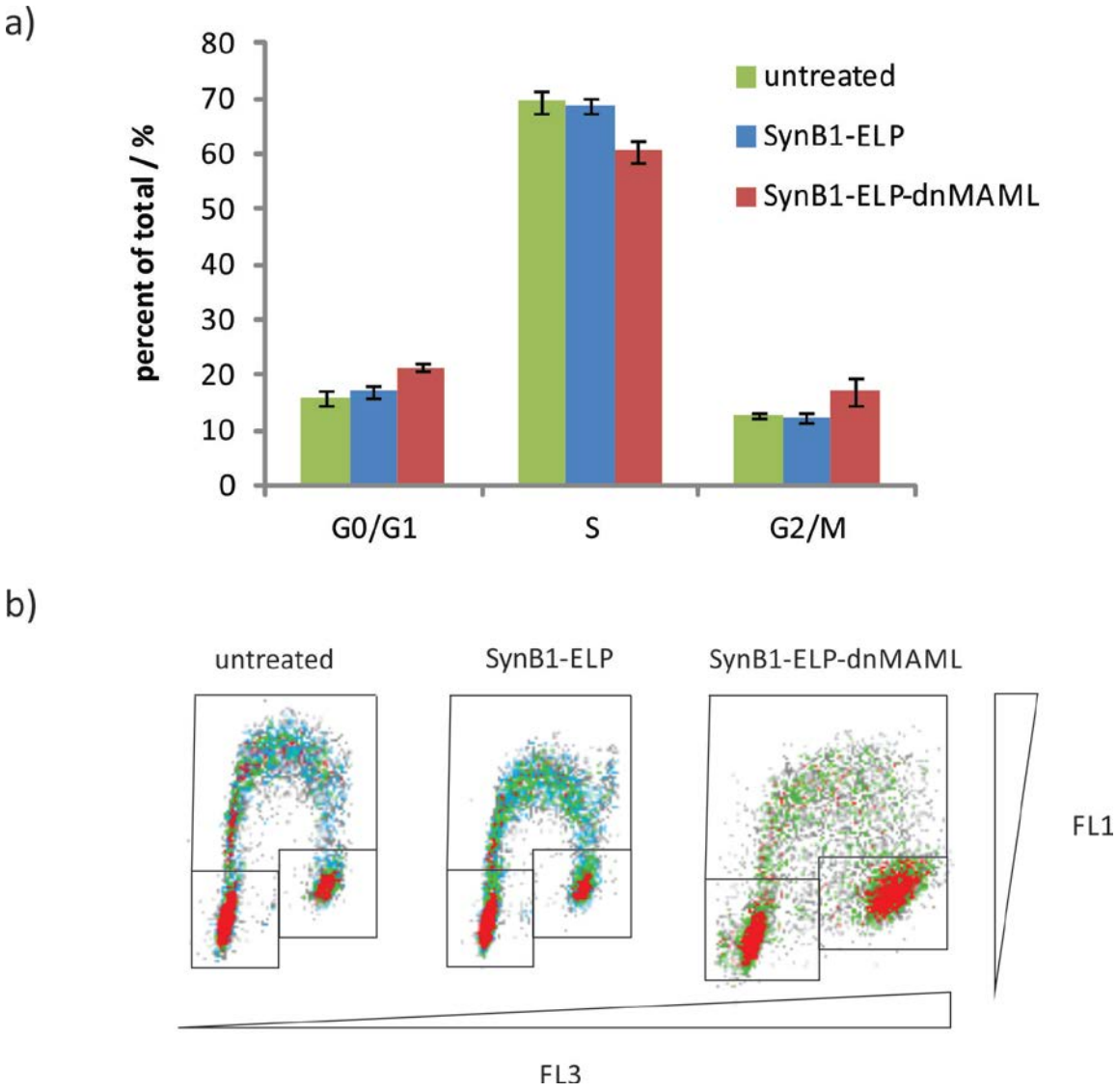

Figure 16. Cell cycle distribution for D54 cell line. Cells were treated with SynB1-ELP and SynB1-ELP-dnMAML, respectively, at 42°C on day 1 and day 4. Cells were pulsed with BrdU before being collected on day 5. Levels of BrdU incorporation were analyzed by flow cytometry. Results in plot **(a)** show percentage of cells in each indicated phase, with respect to treatment applied. Plot **(b)** shows representative raw data from flow cytometry (FL1 is the BrdU intensity and FL3 in PI intensity).

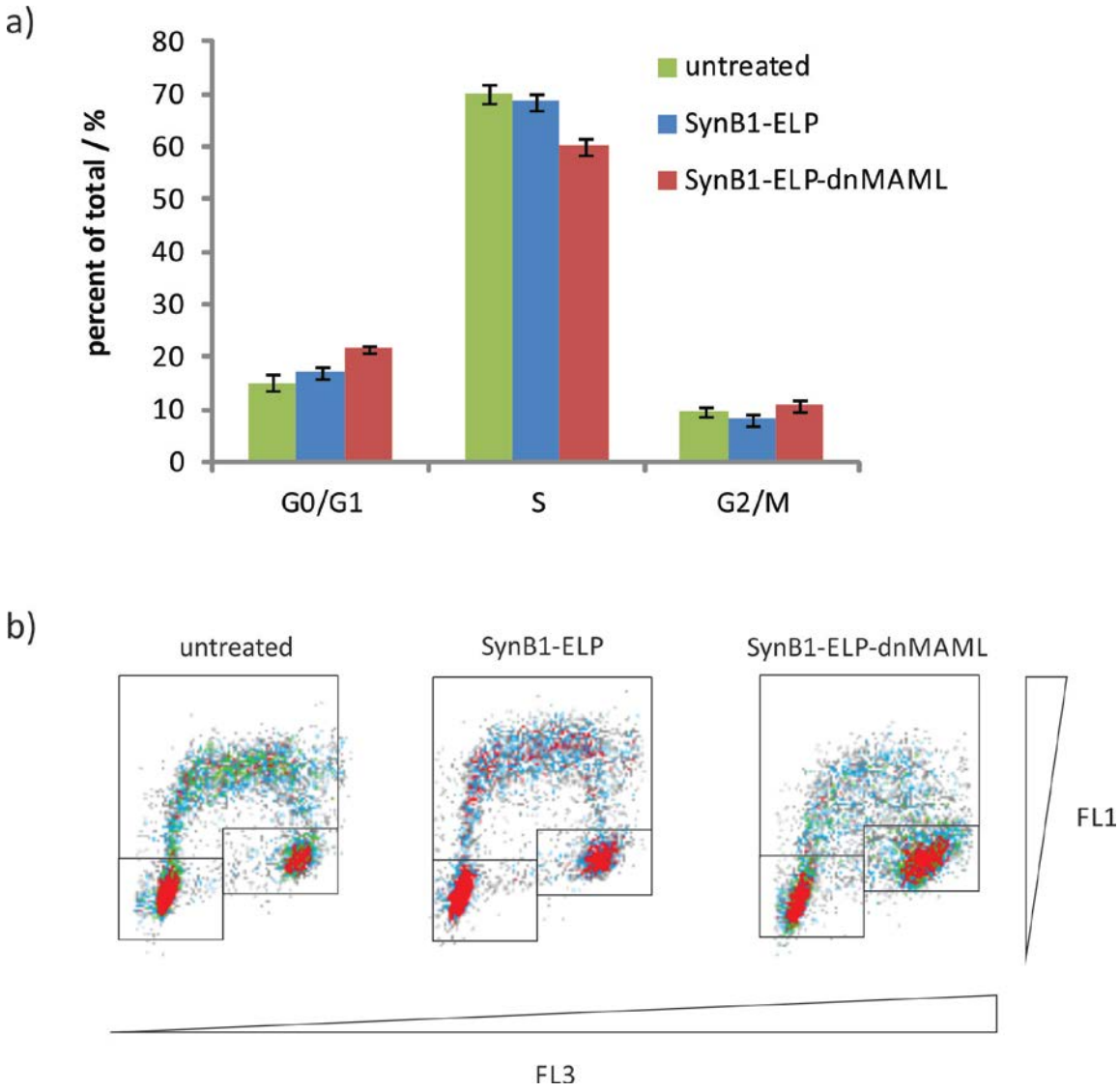

Figure 17. Cell cycle distribution for U251 cell line. Cells were treated with SynB1-ELP and SynB1-ELP-dnMAML, respectively, at 42°C on day 1 and day 4. Cells were pulsed with BrdU before being collected on day 5. Levels of BrdU incorporation were analyzed by flow cytometry. Results in plot **(a)** show percentage of cells in each indicated phase with respect to treatment applied. Plot **(b)** shows representative raw data from flow cytometry (FL1 is the BrdU intensity and FL3 in PI intensity).

4.8. SYNB1-ELP-DNMAML EFFECT ON EXPRESSION OF HES-1 AND HEY-L

Hes-1 and Hey-L are two well known targets of Notch. As Hes-1 can be activated by some other complexes, Hey-L is also used as an additional indicator of canonical Notch inhibition. Both are important proof that SynB1-ELP-dnMAML can really replace MAML1 in cellular function. Results show that no inhibition was achieved (Figure 18.). Expression levels of Hes-1 show no change after SynB1-ELP-dnMAML treatment (Figure 18. a). In D54 cells treatment lowers Hey-L levels slightly but not significantly ($p=0.052$). U251 cells show no change in expression of Hey-L (Figure 18. b)

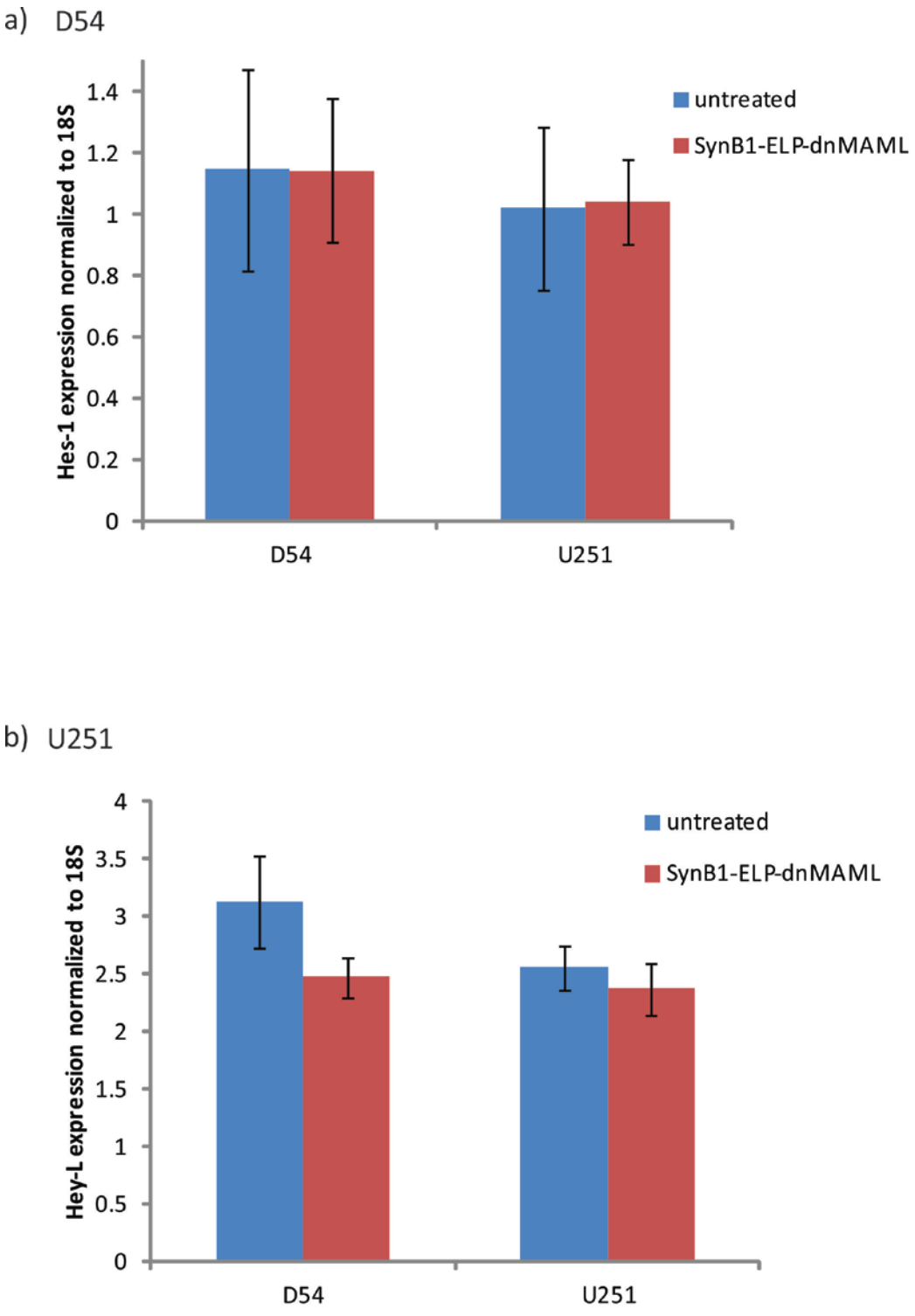

Figure 18. Expression levels of canonical Notch targets in D54 and U251 cells. D54 and U251 cells were treated with 30 µM SynB1-ELP-dnMAML overnight. Total RNA was extracted and cDNA transcribed with specific primers for target genes Hes-1 and Hey-L, as well as 18S RNA as reference gene. Graphs show expression levels of Hes-1 (a) and Hey-L (b) normalized to 18S. All results are presented as mean ± SD.

4.9. SYNB1-ELP-DNMAML EFFECT ON LEVELS OF NON-CANONICAL NOTCH TARGETS AND NOTCH-INDEPENDENT TARGETS OF MAML

MAML1 participates in signaling cross-talk in the cell. Effects on the modulation of the expression of p53, pAKT and MAPK proteins after the treatment with both SynB1-ELP and SynB1-ELP-dnMAML are shown in Figure 19. Interestingly, although D54 cells have been more affected in all experiments until this point, the effect on non-canonical Notch targets, MAPK and pAKT, is much more evident in U251 cell line. Effect is the consequence of dnMAML expression and it cannot be seen in the samples treated with SynB1-ELP.

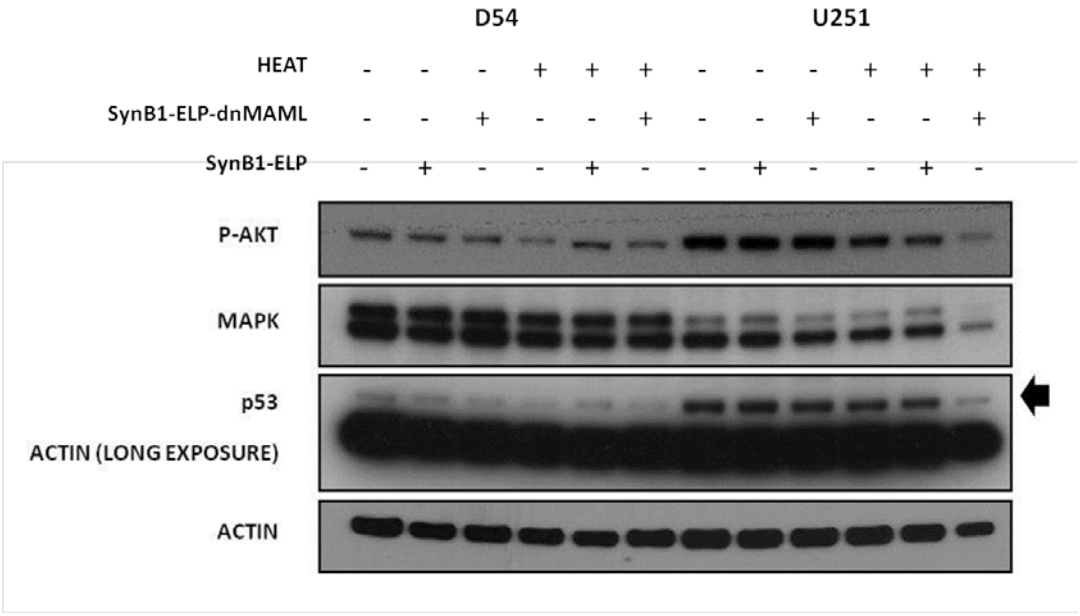

Figure 19. Western blot of selected proteins interacting with MAML1. Non-canonical targets of Notch and MAML1: MAPK and pAKT along with p53 show changes in expression levels upon treatment with 30 μM SynB1-ELP-dnMAML. Western blot was performed on whole cell lysates.

Hyperthermia alone causes a slight decrease in levels of most tested proteins. Levels of pAKT in D54 cells decrease more upon application of heat than with any other treatment. MAPK levels in D54 cells are reduced in hyperthermia and show minimum change when SynB1-ELP-dnMAML is added. In U251 cells levels of both pAKT and MAPK decrease somewhat in hyperthermic conditions. Treatment with SynB1-ELP-dnMAML in

combination with heat results in almost complete inhibition of expression of these two targets in U251 cells.

MAML1 interacts with p53 independently of Notch. Endogenous levels of wt p53 in D54 cells are very low. Separately, heat and SynB1ELP-dnMAML appear to lower those levels further. p53 levels in cells treated with the combination of heat and SynB1-ELP-dnMAML are similar to levels found in samples treated with heat or SynB1-ELP-dnMAML alone. U251 cells have mutant p53. Levels of p53 protein in U251 cells is reduced significantly with the SynB1-ELP-dnMAML treatment combined with hyperthermia. The difference in p53 levels observed between wt p53 in D54 cells and mutant p53 in U251 cells could indicate discrimination between the two by Synb1-ELP-dnMAML.

5. DISCUSSION

Targeted molecular medicine is considered direction of the future moving towards the final goal of personalized medicine. One of the aspects of this development is the ability to target and treat diseases in the affected area without intruding on the rest of the organism.

Notch pathway belongs to a group of highly conserved signaling mechanisms that control a vast number of different development and maintenance related paths. The reasoning behind targeting such a pathway is in its high hierarchical position, which implies the possibility to disrupt more than one signaling cascades with only one agent. Currently γ-secretase inhibitors (GSI) are the choice of inhibitors for Notch. They are cheap, easy to produce and administer in clinic, however have several drawbacks. GSI block all Notch signaling regardless of receptor specificity and administering them systemically has some severe side effects. Also, there are considerable off-target effects coming from the fact that GSI have a wide array of targets apart from Notch (Rizzo et al. 2008).

Alternative ways to target Notch have focused on nucleic acids and various ways to block Notch by either using siRNAs for Notch or transfecting the cells with expression vector containing NICD or MAML1 (Gilbert et al. 2010; Wu et al. 2000; Perumalsamy et al. 2009). Protein use in this research was limited to synthesized Notch soluble ligands to mimic activation (Zhao et al. 2007).

In the work published by Chen and coworkers, dnMAML was used as an alternative to classical GSI treatment. Obtained results show effective inhibition of Notch signaling by increased expression of dnMAML using a lentiviral expression vector (Chen et al. 2010). Glioblastoma cell lines they used exhibited high levels of both Notch receptors and their target genes. In 2009 Moellering and his colleagues published a paper describing the use of a peptide fragment of dnMAML to directly inhibit assembly of Notch activation complex (Moellering et al. 2009). Unlike Chen's results their work was done in T-ALL, the most researched Notch related cancer, in which the role of this pathway was much better known than in GBMs, but it was the first peptide based direct inhibitor of Notch and a perfect candidate for ELP targeting. Structural requirement in Notch activation complex assembly is an intact helical secondary structure of MAML (Nam et al. 2003).

Stapling amino acids at critical positions ensure a rigid and resistant structure since modified amino acids are less susceptible to proteolysis.

After considering all the previously mentioned, SynB1-ELP-dnMAML was the final choice of Notch inhibitor to base this work on. Thermal characterization confirmed that ELP is unaffected by the addition of SynB1 and dnMAML. The transition temperature of the complete construct is lower than that of pure ELP. Construct is thermally responsive but its functionality depends on dnMAML being able to fold correctly while attached to ELP. Structural integrity of dnMAML is essential for its function since the assembly of the transcription activation complex is guided by strict geometrical arrangements of all proteins involved (Nam et al. 2006). Shorter peptides and chemotherapeutics have been successfully attached to ELP without their function being compromised by this macromolecular carrier (Moktan and Raucher 2012; Massodi et al. 2010). In this case, it was decided to use a protein fragment of over 60 amino acids. The hypothesis was that this is functional length of dnMAML and it might just be long enough to insure proper secondary structure without the need for stapling. It was also an opportunity to test one more possible type of cargo for the ELP system since it was not done before.

SynB1 is a potent CPP with good cytoplasmic localization that gave entire construct the possibility to interact with more targets besides Notch. Since very small amounts of NICD can actually be found in the nucleus, we hypothesized that the amount of SynB1-ELP-dnMAML that enters the nucleus passively, as a result of cytoplasmic accumulation, will suffice to block nuclear NICD (Sebbage 2009). Also, emerging information about the role of endosomal trafficking in activation of Notch receptor (Kopan and Ilagan 2009) led to the choice of cytoplasmic CPP.

Choice of an adequate model system is of great importance in determining work progression. All of the protein-based therapeutics developed both in this work and the previously studied ones, are destined to be translated into clinical application and choice of in vitro cell lines is lead by that condition in mind. D54 and U251 cell lines presented as good candidates on several bases. Both cell lines are well established and have been in use for more than 30 years now. Both D54 and U251 cell lines were established from primary samples of high grade gliomas at Duke University and the

University of Uppsala, respectively. Both cell lines are tumorigenic in nude mice and were capable of colony forming in soft agar (Bigner et al. 1981; Pontén and Westermark 1978). Additional cell line considered was U87 but, it was ruled out because it is not considered a good GBM model in vivo (Jacobs et al. 2011). Both of the considered cell lines were transfected with luciferase. Luciferase is important in *in vivo* imaging and testing the exactly same cells *in vitro* gives more reliable data correlation. D54 and U251 differ in their p53 status, with D54 having wild-type p53 and U251 mutant p53 with arginine to histidine mutation (Gomez-Manzano et al. 1996; Van Meir et al. 1994). Both cell lines have been analyzed on proteomic level and have differential expression of CDKs, cyclin D3, PI3-K, MAPK and AKT (Jacobs et al. 2011; Mendes et al. 2007). On the other hand, these cell lines share certain main characteristics, such as histological grade, origin and growing conditions and are good translation models. Differences in expression of fore-mentioned proteins provide more insight into the effects of the tested Notch inhibitor.

SynB1-ELP-dnMAML was successfully cloned in blocks from separate sequences with their distinct functions. Yield and purity of each construct appears to depend on the N-terminal sequence. SynB1 constructs have better yield and are more easily purified than constructs bearing other CPPs (for example, Bac) on N-terminus (unpublished data). Why this happens has not yet been explained. Thermal transition of the newly synthesized protein was checked and effective transition was observed. As expected, additional sequences lower the temperature of SynB1-ELP-dnMAML when compared to the same concentration of pure ELP. Most tested concentrations, however, remained within the range of treatment conditions (Figure 8.). Length of dnMAML fragment did not affect ELP's aggregation, key characteristic needed for successful thermal targeting. Inserting ELP's high transition temperature version termed ELP2, gave the same construct with T_t of about 60° C (Figure 9.). The only difference between the two is the amino acid composition of the ELP, namely the ratio of the guest residue in the fourth position in the repeat (V:G:A ratio of 5:2:3 of ELP versus 1:7:8 for ELP2). High transition temperature of SynB1-ELP2-dnMAML ensures minimal aggregation in experimental conditions, making it a good control to distinguish the effects of aggregation on the overall efficiency of SynB1-ELP-dnMAML. SynB1-ELP was chosen as functional control

for SynB1-ELP-dnMAML, corresponding to empty vector controls in similar experiments with siRNA or antisense DNA based inhibition.

The results of confocal microscopy (Figure 10.) show distinct cytoplasmic localization with no protein accumulating on the membrane and no protein detected in the nucleus. This is consistent with reported data for SynB1 (Moktan and Raucher 2012; Bidwell et al. 2010). NICD in the nucleus is present in such small quantities that it cannot be detected by immunochemical methods, so any protein bound to NICD in the nucleus would be below the level of detection by this method. Confirming the ability of SynB1 to take the protein into the cell successfully is indispensable for further experiments.

Cellular uptake results show the combined effect of aggregation and CPP induced cellular intake. These experiments were done with trypan to quench membrane bound fluorescence (Vives et al. 2003) and can consequently be considered reliable in quantifying the total amount of protein in the cell. The difference in uptake between D54 and U251 cells can possibly be explained by different mechanisms of entry that results in variation to receptiveness to protein transduction. It is important to note that the heat effect remains an important contributor to the overall uptake in both cell lines. Cells treated with SynB1-ELP2-dnMAML, the thermally unresponsive control, have minor amounts of protein detected in the cell. Increase due to heat can be attributed to higher membrane fluidity in hyperthermia conditions (Balogh et al. 2011). In spite of the difference between the cell lines, the increase of protein amounts inside the cells is significant for both (5-fold and 3-fold for D54 and U251, Figure 11.) and justify the use of hyperthermia for optimization of delivery.

Previous work done in the field of peptide delivery and targeting by dr. Raucher and his associates has been focused on short peptides (Bidwell et al. 2012; Massodi et al. 2010) and chemotherapeutics (Bidwell et al. 2007; Moktan et al. 2010) bound to ELP. The work presented here deals, for the first time, with a sequence longer than a dozen amino acids attached to ELP. The intent was to show that even full length proteins can be successfully attached to ELP and maintain their secondary structure and function. In the case of dnMAML, this is of particular importance due to the fact that its alpha

helical structure is crucial in binding NICD and other transcription factor proteins (Nam et al. 2006). Synthesis of protein by hyper-expression in bacteria limits the modification of amino acids incorporated into the protein. Stapling dnMAML by inserting not naturally occurring amino acids into the sequence to be chemically cross-linked (Moellering et al. 2009) is not possible. Structural stability and protection from proteolysis provided by stapling is in this model ensured by macromolecular ELP. ELP could also interfere with function or prevent proper folding therefore impairing function of dnMAML.

The ability to bind NICD is a sure way to test whether or not proper structure of dnMAML is maintained after the attachment of ELP. Activation of Notch signaling, with corresponding high levels of Hes-1 was found in secondary GBM samples and high grade tumors (Stockhausen et al. 2010). Since both cell lines are derived from grade IV tumors there should be enough endogenous Notch protein to confirm binding to SynB1-ELP-dnMAML. Cell lysate was incubated with SynB1-ELP-dnMAML. SynB1-ELP was used as control because ELP itself can pull some protein randomly upon aggregation. In Figure 12. membrane after transfer shows equal levels of coloration with obvious overload due to high concentration of ELP constructs used. When probed with Notch 1 specific antibody, on the other hand, only one visible band is seen. SynB1-ELP-dnMAML pulled a considerable amount of Notch 1 from the cell lysate. The amount bound to SynB1-ELP is below detection level, showing clearly that dnMAML is responsible for Notch binding. These results confirm that function of dnMAML is preserved. It should be noted that the antibody used in this pull down experiment detects total Notch 1 protein and cannot be used to assess the level of Notch signaling activity.
All experiments confirmed that the first aim of producing a functional thermally responsive protein with the structure of dnMAML domain preserved can be accomplished in a bacterial system without additional structure stabilization.

Effectiveness of SynB1-ELP-dnMAML depends on different factors. Levels of activated Notch in the form of NICD, as well as the availability of non-canonical targets, are all going to affect final result. Additional issues could come from insufficient delivery to the nucleus and the effect that each of the Notch controlled components has on the overall

growth rate. These problems would result in inability to inhibit cell growth. Results of growth inhibition in both D54 and U251 cells show that in the case of SynB1-ELP-dnMAML considerable growth inhibition can be achieved (Figure 13. a and 14. a). Only 20% of D54 cells survive at the highest protein concentration used and the lowest 10 µM concentration kills 40% of treated cells. U251 cells are less affected and show between 70% and 45% cell survival at treatment concentrations of 10 µM and 30 µM respectively. Aside from these differences both cell lines show significant growth inhibition (with p values below 0.0001) in all treated samples when compared to controls. D54 cells exhibit significant heat effect if heated and unheated samples at the same protein concentrations are compared. In U251 cells heat doesn't have significant effect on the growth of treated cells. There is little cell line specific data for growth inhibition of D54 cells to compare with. The results observed in heated U251 are very similar to growth inhibition results (40% to 60% survival) reported in the same cell line after transfection with dnMAML carrying vector (Chen et al. 2010). The same article shows that dnMAML successfully blocks growth of different glioma derived cell lines (U87, LN827, LN428) besides U251. Non peptide based approaches for Notch inhibition such as transfecting the cells with Notch1 siRNA gave positive results in U251 xenograft tumors as well (Xu et al. 2010). SynB1-ELP2-dnMAML treatment results show inhibition but when compared with same concentration of SynB1-ELP-dnMAML prove once more the importance of hyperthermia and confirm that inhibition is directly linked to the protein levels in the cell. SynB1-ELP has no effect on the growth of both cell lines and can hence be used as control for all following experiments.

After confirming the hypothesis that SynB1-EL-dnMAML can significantly inhibit growth of glioblastoma cells, a further look into the mechanism is necessary to confirm Notch inhibition.

Apoptosis induction and cell cycle arrest are two common ways looked for in most cancer therapeutic candidates (Evan and Vousden 2001). Potent apoptosis induction is a landmark of every good anti-cancer agent and it was the first cell death mechanism investigated. Apoptosis was induced using SynB1-ELP-dnMAML and this time a functional control was used, SynB1-ELP, vehicle protein without the functional moiety. As indicated by the growth inhibition experiments apoptosis was much better induced

in D54 cells than in U251 cells. SynB1-ELP induced apoptosis in less than 5% of D54 cells and in 10% of U251 cells consistent with the number of apoptotic cells present in untreated samples (Figure 15. a and b). This showed that the vehicle itself is not cytotoxic and does not contribute to the overall observed effect. Induction of apoptosis is supposed to be one of the effects of Notch inhibition and has been confirmed in glioblastoma (Chen et al. 2010; Xu et al. 2010) as well as in T-ALL (Moellering et al. 2009). In the above listed articles Notch inhibition was confirmed by expression levels of main targets like Hes-1 and Hey-L and correlated to percentage of apoptotic cells and caspase activity in treated samples. In D54 cells both heated and unheated cells treated with SynB1-ELP-dnMAML show significant levels of apoptosis (Figure 15. a). According to these results underlying mechanism of cell killing in D54 cells is induction of apoptosis. That does not seem to be the case with U251 cells where the difference between dnMAML treated samples and samples treated with vehicle protein doesn't exceed 10%. Unheated SynB1-ELP-dnMAML does not seem to induce apoptosis any better than heated SynB1-ELP. Observed percentage of apoptotic cells for U251 cell line does not correspond to the levels of inhibition and cannot be the principal mechanism involved.

Cell cycle block can be the reason for increased apoptosis induction or it can slow cell growth on its own. Giving the cells more time to control the results of DNA replication and the overall multiplication process can activate apoptosis if damage is too great or just prolong the period between replications to assure corrections are made. Cells that are in one of the dormant cycle phases present an obstacle to efficient cancer eradication because of their ability to restart dividing at any given moment (Maddika et al. 2007).

Elevated dnMAML levels in LN827 cell line cause a G0/G1 phase arrest according to work done by Chen and coworkers where they over expressed dnMAML by transfecting the cell with a lentiviral vector. Similar results are reported in U251 cells by Xu and colleagues who used Notch siRNA approach. Purrow et al. used siRNA approach and U251 cells as well, but reports G2/M phase arrest. Obtained results do not show an obvious bias towards any of the phases. In this case, D54 and U251 cells behave in a very similar manner, showing a decrease in actively dividing cells and more cells in both

G0/G1 and G2/M. Percent-wise numbers are not substantial but are still statistically significant (S phase decrease with p values of 0.002 and 0.001 for D54 and U251 cells respectively). The reason for retardation of cell cycle progression in both G0/G1 and G2/M may the fact that samples used in this experiment were not synchronized.
Apoptosis and cell cycle results show that SynB1-ELP-dnMAML can indeed inhibit the growth of glioblastoma cells but the main goal is not achieved till the Notch pathway is proved to be the cause of the proliferation inhibition.

Importance of inhibiting Notch in glioblastoma has an even greater impact. Constitutive Notch activation results in resistance of GBMs to radiation and more importantly regulates invasiveness and stem cell renewal (Wang et al. 2010; Tchorz et al. 2012). In astrocytic GBMs these characteristics are controlled by differential expressions of Notch 1 and Notch 2 (Xu et al. 2013). Secondary GBMs that develop from primary low grade astrocytomas progress from small populations of neural stem cells with high levels of active Notch (Notch 2 in this case) that directs cell away from differentiation (Tchorz et al. 2012). Inhibition of Notch with GSIs increased efficacy of temozolomide treatment and more importantly prevented recurrence and neurosphere repopulation. Complete renewal of neurospheres overexpressing NICD confirms Notch is the main regulator of stem cell growth (Gilbert et al. 2010). Main culprits for recurrence and resistance are stem cells that can be found in small percentages. Hypoxic conditions within the tumor are conducive to stem cell generation (Bar et al. 2010). These processes have been repeatedly linked to Notch (Qiang et al. 2012; Gustafsson et al. 2005).

Because of versatile nature of the network and multiple ways to generate signal inhibition has to be confirmed in both canonical and non-canonical targets. Canonical targets are downstream gene families Hes and Hey. Expression levels of representative genes for these two families, Hes-1 and Hey-L, were measured in D54 and U251 cells. The decision to measure both instead of only Hes-1 although it is prevalent in literature was made because expression of Hes-1 can be enhanced independently of Notch by environmental factors such as hypoxia (Poellinger and Lendahl 2008). Unfortunately, SynB1-ELP-dnMAML does not seem to affect canonical Notch targets (Figure 18.). Inhibition observed is negligible for both cell lines. Hey-L seems to respond a little

better, in D54 cells, but still below significance level. This kind of result is unexpected since the pull down showed good binding between Synb1-ELP-dnMAML and his intended target (Figure 12.). There are several ways to explain these two observed results. First, there is a difference between binding in the cell and in the cell lysate. Although structurally compatible, two molecules may not come into contact in the cell. Second, canonical pathway ends in the nucleus. SynB1-ELP-dnMAML was made to localize in the cytoplasm. According to published theories on CPP transport, nuclear entry is thought to be passive, the result of CPP-linked cargo accumulating in the cytoplasm (Sebbage 2009). With SynB1-ELP-dnMAML there is no nuclear protein detected (Figure 10.), although cytoplasmic accumulation of protein is substantial. It can be easily concluded that insufficient delivery to the nucleus is the main reason for lack of inhibition of Notch canonical signal. Since, ELP peptide constructs are very easy to optimize, this particular problem could be resolved by attaching a nuclear localization sequence to the end of the existing construct or cleavage site in the linker between dnMAML and ELP. These changes, that are necessary to make an efficient Notch inhibitor, should be the basis for further investigation. D54 cells, that show minimum inhibition of Hey-L and minimal non canonical inhibition, should be further investigated to find the cause of inhibition and apoptosis observed. A wider array of non-canonical targets of both NICD and MAML should provide additional insight into the mechanism of inhibition of these cells.

Non-canonical Notch targets are cellular processes that are controlled by NICD or MAML1 independently of their role in the transcription activation complex. These interactions make basis for cross talk between different signaling cascades in the cell. Notch has been linked to numerous pathways: Wnt/β-catenin, Sonic Hedgehog, Ras/MAPK, pAkt, NF-κB to name just a few (Perumalsamy et al. 2009; Zhang et al. 2012; Liu et al. 2006; Mittal et al. 2009).

AKT activation is often detected in cancer cells and aberrations help cells avoid neglect-induced cell death due to lack of cytokines or nutrients. Notch controls levels of pAKT through NICD independently of CSL (Perumalsamy et al. 2009). Cooperation between Notch and AKT was confirmed in various cell lines and is also found in glioblastoma (Guo et al. 2009; Liu et al. 2006). Notch can either activate phosphorylation of AKT, or it

can down-regulate it. Regulation of pAKT links Notch to NF-κB and β-catenin that are in turn regulated by AKT activity (Zhang et al., 2012). SynB1-ELP-dnMAML significantly decreases the levels of pAKT in U251 cells, while having negligible effect on D54 cells (Figure 19.). It is important to note that the only sample showing this decrease in pAKT is the sample treated with SynB1-ELP-dnMAML at 42 °C. Since pAKT levels of the 37 °C-treated samples are comparable to control, threshold value may need to be overcome in order to show effect. This effect is contrary to the effect observed after transfecting the cells with a vector carrying NICD to mimic activation of Notch pathway (Zhang et al. 2012; Perumalsamy et al. 2009). Endogenous high pAKT are also found in GBM tissue samples. This however, is not valid for all cell types. In epithelial cells Notch activation leads to negative regulation of pAKT (Liu et al. 2006) indicating the above-mentioned dual role and context-dependent effects of Notch.

In a manner much like to the one just described for pAKT Notch regulates MAPK. Positive correlation between activities of Notch and MAPK was found in breast cancer and same as for pAKT negative regulation takes place in epithelial cells (Mittal et al. 2009; Liu et al. 2006). Again, as was the case with pAKT U251 cells show significant effect, while in SynB1-ELP-dnMAML did not induce any change in levels of MAPK in D54 cells (Figure 19.). MAPK activation is an important step in signal transduction and Notch control over MAPK levels increases the number of targets that can be reached by effective Notch inhibition.

SynB1-ELP-dnMAML results are comparable to those obtained by inhibition of Notch with siRNA showing that it can efficiently block non-canonical Notch signaling. With all the additional cross talk involved in this aspect of Notch signaling and the fact that non canonical pathway cannot be blocked by using GSIs validates the use of peptide-based Notch inhibitors even more.

There are functions of MAML1 completely independent of Notch, meaning that those functions are a result of direct MAML1 interaction with targets without NICD or CSL. One of such is MAML1 interaction with p53. If SynB1-ELP-dnMAML is indeed fully functional MAML1 domain it should be able to replace MAML1 in those aspects as well.

MAML1 was identified as a co-activator of p53 by Zhao and coworkers in a paper published in 2007 (Zhao et al. 2007). That same paper shows that N-terminal region of MAML1 comes in direct contact with DNA binding domain of p53 and stabilizes p53 consequently increasing p53 levels in the cell. D54 cells have wt p53 and U251 cells have mutant p53. In D54 cells, SynB1-ELP-dnMAML does not increase p53 level, what is more, it is slightly decreased. The article does not explore the interaction of mutant p53 and MAML1 which is what should happen with SynB1-ELP-dnMAML in U251 cells. SynB1-ELP-dnMAML, along with hyperthermia lowers the level of p53 in those cells to barely detectable (Figure 19.). This point warrants further research. p53 control is shared by other developmental pathways present in cancer, like Hedgehog (Stecca and Altaba 2009), so results can be conflicting. There is very little data on the effect of MAML on p53, especially on various mutants present in cancer, and these results show that it is a subject worth exploring.

Taken together all these results impress once more the complexity and importance of Notch in cell proliferation and differentiation. It also proves as a very valid target for future development of therapeutics. As for SynB1-ELP-dnMAML, it can be made to inhibit both canonical and non-canonical Notch pathway which is an obvious advantage compared to GSIs when the impact of non-canonical Notch cross talk with other signaling pathways is taken into consideration. Other advantages that need to be considered is the possibility of active thermal targeting and facilitated delivery across the blood-brain barrier since systemic Notch inhibition can have serious consequences due to context dependent Notch effects in various cell types.

Overall SynB1-ELP-dnMAML has shown that ELP based therapeutics can be expanded to include full size proteins. It is a good first step in trying to find effective ways to target pathways with such diverse roles.

GBM therapy is in dire need of something similar to what SynB1-ELP-dnMAML can do and that is why with further research, optimization and the technology required to implement thermal targeting already in place approaches like this hold great promise for the future of peptide based personalized medicine.

6. CONCLUSION

From the presented results we can conclude the following:

- dnMAML can be successfully attached to ELP
- complete protein construct displays a lower transition temperature than ELP alone but still provides an acceptable range of concentrations within the designated temperature window
- dnMAML efficiently inhibits both D54 and U251 cell lines with inhibition more pronounced in D54 cells
- application of heat enhances effects of SynB1-ELP-dnMAML as well as protein uptake into the cells proving that the combination of ELP and hyperthermia is important in achieving therapeutic concentrations of protein in the cells
- cytosolic localization determined by the presence of SynB1 in the construct does not impede dnMAML function but it does not affect Notch canonical targets

As far as cell behavior after treatment is concerned the conclusion are:

- D54 and U215 cells respond differently to Notch inhibition displaying variations in effect that Notch has on each cell line
- both cell lines cells undergo apoptosis after treatment with SynB1-ELP-dnMAML, while it is lower in U251 cells
- both cell lines respond to treatment by lowering the rate of actively replicating cells as cell cycle distribution shows decrease in S phase with corresponding increase of G0/G1 and G2/M phase
- U251 cells show greater inhibition of non-canonical Notch targets MAPK and pAKT than D54 cells
- dnMAML seems to have no effect on wt p53 levels in D54 but greatly reduces levels of mutated p53 in U251 cells

Overall conclusion is that in SynB1-ELP-dnMAML ELP did not impede proper folding of dnMAML essential for its function. Non-canonical Notch targets MAPK and pAKT levels in SynB1-ELp-dnMAML treated cells are similar to levels observed in systems with efficient Notch inhibition. With optimizations to improve nuclear entry, this construct could replace GSIs in treatment of GBMs. Being the first construct with a full length protein attached in place of much shorter peptides it opens a new direction and new possibilities for application of ELP based systems in both therapy and research.

7. LITERATURE

Agrawal, N., Frederick, M. J., Pickering, C. R., Bettegowda, C., Chang, K., Li, R. J., Fakhry, C., Xie, T.-X., Zhang, J., Wang, J., Zhang, N., El-Naggar, A. K., Jasser, S. A., Weinstein, J. N., Trevino, L., Drummond, J. A., Muzny, D. M., Wu, Y., Wood, L. D., Hruban, R. H., Westra, W. H., Koch, W, M., Califano, J. A., Gibbs, R. A., Sidransky, D., Vogelstein, B., Velculescu, V. E., Papadopoulos, N. Wheeler, D. A., Kinzler, K. W., Myers, J. N., 2011. Exome Sequencing of Head and Neck Squamous Cell Carcinoma Reveals Inactivating Mutations in NOTCH1. *Science*, 333(6046), pp.1154–1157.

Andersen, P., Uosaki, H., Shenje, L. T., Kwon, C., 2012. Non-canonical Notch signaling: emerging role and mechanism. *Trends in cell biology*, 22(5), pp.257–265.

Arkin, H. and Bilsel, M., 2010. How conformational transition depends on hydrophobicity of elastin-like polypeptides. *The European physical journal. Soft matter*, 31(3), pp.327–332.

Artavanis-Tsakonas, S., Rand, M.D., Lake, R.J., 1999. Notch signaling: cell fate control and signal integration in development. *Science*, 284(5415), pp.770–776.

Balogh, G., Maulucci, G., Gombos, I., Horváth, I., Török, Z., Péter, M., Fodor, E., Páli, T., Benkő, S., Parasassi, T., De Spirito, M., Harwood, J. L., Vígh, L., 2011. Heat stress causes spatially-distinct membrane re-modelling in K562 leukemia cells. *PLoS ONE*, 6(6), p.e21182.

Bar, E.E., Lin, A., Mahairaki, V., Matsui, W., Eberhart, C. G., 2010. Hypoxia increases the expression of stem-cell markers and promotes clonogenicity in glioblastoma neurospheres. *The American journal of pathology*, 177(3), pp.1491–502.

Bidwell 3rd, G.L., Davis, A. N., Fokt, I., Priebe, W., Raucher, D., 2007. A thermally targeted elastin-like polypeptide-doxorubicin conjugate overcomes drug resistance. *Investigational New Drugs*, 25(4), pp.313–326.

Bidwell 3rd, G.L. and Raucher, D., 2005. Application of thermally responsive polypeptides directed against c-Myc transcriptional function for cancer therapy. *Molecular cancer therapeutics*, 4(7), pp.1076–85.

Bidwell 3rd, G.L. and Raucher, D., 2010. Cell penetrating elastin-like polypeptides for therapeutic peptide delivery. *Advanced Drug Delivery Reviews*, 62(15), pp.1486–1496.

Bidwell 3rd, G.L., Whittom, A., Thomas, E., 2010. A thermally targeted peptide inhibitor of symmetrical dimethylation inhibits cancer-cell proliferation. *Peptides*, 31(5), pp.834–841.

Bidwell 3rd, G.L., Fokt, I., Priebe, W., Raucher, D., 2007. Development of elastin-like polypeptide for thermally targeted delivery of doxorubicin. *Biochemical pharmacology*, 73(5), pp.620–31.

Bidwell 3rd, G.L., Perkins, E., Raucher, D., 2012. A thermally targeted c-Myc inhibitory polypeptide inhibits breast tumor growth. *Cancer letters*, 319(2), pp.136–43.

Bigner, D.D., Bigner, S. H., Pontén, J., Westermark, B., Mahaley, M. S., Ruoslahti, E., Herschman, H., Eng, L. F., Wikstrand, C. J., 1981. Heterogeneity of Genotypic and phenotypic characteristics of fifteen permanent cell lines derived from human gliomas. *Journal of neuropathology and experimental neurology*, 40(3), pp.201–229.

De Bruin, E.C. and Medema, J.P., 2008. Apoptosis and non-apoptotic deaths in cancer development and treatment response. *Cancer treatment reviews*, 34(8), pp.737–749.

Campa, V.M., Gutiérrez-Lanza, R., Cerignoli, F., Díaz-Trelles, R., Nelson, B.,Tsuji, T., Barcova, M., Jiang, W., Mercola, M., 2008. Notch activates cell cycle reentry and progression in quiescent cardiomyocytes. *The Journal of cell biology*, 183(1), pp.129–41.

CBTRUS, 2012. The Central Brain Tumor Registry of the United States of America factsheet. Available at: http://www.cbtrus.org/factsheet/factsheet.htlm

Charles, N., Ozawa, T., Squatrito, M., Bleau, A.-M., Brennan, C. W., Hambardzumyan, D., Holland, E. C., 2010. Article Perivascular Nitric Oxide Activates Notch Signaling and Promotes Stem-like Character in PDGF-Induced Glioma Cells. *Stem Cell*, 6(2), pp.141–152.

Chen, J., Kesari, S., Rooney, C., Strack, P. R., Shen, H., Wu, L., Griffin, J. D., 2010. Inhibition of notch signaling blocks growth of glioblastoma cell lines and tumor neurospheres. *Genes Cancer*, 1(8), pp.822–835.

Chen, T.-H.H., Bae, Y., Furgeson, D. Y., 2008. Intelligent biosynthetic nanobiomaterials (IBNs) for hyperthermic gene delivery. *Pharmaceutical research*, 25(3), pp.683–91.

Cheng, H.-T., Kim, M., Valerius, M. T., Surendran, K., Schuster-Gossler, K., Gossler, A., McMahon, A. P., Kopan, R., 2007. Notch2, but not Notch1, is required for proximal fate acquisition in the mammalian nephron. *Development* , 134 (4), pp.801–811.

Cohen, Z.R., Zaubermann, J., Harnof, S., Mardor, Y., Nass, D., Zadicario, E., Hananel, A., Castel, D., Faibel, M., Ram, Z., 2007. Magnetic resonance imaging-guided focused ultrasound for thermal ablation in the brain: a feasibility study in a swine model. *Neurosurgery*, 60(4), pp.593–600.

Daniell, H., Guda, C., McPherson, D. T., Zhang, X., Xu, J., Urry, D. W., 1997. Hyperexpression of a synthetic protein-based polymer gene. *Methods in Molecular Biology*, 63, pp.359–371.

Dewhirst, M. W., Prosnitz, L., Thrall, D., Prescott, D., Clegg, S., Charles, C., MacFall, J., Rosner, G., Samulski, T., Gillette, E., LaRue, S., 1997. Hyperthermic treatment of

malignant diseases: current status and a view toward the future. *Seminars in Oncology*, 24(6), pp.616–625.

Dickson, B.C., Mulligan, A. M., Zhang, H., Lockwood, G., O'Malley, F. P., Egan, S. E., Reedijk, M., 2007. High-level JAG1 mRNA and protein predict poor outcome in breast cancer. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology*, 20(6), pp.685–93.

Dreher, M. R., Liu, W., Michelich, C. R., Dewhirst, M. W., Chilkoti, A., 2007. Thermal cycling enhances the accumulation of a temperature-sensitive biopolymer in solid tumors. *Cancer research*, 67(9), pp.4418–24.

Drin, G., Rousselle, C., Scherrmann, J.-M. M., Rees, A. R., Temsamani, J., 2002. Peptide delivery to the brain via adsorptive-mediated endocytosis: advances with SynB vectors. *American Association of Pharmaceutical Sciences: PharmSci*, 4(4), p.E26.

Egloff, A.M. and Grandis, J.R., 2012. Molecular Pathways: Context-dependent approaches to Notch targeting as cancer therapy. *Clinical cancer research*, 18(19), pp.5188–5195.

Evan, G.I. and Vousden, K.H., 2001. Proliferation, cell cycle and apoptosis in cancer. *Nature*, 411(6835), pp.342–348.

Falk, M.H. and Issels, R.D., 2001. Hyperthermia in oncology. *International Journal of Hyperthermia*, 17(1), pp.1–18.

Feyerabend, T., Steeves, R., Wiedemann, G. J., Richter, E., Robins, H. I., 1997. Rationale and clinical status of local hyperthermia, radiation, and chemotherapy in locally advanced malignancies. *Anticancer Research*, 17(4B), pp.2895–2897.

Fischer, A. and Gessler, M., 2007. Delta–Notch—and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors. *Nucleic Acids Research* , 35 (14), pp.4583–4596.

Fiúza, U.-M. and Arias, A.M., 2007. Cell and molecular biology of Notch. *The Journal of endocrinology*, 194(3), pp.459–474.

Gilbert, C. A., Daou, M.-C. Moser, R. P., Ross, A. H., 2010. Gamma-secretase inhibitors enhance temozolomide treatment of human gliomas by inhibiting neurosphere repopulation and xenograft recurrence. *Cancer research*, 70(17), pp.6870–6879.

Gomez-Manzano, C., Fueyo, J., Kyritsis, A. P., Steck, P. A., Roth, J. A., McDonnell, T. J., Steck, K. D., Levin, V. A., Yung, W. K., 1996. Adenovirus-mediated transfer of the p53 gene produces rapid and generalized death of human glioma cells via apoptosis. *Cancer research*, 56(4), pp.694–699.

Guarneri, V., Dieci, M.V. and Conte, P., 2012. Enhancing intracellular taxane delivery: current role and perspectives of nanoparticle albumin-bound paclitaxel in the treatment of advanced breast cancer. *Expert Opinion on Pharmacotherapy*, 13(3), pp.395–406.

Guo, D., Ye, J., Dai, J., Li, L., Chen, F., Ma, D., Ji, C., 2009. Notch-1 regulates Akt signaling pathway and the expression of cell cycle regulatory proteins cyclin D1, CDK2 and p21 in T-ALL cell lines. *Leukemia Research*, 33(5), pp.678–685.

Gustafsson, M. V., Zheng, X., Pereira, T., Gradin, K., Jin, S., Lundkvist, J., Ruas, J. L., Poellinger, L., Lendahl, U., Bondesson, M., 2005. Hypoxia Requires Notch Signaling to Maintain the Undifferentiated Cell State. *Developmental cell*, 9(5), pp.617–628.

Haider, M., Megeed, Z., Ghandehari, H., 2004. Genetically engineered polymers: status and prospects for controlled release. *Journal of controlled release : official journal of the Controlled Release Society*, 95(1), pp.1–26.

Hed, J., Hallden, G., Johansson, S. G., Larsson, P., 1987. The use of fluorescence quenching in flow cytofluorometry to measure the attachment and ingestion phases in phagocytosis in peripheral blood without prior cell separation. *Journal of Immunological Methods*, 101(1), pp.119–125.

Hochberg, F.H. and Pruitt, A., 1980. Assumptions in the radiotherapy of glioblastoma. *Neurology*, 30 (9), p.907.

Hokland, S.L., Nielsen, T., Busk, M., Horsman, M. R., 2010. Imaging tumour physiology and vasculature to predict and assess response to heat. *International Journal of Hyperthermia*, 26(3), pp.264–272.

Hu, X. and Jing, X., 2009. Biodegradable amphiphilic polymer–drug conjugate micelles. *Expert Opinion on Drug Delivery*, 6(10), pp.1079–1090.

Hulleman, E., Quarto, M., Vernell, R., Masserdotti, G., Colli, E., Kros, J. M., Levi, D., Gaetani, P., Tunici, P., Finocchiaro, G., Baena, R. R. Y., Capra, M., Helin, K., 2009. A role for the transcription factor HEY1 in glioblastoma. *Journal of cellular and molecular medicine*, 13(1), pp.136–46.

Hurwitz, M.D., 2010. Today's thermal therapy: not your father's hyperthermia: challenges and opportunities in application of hyperthermia for the 21st century cancer patient. *American Journal of Clinical Oncology*, 33(1), pp.96–100.

Igney, F.H. and Krammer, P.H., 2002. Death and anti-death: tumour resistance to apoptosis. *Nature reviews. Cancer*, 2(4), pp.277–88.

Issels, R.D., 1995. Regional hyperthermia combined with systemic chemotherapy of locally advanced sarcomas: preclinical aspects and clinical results. *Recent Results in Cancer Research*, 138, pp.81–90.

Jacobs, V.L., Valdes, P. A., Hickey, W. F., Leo, J. A. D., 2011. Current review of in vivo GBM rodent models : emphasis on the CNS-1 tumour model. *American society for neurochemistry: Neuro*, 3(3), pp.171–181.

Saint Just Ribeiro, M. and Wallberg, A.E., 2009. Transcriptional mechanisms by the coregulator MAML1. *Current protein and peptide science*, 10(6), pp.570–576.

Kanamori, M., Kawaguchi, T., Nigro, J. M., Feuerstein, B. G., Berger, M. S., Miele, L., Pieper, R. O., 2007. Contribution of Notch signaling activation to human glioblastoma multiforme. *Journal of neurosurgery*, 106(3), pp.417–427.

Khare, P., Jain, A., Gulbake, A., Soni, V., Jain, N. K., Jain, S. K., 2009. Bioconjugates: harnessing potential for effective therapeutics. *Critical Reviews in Therapeutic Drug Carrier Systems*, 26(2), pp.119–155.

Kong, G. and Dewhirst, M. W., 1999. Hyperthermia and liposomes. *International Journal of Hyperthermia*, 15(5), pp.345–370.

Kopan, R. and Ilagan, M., 2009. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell*, 137(2), pp.216–233.

Kopecek, J., 2003. Smart and genetically engineered biomaterials and drug delivery systems. *European journal of pharmaceutical sciences*, 20, pp.1–16.

Koren, E. and Torchilin, V.P., 2012. Cell-penetrating peptides: breaking through to the other side. *Trends in molecular medicine*, 18(7), pp.385–93.

Kratz, F., Senter, P., Steinhagen, H., 2011. *Drug Delivery in Oncology: From Basic Research to Cancer Therapy* 1st ed. Wiley-VCH.

Krebs, L.T., Iwai, N., Nonaka, S., Welsh, I. C., Lan, Y., Jiang, R., Saijoh, Y., O'Brien, T. P., Hamada, H., Gridley, T., 2003. Notch signaling regulates left–right asymmetry determination by inducing Nodal expression. *Genes and Development*, 17 (10), pp.1207–1212.

De Las Heras Alarcon, C., Pennadam, S., Alexander, C., 2005. Stimuli responsive polymers for biomedical applications. *Chemical Society reviews*, 34(3), pp.276–85.

Li, B., Alonso, D. O., Bennion, B. J., Daggett, V., 2001. Hydrophobic hydration is an important source of elasticity in elastin-based biopolymers. *Journal of the American Chemical Society*, 123(48), pp.11991–11998.

Lino, M.M., Merlo, A., Boulay, J.-L., 2010. Notch signaling in glioblastoma: a developmental drug target? *BMC medicine*, 8(1), p.72.

Liu, W., Dreher, M. R., Furgeson, D. Y., Peixoto, K. V., Yuan, H., Zalutsky, M. R., Chilkoti, A., 2006. Tumor accumulation, degradation and pharmacokinetics of elastin-like polypeptides in nude mice. *Journal of Controlled Release*, 116(2), pp.170–178.

Liu, Z.J., Xiao, M., Balint, K., Soma, A., Pinnix, C. C., Capobianco, A. J., Velazquez, O. C., Herlyn, M., 2006. Inhibition of endothelial cell proliferation by Notch1 signaling is mediated by repressing MAPK and PI3K/Akt pathways and requires MAML1. *Faseb Journal*, 20(7), pp.1009–1011.

Lobry, C., Oh, P., Aifantis, I., 2011. Oncogenic and tumor suppressor functions of Notch in cancer: it's NOTCH what you think. *The Journal of experimental medicine*, 208(10), pp.1931–1935.

Louis, D.N., Ohgaki, H., Wiestler, O. D., Cavenee, W. K., Burger, P. C., Jouvet, A., Scheithauer, B. W., Kleihues, P., 2007. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathologica*, 114(2), pp.97–109.

Maddika, S., Ande, S. R., Panigrahi, S., Paranjothy, T., Weglarczyk, K., Zuse, A., Eshraghi M., Manda, K. D., Wiechec, E., Los, M., 2007. Cell survival, cell death and cell cycle pathways are interconnected: Implications for cancer therapy. *Drug Resistance Updates*, 10(1–2), pp.13–29.

Maeda, H., 2010. Tumor-Selective Delivery of Macromolecular Drugs via the EPR Effect: Background and Future Prospects. *Bioconjugate Chemistry*, 21(5), pp.797–802.

Martinez, L., Goluszko, E., Chen, H.-Z., Leone, G., Post, S., Lozano, G., Chen, Z., Chauchereau, A., 2010. E2F3 is a mediator of DNA damage-induced apoptosis. *Molecular and cellular biology*, 30(2), pp.524–536.

Massodi, I., Moktan, S., Rawat, A., Bidwell 3rd, G. L., Raucher, D., 2010. Inhibition of ovarian cancer cell proliferation by a cell cycle inhibitory peptide fused to a thermally responsive polypeptide carrier. *International Journal of Cancer*, 126(2), pp.533–544.

Massodi, I., Thomas, E., Raucher, D., 2009. Application of thermally responsive elastin-like polypeptide fused to a lactoferrin-derived peptide for treatment of pancreatic cancer. *Molecules*, 14(6), pp.1999–2015.

Matsumura, Y. and Maeda, H., 1986. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer research*, pp.6387–6392.

McElhinny, A.S., Li, J.-L., Wu, L., 2008. Mastermind-like transcriptional co-activators: emerging roles in regulating cross talk among multiple signaling pathways. *Oncogene*, 27(38), pp.5138–5147.

Mendes, K.N., Nicorici, D., Cogdell, D., Tabus, I., Yli-harja, O., Guerra, R., Hamilton, S. R., Zhang, W., 2007. Analysis of signaling pathways in 90 cancer cell lines by protein lysate array. *Journal of Proteome Research*, 6(7), pp.2753–2767.

Meyer, D. E. and Chilkoti, A., 1999. Purification of Recombinant Proteins by Fusion with Thermally Responsive Polypeptides. *Nature Biotechnology*, 17, pp.1112–1115.

Meyer, D. E. and Chilkoti, A., 2002. Genetically encoded synthesis of protein-based polymers with precisely specified molecular weight and sequence by recursive directional ligation: examples from the elastin-like polypeptide system. *Biomacromolecules*, 3(2), pp.357–367.

Meyer, D. E. and Chilkoti, A., 2004. Quantification of the effects of chain length and concentration on the thermal behavior of elastin-like polypeptides. *Biomacromolecules*, 5(3), pp.846–851.

Meyer, D. E., Shin, B. C., Kong, G. A., 2001. Drug targeting using thermally responsive polymers and local hyperthermia. *Journal of Controlled Release*, 74(1-3), pp.213–224.

Miele, L., 2006. Notch signaling. *Clinical cancer research*, 12(4), pp.1074–1079.

Miele, L. and Osborne, B., 1999. Arbiter of differentiation and death: Notch signaling meets apoptosis. *Journal of cellular physiology*, 409, pp.393–409.

Mittal, S., Subramanyam, D., Dey, D., Kumar, R. V., Rangarajan, A., 2009. Cooperation of Notch and Ras/MAPK signaling pathways in human breast carcinogenesis. *Molecular cancer*, 8, p.128.

Moellering, R.E., Cornejo, M., Davis, T. N., Del Bianco, C., Aster, J. C., Blacklow, S. C., Kung, A. L., Gilliland, D. G., Verdine, G. L., Bradner, J. E., 2009. Direct inhibition of the NOTCH transcription factor complex. *Nature*, 462(7270), pp.182–188.

Moktan, S., Ryppa, C., Kratz, F., Raucher, D., 2010. A thermally responsive biopolymer conjugated to an acid-sensitive derivative of paclitaxel stabilizes microtubules, arrests cell cycle, and induces apoptosis. *Investigational New Drugs*. 30(1), pp.236-248

Moktan, S. and Raucher, D., 2012. Anticancer Activity of Proapoptotic Peptides is Highly Improved by Thermal Targeting using Elastin-like Polypeptides. *International Journal of Peptide Research and Therapeutics*, 18(3), pp.227–237.

Nam, Y., Sliz, P., Song, L., Aster, J. C., Blacklow, S. C., 2006. Structural basis for cooperativity in recruitment of MAML coactivators to Notch transcription complexes. *Cell*, 124(5), pp.973–83.

Nam, Y., Weng, A. P., Aster, J. C., Blacklow, S. C., 2003. Structural requirements for assembly of the CSL.intracellular Notch1.Mastermind-like 1 transcriptional activation complex. *Journal of Biological Chemistry*, 278(23), pp.21232–21239.

Perumalsamy, L.R., Nagala, M., Banerjee, P., Sarin, A., 2009. A hierarchical cascade activated by non-canonical Notch signaling and the mTOR-Rictor complex regulates neglect-induced death in mammalian cells. *Cell death and differentiation*, 16(6), pp.879–89.

Poellinger, L. and Lendahl, U., 2008. Modulating Notch signaling by pathway-intrinsic and pathway-extrinsic mechanisms. *Current opinion in genetics and development,* 18, pp.449-454.

Pontén, J. and Westermark, B., 1978. Properties of human malignant glioma cells in vitro. *Medical biology*, 56(4), pp.184–193.

Porter, K. R., McCarthy, B. J., Freels, S., Kim, Y., Davis, F. G., 2010. Prevalence estimates for primary brain tumors in the United States by age, gender, behavior, and histology. *Neuro-oncology*, 12(6), pp.520–527.

Purow, B. W., Haque, R. M., Noel, M. W., Su, Q., Burdick, M. J., Lee, J., Sundaresan, T., Pastorino, S., Park, J. K., Mikolaenko, I., Maric, D., Eberhart, C. G., Fine, H., 2005. Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation. *Cancer research*, 65(6), pp.2353–2363.

Qiang, L., Wu, T., Zhang, H.-W., Lu, N., Hu, R., Wang, Y.-J., Zhao, L., Chen, F.-H., Wang, X.-T., You, Q.-D., Guo, Q.-L., 2012. HIF-1α is critical for hypoxia-mediated maintenance of glioblastoma stem cells by activating Notch signaling pathway. *Cell death and differentiation*, 19(2), pp.284–294.

Rakowski, J.A., Ahmad, S. and Holloway, R.W., 2011. Use of pegylated liposomal doxorubicin in the management of platinum-sensitive recurrent ovarian cancer: current concepts. *Expert Review of Anticancer Therapy*, 12(1), pp.31–40.

Ram, Z., Cohen, Z. R., Harnof, S., Tal, S., Faibel, M., Nass, D., Maier, S. E., Hadani, M., Mardor, Y., 2006. Magnetic resonance imaging-guided, high-intensity focused ultrasound for brain tumor therapy. *Neurosurgery*, 59(5), pp.946–949.

Ranganathan, P., Weaver, K.L., Capobianco, A. J., 2011. Notch signalling in solid tumours: a little bit of everything but not all the time. *Nature Reviews Cancer*, 11(5), pp.338–351.

Rangarajan, A., Talora, C., Okuyama, R., Nicolas, M., Mammucari, C., Oh, H., Aster, J. C., Krishna, S., Metzger, D., Chambon, P., Miele, L., Aguet, M., Radtke, F., Dotto, G. P., 2001. Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. *EMBO Journal*, 20(13), pp.3427–3436.

Raucher, D. and Chilkoti, A., 2001. Enhanced uptake of a thermally responsive polypeptide by tumor cells in response to its hyperthermia-mediated phase transition. *Cancer research*, pp.7163–7170.

Reedijk, M., Odorcic, S., Chang, L., Zhang, H., Miller, N., McCready, D. R., Lockwood, G., Egan, S. E., 2005. High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. *Cancer research*, 65(18), pp.8530–8537.

Rizzo, P., Osipo, C., Foreman, K., Golde, T., Osborne, B., Miele, L., 2008. Rational targeting of Notch signaling in cancer. *Oncogene*, 27(38), pp.5124–5131.

Ronchini, C. and Capobianco, A., 2001. Induction of cyclin D1 transcription and CDK2 activity by Notchic: implication for cell cycle disruption in transformation by Notchic. *Molecular and cellular biology*, 21(17), pp.5925–5934.

Rousselle, C., Clair, P., Lefauconnier, J. M., Kaczorek, M., Scherrmann, J. M., Temsamani, J., 2001. Enhanced delivery of doxorubicin into the brain via a peptide-vector-mediated strategy: saturation kinetics and specificity. *The Journal of pharmacology and experimental therapeutics*, 296(1), pp.124–31.

Roy, M., Pear, W.S., Aster, J. C., 2007. The multifaceted role of Notch in cancer. *Curr Opin Genet Dev*, 17(1), pp.52–59.

Sanalkumar, R., Dhanesh, S.B., James, J., 2010. Non-canonical activation of Notch signaling/target genes in vertebrates. *Cellular and molecular life sciences*, 67(17), pp.2957–68.

Schildkopf, P., Ott, O. J., Frey, B., Wadepohl, M., Sauer, R., Fietkau, R., Gaipl, U. S., 2010. Biological rationales and clinical applications of temperature controlled hyperthermia--implications for multimodal cancer treatments. *Current Medicinal Chemistry*, 17(27), pp.3045–3057.

Sebbage, V., 2009. Cell-penetrating peptides and their therapeutic applications. *Bioscience Horizons*, 2(1), pp.64–72.

Sherr, C.J., 2000. The Pezcoller Lecture : Cancer Cell Cycles Revisited The Pezcoller Lecture : Cancer Cell Cycles Revisited. , pp.3689–3695.

Sriuranpong, V., Borges, M. W., Ravi, R. K., Arnold, D. R., Nelkin, B. D., Baylin, S. B., Ball, D. W., 2001. Notch Signaling Induces Cell Cycle Arrest in Small Cell Lung Cancer Cells. *Cancer Research* , 61 (7), pp.3200–3205.

Stecca, B. and Altaba, A., 2009. A GLI1-p53 inhibitory loop controls neural stem cell and tumour cell numbers. *EMBO journal*, 28(6), pp.663–676.

Stockhausen, M.-T., Kristoffersen, K., Poulsen, H.S., 2010. The functional role of Notch signaling in human gliomas. *Neuro-Oncology* , 12 (2), pp.199–211.

Strebhardt, K. and Ullrich, A., 2008. Paul Ehrlich's magic bullet concept: 100 years of progress. *Nature Reviews Cancer*, 8(6), pp.473–480.

Takahashi, I., Emi, Y., Hasuda, S., Kakeji, Y., Maehara, Y., Sugimachi, K., 2002. Clinical application of hyperthermia combined with anticancer drugs for the treatment of solid tumors. *Surgery*, 131(1 Suppl), pp.S78–84.

Tamm, I., Schriever, F., Dörken, B., 2001. Apoptosis: implications of basic research for clinical oncology. *The Lancet Oncology*, 2(1), pp.33–42.

Tchorz, J.S., Tome, M., Cloëtta, D., Sivasankaran, B., Grzmil, M., Huber, R. M., Rutz-Schatzmann, F., Kirchhoff, F., Schaeren-Wiemers, N., Gassmann, M., Hemmings, B., Merlo, A., Bettler, B., 2012. Constitutive Notch2 signaling in neural stem cells promotes tumorigenic features and astroglial lineage entry. *Cell death and disease*, 3, p.e325.

Urry, D. W., 1988. Entropic elastic processes in protein mechanisms. I. Elastic structure due to an inverse temperature transition and elasticity due to internal chain dynamics. *Journal of Protein Chemistry*, 7(1), pp.1–34.

Urry, D. W., 1992. Free energy transduction in polypeptides and proteins based on inverse temperature transitions. *Progress in Biophysics and Molecular Biology*, 57(1), pp.23–57.

Urry, D. W., Luan, C.-H., Parker, T. M., Gowda, D. C., Prasad, K. U., Reid, M. C., Safavy, A., 1991. Temperature of Polypeptide Inverse Temperature Transition Depends on Mean Residue Hydrophobicity. *Journal of the American Chemical Society*, 113, pp.4346–4348.

Viatour, P., Ehmer, U., Saddic, L. A., Dorrell, C., Andersen, J. B., Lin, C., Zmoos, A.-F., Mazur, P. K., Schaffer, B. E., Ostermeier, A., Vogel, H., Sylvester, K. G., Thorgeirsson, S. S., Grompe, M., Sage, J., 2011. Notch signaling inhibits hepatocellular carcinoma following inactivation of the RB pathway. *Journal of experimental medicine*, 208(10), pp.1963–76.

Vives, E., Richard, J. P., Rispal, C., Lebleu, B., 2003. TAT peptide internalization: seeking the mechanism of entry. *Current Protein and Peptide Science*, 4(2), pp.125–132.

van Meir, E. G., Kikuchi, T., Tada, M., Van Meir, G., Huang, H.-J. S., Friedmann, T., Thbolet, N. D., Cavenee, W. K., 1994. Analysis of the p53 Gene and Its Expression in Human Glioblastoma Cells Analysis of the p53 Gene and Its Expression. *Cancer Research* 54(3) , pp.649–652

van Vulpen, M., Raaymakers, B. W., de Leeuw, A. A., van de Kamer, J. B., van Moorselaar, R. J., Hobbelink, M. G., Battermann, J. J., Lagendijk, J. J., 2002. Prostate perfusion in patients with locally advanced prostate carcinoma treated with different hyperthermia techniques. *Journal of Urology*, 168(4 Pt 1), pp.1597–1602.

Wang, J., Wakeman, T. P., Lathia, J. D., Hjelmeland, A. B., Wang, X.-F., White, R. R., Rich, J. N., Sullenger, B. A., 2010. Notch promotes radioresistance of glioma stem cells. *Stem cells*, 28(1), pp.17–28.

Wen, P. and Kesari, S., 2008. Malignant gliomas in adults. *New England Journal of Medicine*, 359, pp.492-507.

Wu, L., Aster, J. C., Blacklow, S. C., Lake, R., Artavanis-Tsakonas, S., Griffin, J. D., 2000. MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors. *Nature genetics*, 26(4), pp.484–489.

Wu, L., Sun, T., Kobayashi, K., 2002. Identification of a family of mastermind-like transcriptional coactivators for mammalian notch receptors. *Molecular and cellular biology*, 22(21), pp.7688–7700.

Xu, P., Qiu, M., Zhang, Z., Kang, C., Jiang, R., Jia, Z., Wang, G., Jiang, H., Pu, P., 2010. The oncogenic roles of Notch1 in astrocytic gliomas in vitro and in vivo. *Journal of neuro-oncology*, 97(1), pp.41–51.

Xu, P., Zhang, A., Jiang, R., Qiu, M., Kang, C., Jia, Z., Wang, G., Han, L., Fan, X., Pu, P., 2013. The different role of notch1 and notch2 in astrocytic gliomas. *PloS one*, 8(1), p.e53654.

Zhang, X., Chen, T., Zhang, J., Mao, Q., Li, S., Xiong, W., Qiu, Y., Xie, Q., Ge, J., 2012. Notch1 promotes glioma cell migration and invasion by stimulating β-catenin and NF-κB signaling via AKT activation. *Cancer science*, 103(2), pp.181–90.

Zhao, N., Guo, Y., Zhang, M., Lin, L., Zheng, Z., 2010. Akt-mTOR signaling is involved in Notch-1-mediated glioma cell survival and proliferation. *Oncology reports*, 23(5), pp.1443–1447.

Zhao, Y., Katzman, R. B., Delmolino, L. M., Bhat, I., Zhang, Y., Gurumurthy, C. B., Germaniuk-Kurowska, A., Reddi, H. V., Solomon, A., Zeng, M. S., Kung, A., Ma, H., Gao, Q., Dimri, G., Stanculescu, A., Miele, L., Wu, L., Griffin, J. D., Wazer, D. E., Band, H., Band, V., 2007. The notch regulator MAML1 interacts with p53 and functions as a coactivator. *Journal of Biological Chemistry*, 282(16), pp.11969–11981.

Zhu, T.S., Costello, M., Talsma, C. E., Flack, C. G., Crowley, J. G., Hamm, L. L., He, X., Hervey-Jumper, S. L., Heth, J., Muraszko, K. M., DiMeco, F., Vescovi, A. L., Fan, X., 2011. Endothelial cells create a stem cell niche in glioblastoma by providing NOTCH ligands that nurture self-renewal of cancer stem-like cells. *Cancer research*, 71(18), pp.6061–72.

8. SUMMARY

Introduction

Today cancer is one of the most investigated diseases in the world. Advances in therapy have been great but remain insufficient. GBMs account for about 70% of the newly diagnosed malignant brain tumors. Current standard treatment includes “the cancer triad”: maximal surgical resection (if possible), radiation therapy with concomitant chemotherapy. In spite of all progress and changes in therapy recurrence rate for GBMs is extremely high with about 90% of the tumors recurring in the original site. Notch signaling pathway is one of the few highly conserved pathways involved in development and homeostasis in multicellular organisms. In cancer Notch has a dual role. In majority of cases activation of Notch is an oncogenic trigger. Some type of Notch activation was found in 80% of GBMs underlining importance of Notch in formation and recurrence of GBMs.

Materials and Methods

Protein based Notch inhibitor dnMAML is actually a portion of MAML1 co-activator protein and is shown to be efficient in blocking Notch function in T-ALL. It is cloned with ELP a thermally reactive polypeptide that can be actively and passively targeted to the tumor site by localized application of heat. Entry into the cells is enhanced by the addition of a CPP, SynB1, which can also carry the cargo over the BBB. Parameters such as growth inhibition, apoptosis induction, cell cycle arrest and levels Notch inhibition are measured in two GBM derived cell lines using flow cytometry and qPCR. Non canonical inhibition is tested through levels of proteins detected by Western blot.

Results

SynB1-ELP-dnMAML can be produced and purified in a bacterial expression system. It enters the cells and localizes in the cytoplasm. Application of heat is essential for the uptake of the protein in the cell. dnMAML retains its structure upon binding to ELP and can successfully pull Notch1 out of the cell lysate. Treating both cell lines with SynB1-ELP dnMAML results in growth inhibition that is additionally increased by hyperthermia. In D54 apoptosis appears to be the main mechanism by which growth is suppressed. Same is not true for U251 cells that undergo apoptosis in a lower percentage. Both cell lines show equal cell cycle dynamics. Cycle lag is observed in G0/G1 and G2/M phases. Canonical targets do not show expected levels of inhibition probably due to small amounts of SynB1-ELP-dnMAML in the nucleus. Non canonical targets AKT, MAPK and

p53 are affected by SynB1-ELP-dnMAML. U251 cells show much lower levels of non-canonical target proteins after treatment indicating that this may be the principal mechanism of Notch activation in that cell line.

Conclusion

Results confirm that dnMAML is effective GBM inhibitor and that combining it with ELP gives effective means of actively targeting and increasing inhibition only in a small area thus avoiding the dangers of systemic side effects. With minor optimization canonical and non canonical targets can be reached in this way so that should give SynB1-ELP-dnMAML an advantage before GSIs. In GBMs this is good alternative approach that can overcome difficulties posed by poor delivery across the blood-brain barrier.

9. SAžETAK

Uvod

Tumori su danas među najistraživanijim bolestima. Napredak u terapiji je velik ali i dalje nedovoljan. Glioblastomi (GBM) čine oko 70% novo dijagnosticiranih malignih tumora mozga. Standardna terapija je maksimalna resekcija (ako je moguća), te zračenje uz kemoterapiju. Usprkos svih napretcima i promjenama u pristupu liječenju stupanj ponovne pojave GBM-a je vrlo visok, oko 90% tumora se ponovi na istom mjestu. Notch signalni put je jedan od vrlo konzerviranih putova koji upravlja razvojem višestaničnih organizama. Sastoji se od transmembranskog receptora i liganda na susjednoj stanici te omogućuje međustaničnu komunikaciju. Uloga Notch-a u tumorima je dvostrana. U većini slučajeva pojačana aktivacija djeluje onkogeno. Pretjerana aktivnost Notch-a je nađena u oko 80% GBM-a što potvrđuje važnu ulogu koju ima u nastanku i ponovnoj pojavi GBM-a

Materijali i metode

dnMAML je proteinski Notch inhibitor koji se pokazao uspješan u blokiranju Notch puta kod T-ALL. Nažalost, kao i većina terapeutskih peptida i proteina ima loša farmako-kinetička svojstva, te ne može prijeći krvno-moždanu barijeru. Kloniranjem na ELP (toplinski osjetljiv polipeptid koji se može aktivno i pasivno dovesti u tumor) uz korištenje translokacijskog peptida, u ovom slučaju SynB1, rješava se problem ulaska u stanicu i prelaska krvno-moždane barijere. Sposobnost inhibicije se testirala na dvije stanične linije izolirane iz GBM, D54 i U251.

Rezultati

SynB1-ELP-dnMAML zaustavlja rast dviju testiranih staničnih linija, D54 i U251 potičući apoptozu i zaustavljajući diobu stanica. 20% do 80% stanica umire ovisno o koncentraciji proteina i tome da li je prisutna hipertermija. Kao što je pretpostavljeno, zagrijavanje povećava količinu proteina u stanicama i pojačava inhibiciju rasta. Kanonske mete nisu inhibirane niti u jednoj liniji dok je inhibicija ne-kanonskih ciljeva jaka u U251 stanicama. p53 koji je u direktnom dodiru s N-krajem MAML1 proteina koji čini dnMAML pokazuje promjene. Wild type p53 koji je prisutan u D54 stanicama ima blago pojačanu ekspresiju dok je razina mutiranog p53, koji je prisutan u U251 stanicama, značajno smanjena. Dvije stanične linije pokazuju različit utjecaj Notch-a. Kod D54 stanica Notch blokira apoptozu, dok je kod U251 stanica veća ovisnost o ne-kanoničkim putevima.

Zaključak

Rezultati potvrđuju da je dnMAML djelotvoran inhibitor GBM te da se u kombinaciji s ELP može uspješno aktivno ciljati te povećati učinkovitost na ograničenom području. Na taj način moguće je izbjeći sistemske nuspojave. Uz male promjene SynB1-ELP-dnMAML može inhibirati obje grane aktivacije Notcha, što bi mu dalo prednosti u usporedbi s GSI. Za liječenje GBM-a ovo je dobar alternativnih pristup koji bi mogao riješiti problem slabog prelaska lijekova preko krvno-moždane barijere.

10. ABBREVIATIONS

ANOVA – analysis of variance

BBB – blood-brain barrier

BCR-ABL – break point cluster region - Abelson murine leukemia viral oncogene fusion protein

BrdU – bromodeoxyuridine

CBTRUS - Central Brain Tumor Registry of the United States

CDK – cyclin dependent kinase

CML – chronic myeloid leukemia

CPP – cell penetrating peptide

CSL - CBF1, Suppressor of hairless and Lag1

DMEM – Dulbecco's modified Eagle medium

DMEM/F12 – Dulbecco's modified Eagle medium/Ham's F12

DMSO – Dimethyl sulfoxide

dnMAML – dominant negative Mastermind like

ELP – elastin-like polypeptide

EPR – enhanced permeability and retention

ERBB2 – erythroblastic leukemia viral oncogene homolog 2

FBS – fetal bovine serum

GBM – gliblastoma multiforme

GSI - γ-secretase inhibitor

HIFU – high intensity focused ultrasound

HPMA – N-(2-hydroxypropyl)methacrylamide

HRP – horseradish peroxidase

MAML – Mastermind – like

MAPK – mitogen activated protein kinase

MRI – magnetic resonance imaging

mTOR – mammalian target of rapamycin

NICD – Notch intracellular domain

NSCLC – non-small cell lung cancer/carcinoma

PBS – phosphate buffered saline

PEG – polyethylene glycol

PEI – polyethylene imine

PFA – paraformaldehyde

PG-1 – protegrin 1

PI – propidium iodide

PVDF – polyvinylidene diflouride

SCLC – small cell lung cancer/carcinoma

SDS-PAGE - sodium dodecyl suflate polyacrylamide gel electrophoresis

T-ALL - T-cell acute lymphoblastic leukemia

TCEP – Tris-(2-caboxyethyl)phosphine

T-PER – tissue protein extraction reagent

T_t – transition temperature

WHO – World Health Organisation

11. BIOGRAPHY

I was born September 28th, 1979 in Osijek. I passed my childhood and teenage years there, finishing both elementary and high school education in my hometown. I started attending college in 1998 as a freshman at University of Zagreb Faculty of Science, Biology Department. My major was molecular biology an engineering program that I graduated from in May 2005 under the mentoring of prof.dr.sc. Ljubica Glavaš-Obrovac and prof.dr.sc. Jasna Ban In my junior year I received a scholarship from the city of Osijek and returned to Osijek upon graduating. In 2006 I started working as a research associate in Agricultural Institute Osijek, Agrochemical laboratory. My duties were to implement molecular methods into laboratory practices for use by all departments. In 2008 I started working at University of Osijek Medical School at the scientific project "Physiochemical and metabolic factors for development of urolithiasis" lead by prof.dr.sc. Vesna Babić-Ivančić. That same year I started attending an interdisciplinary PhD program "Molecular biosciences". In 2009 I applied for and was awarded a fellowship for PhD students from the Croatian Science Foundation to visit dr.sc. Dražen Raucher laboratory at the University of Mississippi Medical Center for 12 months which allowed me to complete most of my work. Upon my return I started actively participating in teaching classes, working at the Department of Medical Chemistry, Biochemistry and Clinical Chemistry, in undergraduate courses of the department. During my stay in the US I presented my work on several international and local conventions and meetings. My work in Croatia was also presented in international and national meetings. Together with my colleagues I have published 3 scientific papers on our results so far. I am married and have a daughter.