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Dedication

I would like to dedicate this dissertation to my parents, Frida and Moshe Oron, for their endless support.

To my brothers Haim and Gadi and my sister Merav, for their help, interest and friendship.

To the Shoshan family for their support and faith

To my friends in Houston and in Israel, for being there at all times, listening and helping.

To my husband Roei and my two bountiful daughters Yael and Tamar, for their love and support over the duration of my graduate career

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NFAT1 CONTRIBUTES TO MELANOMA TUMOR GROWTH AND METASTASIS BY REGULATING IL-8 AND MMP-3

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Studies from our laboratory have recently demonstrated that Gal-3 regulates autotaxin through NFAT1 and support melanoma progression. These findings prompted us to further study the role of NFAT1 in melanoma progression and metastasis. NFAT1 is a transcription factor that was first identified in immune cells, acting as a positive regulator of interleukin-2 by binding to its promoter during T cell activation. NFAT1 has an important role in the innate and adaptive immune response. In this dissertation I studied the mechanisms by which NFAT1 contributes to the acquisition of the melanoma metastatic phenotype.

To identify the role of NFAT1 in melanoma progression we stably silenced NFAT1 expression in the highly metastatic cell line, A375SM, and subjected the cells to gene expression microarray analysis. We identified and validated two downstream targets of NFAT1, i.e; IL-8 and MMP-3 to be downregulated following silencing NFAT1. While silencing of NFAT1 reduced IL-8 and MMP-3 in highly metastatic cell lines, A375SM and WM902B, overexpression of NFAT1 in the low metastatic cell line, SB2 induced the expression of both IL-8 and MMP-3. We further demonstrated that silencing NFAT1

significantly reduced the promoter activity of IL-8 and MMP-3 and mutations of the NFAT1 binding sites at either promoter reduced the promoter activity. Rescue of NFAT1 increased both IL-8 and MMP-3 expression back to their initial levels, indicating that they are directed targets of NFAT1. Importantly, we demonstrated in melanoma patient specimens and cell lines that overexpression of NFAT1 is correlated with disease progression and staging. Moreover, our *in vivo* studies demonstrated that NFAT1 is a major contributor of tumor growth and lung metastasis. The role of MMP-3 in melanoma progression has not been previously described. Therefore, we next decided to elucidate the role of MMP-3 in melanoma. Our *in vivo* studies demonstrated that MMP-3 contributes to melanoma tumor growth and metastasis.

Collectively, our data assign a previously undescribed role for NFAT1in melanoma progression through the regulation of IL-8 and MMP-3.

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CHAPTER 3: To Determine the Status and Contribution of NFAT1 in Melanoma Progression

Chapter 1: Introduction and Background

Melanoma Occurrence

Melanoma is considered to be the deadliest and most aggressive form of skin cancer. The annual death incidence of this disease is higher than any other skin cancer [1]. The current statistics are evidence that malignant melanoma presents a major clinical challenge due to limited treatment options. In the United States, melanoma is documented as the fifth and seventh most common form of cancer in men and women respectively [2]. For 2014, there are 76,100 (43,890 in men, 32,210 in women) expected new cases of melanoma with 9,710 of those cases resulting in death [2]. Although melanoma cases represent less than 5% of all skin cancers, it is responsible for more than 80% of deaths from skin cancer [3]. It seems that cutaneous melanoma is relevant for all major ethnic groups and races in the USA, however there are variations by race and ethnicity. In the USA non-hispanic white individuals are the most likely to develop melanoma in their life period [4]. To identify novel therapeutic molecular targets and successfully cure this disease, it is critical to understand the molecular events that lead to melanoma metastasis.

Melanoma Development

The onset of melanoma is largely due to a cohort of cellular, tissue, and/or environmental factors that lead to genetic and epigenetic alterations influencing the proliferation of normal melanocytes [5]. Melanocytes are cells of the skin that are responsible for producing the pigment melanin which in turn helps protect against the harmful effects of ultraviolet light. Human melanocytes arise from neural crest (ectoderm)

cells that are only present during embryonic development and migrate in a coordinated fashion to be effectively incorporated into every follicle of hair and every unit of the human epidermis [6]. Over three decades ago, a study focused on the progression of tumor development led Clark et al. to propose that melanoma develops in a stepwise manner of five steps [7]. The first step to the model, Benign Nevus, involves the formation of a benign nevus due to an abnormal increase in proliferation of normal melanocytes. In standard clinical observation, these nevi will appear as either flat or slightly raised lesions on the surface of the skin. The pigmentation of these lesions can be either completely uniform or with a dot-like pattern [3]. In step two, Dysplastic Nevus, due to uncontrolled growth, the development of cytologic atypia arise from these newly formed benign nevi or in an entirely new location. Clinically, these lesions lack uniformity, such that they are typically seen with irregularities in the borders and may contain multiple pigments [3]. Primary melanoma develops in the third stage termed the Radial Growth Phase (RGP). In this step, cells now have the limited ability to only penetrate the epidermis and proliferate to form tumors [3]. With progression of the tumor comes the ability of cells to fully invade the dermis to form lesions beyond the basement membrane. This is the fourth step and it is termed the Vertical Growth Phase (VGP). At this step, cells gain the ability to form tumors when implanted in nude mice [3]. The fifth and final stage of the primary tumor is to form distant metastasis by dissociating from the primary tumor, entering the lymphatic system, and transporting into outlying organ sites to proliferate and form new tumors [3]. Among the most common sites for melanoma metastasis are bone, lung, liver and brain. There is a second model for the development of melanoma that suggests that melanoma does not arise from molecular disturbances of a preexisting Dysplastic Nevus but rather as completely de novo [8]. The

transformation of melanocytes to melanoma is promoted through the activation of different cellular pathways that induce the genetic and epigenetic changes necessary for the development of melanoma [9]. Although both models are plausible for occurring in humans, the model that includes the five defined sequential steps is most widely accepted (Figure 1).

Melanoma Staging and Survival

Currently there are four distinct clinical classifications for melanoma. The factors for prognosis are: the thickness and presence or absence of ulceration of the primary tumor, the expression of lymph node metastasis, and the presence of distant metastatic disease [10]. Those patients who are diagnosed within the first two stages have primary tumors with no identifiable metastasis. Specifically, stage I melanoma is identified by the presence of primary tumors with a thickness of less than 2mm. Furthermore, a sub-classification of stage I, stage IA and IB, distinguishes between tumors that are less than 1mm thick with no visible ulcerations (IA) and tumors that are also less than 1mm thick but are either ulcerated or have greater than one mitotic cell per mm^2 (IB). A tumor with thickness of up to 2mm with no ulceration (termed T2a melanoma) is also classified as stage IB [11]. Patients with stage II melanoma are sub-classified into either IIA or IIB depending on the tumor thickness which ranges from 1mm to greater than 4mm, and the presence or absence of ulcerations [10]. The stage of the disease is a critical factor in determining a patient's prognosis, since the five year rate of survival in patients presenting an early stage of melanoma is 90% but those in stage IV of the disease (wide spread metastasis) only have a 10-20% chance of survival [12-16]. Crucial independent predictors for patient outcome and those that also contribute to the variation in survival are tumor thickness, mitotic rate, and presence of ulcerations [10, 17,

18]. Results from a 2011 study found a correlation between mitotic rate and the development of ulcerations and concluded that at ten years, there is a 33% chance of survival for patients with ulcerated tumors greater than 5mm as opposed to patients with ulcerations less than 5mm in diameter who have a 69% chance of survival [17, 19]. At stage III melanoma, regional lymph node metastasis is present. Three additional subcategories to stage III melanoma are IIIA, IIIB, and IIIC respectively. The classification criteria depends on the number of regional lymph nodes present, the size of the lesion within the node (micro vs, macrometastasis), and if in transit metastasis is seen. There is a 25-35% 10 year survival rate for patients with multiple affected lymph nodes (macrometastasis) opposed to 45-65% survival for patients with micrometastasis, one or two lymph nodes affected [11]. Stage IV melanoma involves patients with distant organ metastasis such as, but not limited, to the bones, brain, and lungs. The ten year survival rate for patients in stage IV melanoma is less than 20% [11]. Recently it was published that age is a prognostic factor in melanoma patients and it can also be used as a predictor of sentinel node metastasis [20, 21]. These reports show that older patients with stage I and II had lower survival rates and higher incidence of nodal metastasis.

Current Treatments for Metastatic Melanoma

Historically, systemic therapy for metastatic melanoma was in the form of a DNA damaging compound named decarbazine (DTIC) and it was approved by the FDA in 1975 [22]. The response rate for the alkylating agent DTIC is low, only 5-12%, with long term response in less than 2% of patients [23], it remains as the standard treatment for many years while new therapies were tested [24]. An oral analogue to decarbazine is temozolemide, another alkylating agent that has a broad spectrum of anti-tumor effect and is much less toxic

than decarbazine [25]. Currently, temozolemide is routinely utilized in place of decarbazine for therapeutic purposes [24]. The first nonspecific immunotherapeutic drug approved before 2011 for the treatment of malignant melanoma was a high dose of Interleukin-2 (IL-2), but it demonstrated only a 15% response rate, and it remains nonspecific whereby treatment requires high dosages leading to intolerability and other side effects [26]. Adjuvant therapy is the term for a treatment that is given after surgical removal of the melanoma tumor. The cytokine, Interferon-alpha (IFN-α) was approved for use as adjuvant therapy for patients with early stage melanoma in order to reduce the risk for disease progression. After primary tumors are resected, and when there is no involvement of the lymph nodes, patients can be treated with high or low doses of IFN-α [27]. Furthermore, because of the significant dose-dependent side effects linked to adjuvant therapies, careful consideration must be taken when deciding whether or not to undergo such treatment.

Within the past years, rigorous research studies focused on understanding the complex interaction between tumors and their microenvironment have led to the development of some newly FDA approved anti-tumor drugs with increased immune specificity [24]. Ipilimumab, a safer T-cell activating agent, is the first among the four most recent drugs approved by the FDA. In 2011, ipilimumab was approved as a therapy for advanced melanoma. Ipilimumab is a monoclonal antibody against cytotoxic T lymphocyte associated antigen 4 (CTLA-4). CTLA-4 is responsible for inhibiting T cells to prevent autoimmune response. Ipilimumab allowed stronger T cell activation against malignant melanocytes, allowing prolonged immune responses to tumor antigens by preventing the down regulation of T-cell activation [28]. A clinical study performed on cases of stage IIIC and IV melanoma with no previous treatment found that the overall two year survival rate

increased by 10% when patients were treated with a combination of ipilimumab and DTIC as compared to DTIC alone [29]. In the clinical setting, the FDA recommends the ipilimumab regimen to consist of a concentration of 3 mg/kg per dose administered 3 weeks apart in 4 cycles [30]. However, a number of auto-immune related adverse effects including adrenal insufficiency, rash, diarrhea/colitis, and hypothyroidism appear in patients several weeks after being treated with ipilimumab [31]. Programmed cell death protein 1 (PD-1) is a protein expressed on T cells and plays a role in tumor cells escaping the immune response. When PD-1 interacts with the ligand PD-L1, it inhibits T cell proliferation and induces apoptosis of T cells [32]. In recent years it was demonstrated that PD-1/PD-L1 plays a role in immunosuppression of tumor cells. Using antibodies against PD-1 exhibited good results in a phase 1 clinical trial that included melanoma [33]. Only patients expressing the PD-L1 ligand on tumor cells were found to be suitable for the trial. Anti PD-1 and anti-PD-L1 are still being tested [33].

The other FDA approved drugs within the last years are therapies designed to target gene mutations of the RAS/RAF/MEK/ERK pathway. Since the most mutated protein in cancer is BRAF, the development of vemurafenib was pivotal as a potent and specific BRAF inhibitor [34, 35]. Currently, 40-60% of metastatic melanomas contain BRAF mutations, where a substitution for valine at the 600th amino acid position (V600E) is the most common, $BRAF^{V600E}$ mutation. To analyze vemurafenib response rate, a randomized phase 3 clinical trial that included 675 patients with stage IIIC or IV melanoma that also was both untreated and resectable demonstrated that after treatment with an oral dosage of 960 mg twice daily, a decrease of ~50% in tumor size was demonstrated with an improvement in progression free survival of ~5 months and an overall 6 month survival of ~85%

[36]. Resistance to vemurafenib is typically seen in all patients. Either the activation of alternative pathways or the reactivation of the mitogen-activated protein kinase creates this resistance thereby creating a need for combination treatments that include downstream and alternative pathway blocking agents [37, 38]. Current insights propose that a more effective therapeutic approach involves the use of individual inhibitors to simultaneously target RAF and MEK rather than targeting either kinase alone [39-41]. Dabrafenib was approved in May 2013 for the treatment of metastatic melanoma. This agent actively works against $BRAF^{V600E}$ mutations and when compared to decarbazine, established a 3 months increase of the progression free survival rate. Interestingly, even though dabrafenib has a reduced blood brain barrier capability, a phase 2 study with cases involving brain metastasis with tumors harboring the V600E mutation had an overall intracranial response rate of ~40% (previously untreated cases) and ~30% (previously treated cases) when administering a dosage of 150 mg dabrafenib 2 times a day [42]. Trametinib is a pharmacological MEK inhibitor that was approved in May 2013 by the FDA, for melanoma patients with $BRAF^{V600E}$ mutations. Patients treated with trametinib showed an increase of ~3 months progression free survival compared to chemotherapy alone [43]. Two notable combination therapies are dabrafenib/trametinib and vemurafenib/cobimetinib. In January 2014 the combination drug dabrafenib/trametinib was FDA approved for use in treating metastatic melanoma cases with unresectable tumors or containing $BRAF^{V600E/V600K}$ mutations [44]. The response rate was ~75% with response duration of ~10 months compared to treatment with dabrafenib alone which yielded a ~55% response rate with ~ 5 month response duration [24]. Another promising therapy is the combination of vemurafenib/with the MEK inhibitor, cobimetinib. Although FDA approval is yet to be obtained, findings from early data suggest

that this combination therapy significantly improves clinical outcomes in patients with advanced $BRAF^{V600}$ mutated metastatic melanoma [45]. The rate of response is increased in more than 20% in the combination group compared to the control group. Furthermore, an increase of ~10% in overall survival rate is observed in the combination group [45].

Genetic Alterations during Melanoma Progression

A wide array of both genetic and epigenetic events takes place throughout the progression of melanoma which leads to the initial formation of cutaneous melanoma and eventually metastasis. There are several genes whose genetic changes play a fundamental role in transitioning the initial stage of melanoma (benign nevi) to premalignant lesions. Some of these important genes includes: NRAS, cyclin D1, PTEN, BRAF, and CDKN2A.

BRAF is an oncogene that when mutated has the potential to cause normal cells to become cancerous. As a member of the RAF family, BRAF acts on the map kinase (MAPK), RAS/RAF/MEK/ERK signaling pathway [46]. An amino acid substitution at position 600 from a valine (V) to a glutamic acid (E) in BRAF results in the V600E mutation. The V600E mutation in BRAF along with NRAS, the upstream molecule that activates BRAF, are indicators of the critical role that the MAPK pathway plays in melanoma progression. While the V600E mutation is seen in approximately 40-60% of melanoma cases, only 20-30% of the upstream NRAS mutations are present [47-50]. These two mutations are mutually exclusive in melanoma. Contradictive to the records that suggest $BRAF^{V600E}$ is essential for melanoma progression is a study that has shown that although 80% of melanocytic nevi contain BRAF mutations, not all progress into primary melanomas [51] and that the introduction of $BRAF^{V600E}$ in melanocytes can induce cell senescence and apoptosis [52]. An explanation for this event comes from the presence of other molecules,

such as the tumor suppressor gene cyclin dependent kinase inhibitor 2A (CDKN2A) which inhibits $BRAF^{V600E}$ and therefore prevents BRAF from releasing cells from senescence and inducing cell growth. CDKN2A is important in melanoma progression since (through alternative mRNA splicing) this gene encodes $p16^{Ink4A}$, a known inhibitor of cyclin D/CDK4 complex, and $p14^{ARF}$, an inhibitor of MDM2, which regulates p53 [46, 53, 54]. The proliferating effects of $BRAF^{V600E}$ are restricted since an increase of $p16^{Ink4A}$ expression is observed in melanocytic nevi in comparison to normal dermis [55]. Primary melanomas have a 7% incidence of mutations in the $p16^{Ink4A}$ gene while it is present in 14% of metastatic lesions [56]. Mutation in the CDKN2A gene is often linked to patients with melanoma in their family history or UV exposure/damage. There are other genetic events that could adopt NRAS and BRAF mutations, which influence a greater chance of developing the disease [57, 58].

The loss of PTEN is also an important determinant in melanoma development. The action of PTEN is that of a phosphatase which removes phosphates from phosphatidylinositol phosphate (PIP_3) which then behaves like an intracellular signal catalyzed by growth factors or other stimuli [3, 59]. PIP_3 recruits the protein phosphoinositide-dependent kinase 1 (PDK1). Then, PDK1 phosphorylates AKT, which acts as a survival factor [60]. The phosphatase PTEN dephosphorylates PIP_3 (PtdIns (3,4,5)) on the 3 position, generating PIP_2 (PtdIns (4,5)) and causes inactivation of the AKT signaling cascade [59]. Early studies indicated that the deletion on chromosome 10q occurs in melanoma in 30-50% of melanomas [61, 62], and later this deletion was connected with the location of the tumor suppressor PTEN which is located within the long arm of chromosome 10, specifically chromosome band 10q23.3 [63, 64]. It was discovered that 60% of melanoma cell lines carry hemizygous

deletions of chromosome 10q [64] and 10% can be observed in primary melanomas [65, 66]. Immunohistochemistry studies revealed that almost all melanomas without PTEN expression showed no deletion or mutation suggesting that the loss of expression can also be a result of transcriptional repression or epigenetic regulation [67]. PTEN loss-mediated AKT activation promotes cell survival and proliferation in melanoma [68]. In the step involving the transition from dysplastic nevus to primary melanoma, the phosphorylation of AKT is increased and multiple processes are affected [68]. One example is the increased interactions between AKT and N-cadherin which leads to inactivation of BAD the pro-apoptotic protein. This interaction promotes melanoma cell survival [69]. AKT activation in melanoma is also associated with the upregulation of NFkB. AKT phosphorylates the protein IKKβ inducing its activity and then IKKb phosphorylates IKβ which is an inhibitor of NFκB. In that way, removing IKβ inhibition allows for NF-kB to be actively transcript. NFκB is responsible for the transcription of angiogenic and pro-tumorogenic genes as VEGF, Cox-2, Bcl-2, MMPs and IL-8 among others [70, 71].

In the last two decades our lab is investigating the molecular and cellular changes that occur during the shift from RGP to VGP in melanoma progression. The transcription factor activator protein 2 alpha (AP2α) is lost during this transition [72-74]. This is a specific characteristic of the melanoma phenotype since less metastatic melanoma cells have higher AP2α expression when compared to highly metastatic cell lines [75]. AP2α is 52 kD and is regulated by cyclic AMP (c-AMP) and retinoic acid (Vitamin A) [76-78]. AP2α expression is inversely correlated with genes like the protease activated G-protein coupled receptor (PAR-1) and the membrane adhesion molecule MCAM/MUC18 that are known to be pro tumorogenic [75, 79]. As a mode of action, AP2α will bind to the promoters of both

MCAM/MUC18 and PAR-1 to suppress their transcriptional activity [72-75, 79]. Loss of expression of nuclear AP2α is connected to melanoma progression through an observable increase in MCAM/MUC18 and PAR-1. MCAM/MUC18 is a cell adhesion molecule, and silencing it leads to a reduction in melanoma tumor growth and metastasis [80].

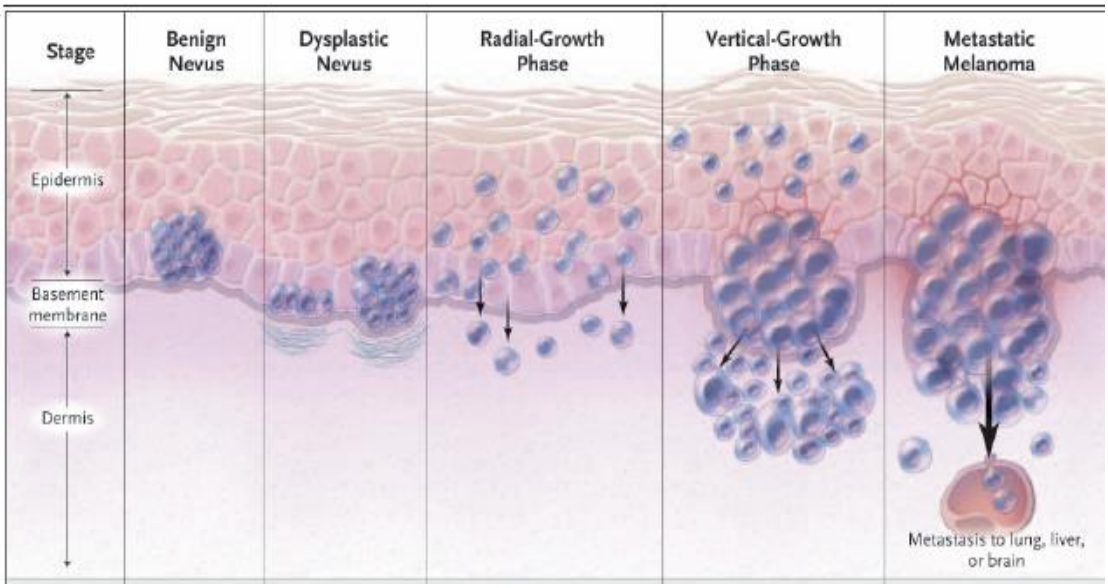
PAR-1 is another important molecule that plays a role in melanoma progression. It is an inflammatory molecule known to be upregulated during the RGP to VGP transition. PAR-1 promotes normal platelet aggregation through its cleavage in the extracellular domain by thrombin. In melanoma, PAR-1 enhances vascular endothelial growth factor (VEGF) and matrix metalloproteinase-2 (MMP2) expression in the microenvironment, thus supporting tumor growth and metastasis. PAR-1 increases the expression of Connexin-43, another pro tumorogenic gene and suppresses maspin, a known tumor suppressor gene [81-83]. Silencing of PAR-1 has shown reduction in melanoma growth and metastasis via Connexin-43 [82].

c-AMP response element binding protein (CREB) is another transcription factor that significantly overexpressed during the transition from RGP to VGP. Previous reports from our laboratory have shown that CREB serves many important functions during this transition [84, 85], including acting as a survival factor and increasing cell invasion by regulating MMP2, IL8, BCL2, MCAM/MUC18, and the tumor suppressor CYR61 [86-89]. Furthermore, CREB regulates other important transcription factors involved in melanoma progression such as, MITF and AP2α [90, 91]. To further evaluate CREB's role during melanoma progression, recently we performed gene expression profiling in metastatic melanoma cells following CREB silencing and identified an important and previously unknown target for CREB, the RNA-editing enzyme adenosine deaminase acting on RNA 1

(ADAR1). The activation of CREB plays an important role in regulating genes that are important for inflammation, invasion, and survival [87, 92, 93]. CREB is activated through phosphorylation at Ser133 and binding to the co-activators CBP and p300 [94, 95]. During melanoma progression, the activation of CREB can both induce the expression of various pro-tumerogenic genes including MCAM/MUC18 and MMP2 [87] as well as inhibit the expression of other genes, like CYR61. CYR61 has been shown to be a regulator of cell proliferation, survival, migration, and extracellular matrix formation [96]. Upon silencing CREB, CYR61 expression is increased and motility and invasion reduced *in vitro*. Silencing CREB reduced tumor growth and metastasis in melanoma *in vivo* [89]. Melanoma cells become susceptible to apoptosis when a dominant negative form of CREB is overexpressed [86]. Silencing CREB increases $p21^{waf1}$, a cell cycle inhibitor, while an increase in CREB activity directly suppresses AP2α expression during melanoma progression. Since AP2α is a positive regulator of $p21^{waf1}$, CREB has a significant effect on melanoma cells through its regulation of other transcription factors that in turn regulate different genes [91]. Past and latest findings emphasize the importance of CREB in melanoma growth and metastasis as the "master switch" in melanoma progression.

Activating transcription factor -2 (ATF-2) which belongs to the CREB family, also has been shown to have a role in melanoma progression [97, 98]. When ATF-2 is activated it promotes melanoma progression and cell growth by inducing such genes as c-Jun and TGFβ. Silencing ATF-2 reduced tumor growth and metastatic potential of melanoma cells [98, 99]. Many other important transcription factors are being deregulated during melanoma progression. SNAIL and SLUG transcription factors are known to inhibit transcription of E-cadherin [100]. Silencing SLUG increased melanoma susceptibility to chemotherapeutics as

cisplastim [101] . The TWIST transcription factor is known to be a metastasis regulator in epithelial cancers responsible for epithelial-to-mesenchymal transitions (EMT). However, in melanoma its role is unclear due to the ambiguous role of EMT in melanoma invasiveness and metastasis [102]. Inspite all these new modalities, further research is needed to understand the biology since BRAF resistance and the fact that only 30-40% response to immunecheckpoint therapies. All these genetic alterations mentioned and many other molecular classifications in melanoma progression are very important for planning new targeted therapies. Another molecule contributing to melanoma growth and metastasis is Galactin-3 (Gal-3). Our studies identified Gal-3 as the major regulator of autotaxin and NFAT1. This thesis will concentrate on the transcription factor, NFAT1 and further investigate the contribution of NFAT1 to the metastatic melanoma phenotype.



Stage	Benign Nevus	Dysplastic Nevus	Radial Growth Phase	Vertical Growth Phase	Metastatic Melanoma
Molecular Events	•BRAF mutations	•N-RAS mutations •INK4A/ARF loss •PTEN loss •p53 mutations •CDK4	•CD1 increase	*Gain of Function* ▪CREB/ATF-1, ATF-2, NF*k*B, SNAIL ▪Integrin $\alpha_v\beta_3$ ▪MCAM/MUC18 ▪Galectin-3, Survivin ▪PAR-1 ▪IL-8, IL-10, MMP-2, iNOS *Loss of Function* •AP-2α •c-KIT •E-cadherin • $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_4\beta_1$	•VEGF •bFGF •IL-8, CXCR2 •EGFR

Figure 1: Molecular and Genetic Changes During Melanoma Progression

The progression of melanoma is a stepwise process. From benign nevus to dysplastic nevus genetic mutations occur within the BRAF or NRAS genes. The loss of PTEN or $p16^{INK4A/ARF}$ expression are early events in a subset of melanomas. The tumor then grows radially throughout the epidermis termed the radial growth phase. The acquisition of multiple factors such as CREB and NFκB activation as well as enhanced expression of MCAM/MUC18, PAR1, Il-8, MMP-2 and galectin-3 induce the degradation of the basement membrane and invasion of melanoma cells termed the vertical growth phase (VGP). Finally, a few select melanoma cells intravasate, circulate, and survive in distant organ sites where metastasis forms. This figure reproduced with permission from (Miller AJ and Mihm MC Jr., N Engl J Med 2006 [3]), Copyright Massachusetts Medical Society, and Melnikova et al. Cancer Biol Ther 2008 [87].

Nuclear Factor of Activated T cell – NFAT Transcription Factor

Nuclear Factor of Activated T cell (NFAT) proteins were first identified in T cells as activators of the transcription of interleukin 2 [103, 104], which serve as a key regulator of T cell immune response. NFAT family members are transcription factors that play an important role in inducing the transcription of central genes during the immune response. All family members have a highly conserved REL-homology domain (RHD), which is a DNA binding domain. They are also involved in the control of T cell development and T cell differentiation [105]. Many years after the discovery of the NFAT gene family, they were found to play many roles in other biological systems besides the immune response. Despite their name, proteins from the NFAT family are expressed also on other cells and not only on T cells.

Calcium flux, calcineurin and NFAT kinases are the regulators of NFAT family. Calcium that is released from intracellular stores increases the levels of intracellular calcium. Calcium binds to calmodulin which then activates the calcineurin phosphatase. In order to be active, NFAT proteins are regulated by calcineurin, a phosphatase that dephosphorylates NFAT proteins to uncover their nuclear localization site (NLS), thus triggering their mobilization from the cytoplasm to the nucleus. In the nucleus, NFAT proteins cooperate with other factors to regulate gene expression for various biological functions [105]. The phosphatase calcineurin responds to a continued rise (not transient) of calcium in the cytoplasm, therefore NFAT is dephosphorylated and imported into the nucleus for the duration of the transcription [106]. In order to dephosphorylate NFAT, calcineurin needs to stably dock on NFAT, usually on a region located on the protein N-terminal. Furthermore, the weak DNA binding of NFAT requires that NFAT partner with other factors to perform transcription regulation. Such

partners can be MEF to control muscle development, GATA in heart development, FOXP3 to regulate immune tolerance and AP-1 to activate T cell response [107, 108].

The NFAT family of proteins contains five family members, NFAT1-NFAT5, that are all evolutionarily related to the Rel family. There are four typical members in the NFAT family (NFAT1-4) that indeed are regulated by calcinurin, in difference to these; NFAT5 does not require calcineurin or a nuclear partner for its activity. The NFAT protein family (NFAT1-4) has few functional modules sites on the protein: phosphorylation sites, nuclear localization site, DNA binding site, and transactivation sites (Figure 2). The N-terminal region includes regulatory domains like, casein kinase 1 (CK1), calcineurin (Cn) docking sites, and transactivation domain (TAD). The C-terminus includes the nuclear localization site (NLS), DNA-binding Rel homology domain (RHD) and an additional calcineurin docking site. In the middle of the protein there are several serine rich domains (SP1-3) that provide phosphorylation sites for kinases targeting NFAT. Furthermore, the NFAT protein contains two signal sequences that regulate its subcellular localization: the nuclear localization signal sequences (NLS1 and NLS2) and the nuclear export signal (NES) (Figure 2). When NFAT proteins are phosphorylated on their serine residues, they are localized in the cytoplasm [108]. The activation of receptors on the cell surface for example: receptor tyrosine kinases (RTKs), T cell receptor (TCR), receptors, and G protein coupled receptors (GPCRs), lead to a signaling cascade that starts causing calcium influx into the cytoplasm. When this influx is sustained, it causes the activation of calcineurin, which then dephosphorylates the cytoplasmic NFAT proteins. Dephosphorylation of NFAT by calcineurin exposes the nuclear localization site (NLS), causing the protein to transfer into the nucleus [109]. Calcineurin can be inhibited by the immunosuppressant cyclosporine A

(CsA) and tacrolimus (FK506), which form binding protein complexes which bind and competitively inhibit calcineurin phosphatase activity [110]. To neutralize NFAT activation, it needs to be rephosphorilated and mobilized outside the nucleus. Several kinases act to phosphorylate NFAT proteins, for example: protein kinase A (PKA), casein kinase 1 (CK1) and more. In the immune system the NFAT family of proteins is well established and their role in activating T cells is clear. There is an established clinical knowledge of NFAT being active in the regulation of T cells and in organ rejection after transplantation. Inhibitors like CsA and FK506 help in preventing this rejection. However the functions of the NFAT family of proteins in other aspects of human diseases and cancer are largely unknown.

In recent years there is more and more evidence that the NFAT family members are also involved in cancer development and metastasis. Phenotypes such as increase cell growth, enhanced proliferation, stimulates angiogenesis and increased resistance were reported in the literature about the NFAT family [111]. In our research we have been concentrating on one NFAT family member the NFAT1. Here in I will test the hypothesis that NFAT1 is not solely involved in T cell activation but could also regulate the melanoma metastatic phenotype.

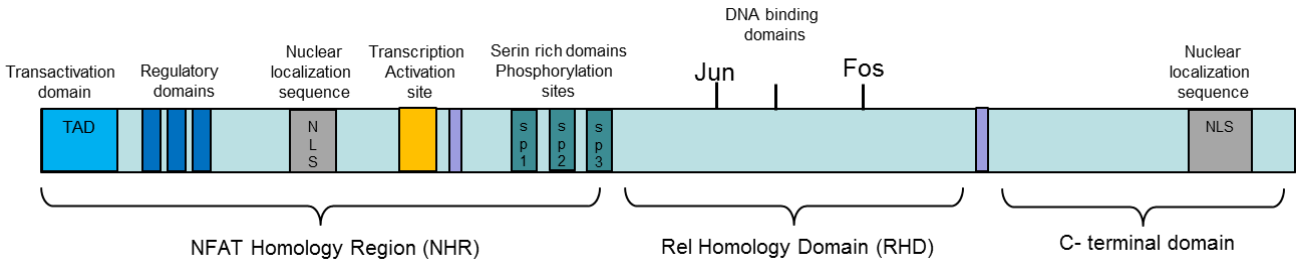


Figure 2: Primary Structure of NFAT

Schematic structure of NFAT. The region of highest homology within NFAT proteins is the DNA-binding Domain, Rel homology domain (RHD), which shows similarity to the Rel homology region of Rel-family transcription factors. A second region of homology is the NFAT homology region (NHR), which contains all regulatory domains. The N-terminal region includes regulatory domains like, casein kinase 1 (CK1), calcineurin (Cn) docking sites, and transactivation domain (TAD). The C-terminus includes the nuclear localization site (NLS), DNA-binding Rel homology domain (RHD) and an additional calcineurin docking site. In the middle of the protein there are several serine rich domains (SP1-3) that provide phosphorylation sites for kinases targeting NFAT. Furthermore, the NFAT protein contains two signal sequences that regulate its subcellular localization: the nuclear localization signal sequences (NLS1 and NLS2) and the nuclear export signal (NES).

Nuclear Factor of Activated T Cell 1 – NFAT1

NFAT1, also known as NFATc2, is the first member of the NFAT family that was discovered on T cells. NFAT1 is a transcription factor bound to the antigen receptor response element on the interleukin-2 (IL-2) promoter. NFAT1 family member is expressed on T lymphocytes but also on many other cells outside the immune system. In equilibrium, NFAT1 is greatly phosphorylated and its activation can be reached through dephosphorylation by calcinurin phosphatase [105]. Similar to the other family members, NFAT1 activation can be blocked using calcineurin inhibitors like tacrolimus (FK506) or cyclosporine (CsA) [110]. The dephosphorylation of NFAT1 helps the protein to be relocated into the nucleus and to be active as a transcription factor, as previously described. In recent years there is more and more evidence that NFAT1 is associated with cancer.

NFAT1 in Cancer

NFAT1 was published to be associated with cancer; it was showen to be associated with a wide range of tumor progression events such as: invasion, migration, tumor cell survival, and apoptosis. In breast cancer, high expression of NFAT1 was found in patients' specimens with primary tumors or lymph node metastasis, when compared to healthy tissue [112]. It was also found that there is a negative cross talk between NFAT1 and Stat5 signaling cascades [112]. These results highlight the effect of NFAT1 on breast tumor formation and metastasis. Primarily in breast cancer, it was demonstrated that NFAT1 enhances cell invasion and cell motility by regulating a variety of downstream genes [113]. One of the mechanisms by which NFAT1 to promote cell invasion in breast cancer was found to be through the induction of Cyclooxygenase-2 (COX-2), which is responsible for

the production of prostaglandins [114]. In 111 clinical samples of glioblastoma, NFAT1 was overexpressed compared with lower grade gliomas. NFAT-1 expression in glioblastoma cell lines was correlated with higher invasion and with the expression of genes which enhance invasion such as: MMP-7, MMP-9 and COX-2 [115]. In the melanoma literature there are few works done on NFAT1. NFAT1 deficient mice (NFAT1-/-) presented less experimental lung metastasis growth after melanoma cell injections when compared to the WT mice [116]. The absence of NFAT1 expression in the microenvironment causes a significant difference in the ability of the B16F10 melanoma cell line to progress [116]. Further research presents NFAT1 as a potential therapeutic target in melanoma, since NFAT1 was found to inhibit melanoma cell apoptosis and promoting proliferation [117]. NFAT1 was found to be an activating transcription factor for the MDM2 oncogene and as a response to DNA damage signals, NFAT1-MDM2 pathway is activated to inhibit p-53 function [118]. NFAT1 was demonstrated to support tumor-induced anergy of $CD4^+$ T cells [119] as well as regulate a set of genes that are responsible for helper T-cell ($CD8^+$) anergy [120]. A recent paper demonstrated that NFAT1 increased CTLA-4 promoter activity at $CD4^+$ T cells compared to $CD8^+$ T cells. This preferential expression of CTLA-4 on CD4, which is mediated by NFAT1, can be important for the anti CTLA-4 therapy [121]. All these data clearly indicate that NFAT1 is a regulator of multiple genes during cancer progression. Yet in melanoma the majority of the downstream genes are still not identified and the effect of NFAT1 on melanoma metastatic phenotype still needs to be elucidated. Our laboratory has previously demonstrated that Gal-3 contributes to melanoma growth and metastasis via the regulation of autotaxin and NFAT1 [122]. In this present thesis I will expand on these results and identify

other targets genes, besides autotaxin, that are regulated by NFAT1 and contributing to melanoma growth and metastasis (see specific aims).

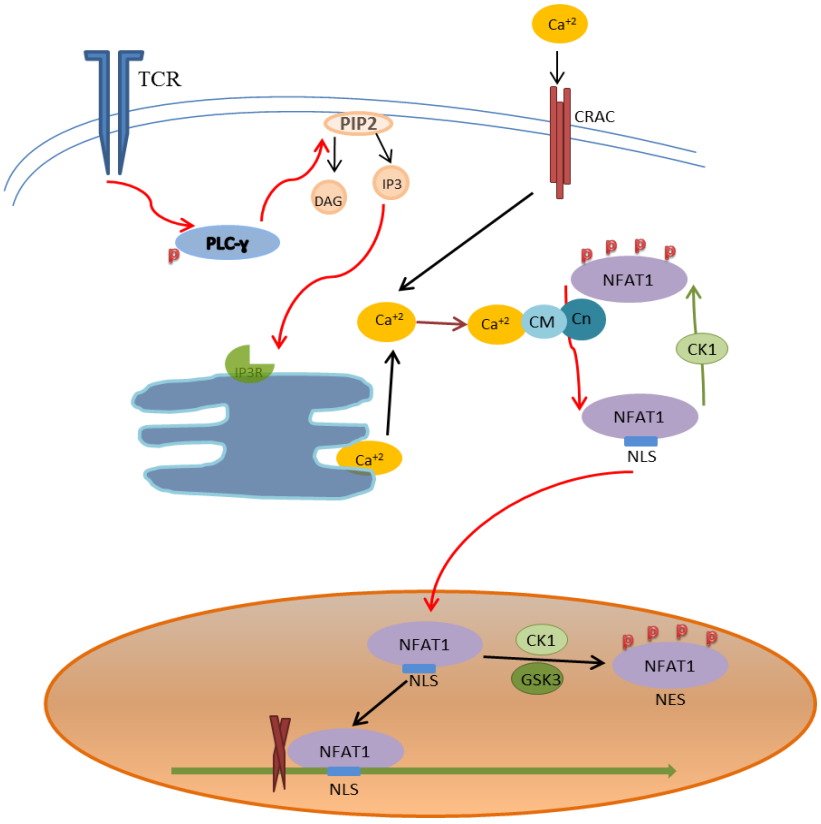


Figure 3: Calcium Signaling and Activation of NFAT1

Activation of the T-cell receptor (TCR) triggers the activation of receptor-associated tyrosine kinases that lead to the activation of phospholipase C-γ (PLC-γ). Activated PLC-γ causes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2), which generates inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds to its receptor and induces an increase in intracellular calcium levels that is caused by the depletion of intracellular stores. This increase triggers the opening of calcium-release-activated calcium channels (CRAC) in the plasma membrane, which leads to a sustained increase in intracellular calcium levels. Calcium binds calmodulin(CM) and activates calcineurin (Cn). Activated calcineurin dephosphorylates nuclear factor of activated T cells (NFAT1) proteins, which exposes their nuclear-localization signal (NLS) and induces their nuclear translocation. After it has entered the nucleus, NFAT1 interacts with activator protein 1 (AP1) and other transcriptional partners to promote gene transcription. The activation of these partners during T-cell stimulation might be elicited by signals that are transmitted through different signalling pathways. The activity of NFAT1 is also regulated by kinases, such as casein kinase 1 (CK1) and glycogen-synthase kinase 3

(GSK3), which help to maintain NFAT1 in a phosphorylated state in the cytosol (maintenance kinases) or induce the rephosphorylation of nuclear NFAT1 to expose a nuclear-export signal (NES) and translocate NFAT1 back to the cytosol (export kinases).

Specific Aims

NFAT1 was historically identified as inducible transcription factor in T cells [103]. In melanoma it was shown that NFAT1 expression in the microenvironment increases the invasive and metastatic phenotype of B16F10 murine melanoma cells [116]. We have previously shown in our lab that Galactin-3 is regulating autotaxin through NFAT1 in melanoma [122]. It is possible that in melanoma there are additional novel genes that could be regulated by NFAT1 to support melanoma growth and metastasis. **Therefore we hypothesized that in addition to its role in the immune system, and to autotaxin regulation in melanoma, NFAT1 regulates the expression of additional downstream targets that contribute to the metastatic melanoma phenotype.**

To test this hypothesis we developed the following specific aims:

<u>Specific Aim 1:</u> To Determine the Status and Contribution of NFAT1 to Melanoma Progression

1.1 Examine the Status of NFAT1 in Melanoma Progression

1.2 Study the *In Vitro* and *In Vivo* Effects of Silencing NFAT1 in Metastatic Melanoma Cell Lines and the Effects of Overexpression NFAT1 in Low Metastatic Cell Lines

<u>Specific Aim 2:</u> To Identify Novel NFAT1 Downstream Target Genes that Contribute to the Metastatic Melanoma Phenotype

2.1 Study NFAT1 as a Potential Regulator of IL-8 Expression in Melanoma Cells

2.2 Study NFAT1 as a Potential Regulator of MMP-3 Expression in Melanoma Cells

2.3 Determine the Contribution of MMP-3 to Melanoma Growth and Metastasis

CHAPTER 2: Materials and Methods

Cell lines and Cell Culture

Human A375SM cells were collected from nude mice that were i.v injected A375P (parental) cells, pooled lung metastasis and grown in culture as described previously [123]. The human SB2 melanoma cell line was isolated from a primary cutaneous lesion and is non-metastatic and poorly tumorigenic in mice [124]. WM902B cell line was isolated from skin malignant melanoma in the vertical growth phase (VGF). All cell lines were cultured in Eagles minimum essential media (MEM) supplemented with 10% fetal bovine serum (FBS), and 1% of HEPES buffer, penicillin-streptomycin, sodium pyruvate and nonessential amino acids, and kept at 37°C in a 5% CO_2 incubator. The human embryonic kidney cells (293FT) were used for lentiviral shRNA and overexpression vectors were maintained in DMEM supplemented with 10% FBS. Sixteen hours before each *in vivo* injection experiment, cells were replenished with fresh 10% FBS MEM. Cell lines in exponential growth phase were harvested by 2-5 minutes exposure to 0.25% trypsin-0.02% EDTA solution. The cells removed from flasks were pipetted to generate a single-cell suspension**.** The cells were resuspended in PBS to the desired cell concentration just before the injecions. Cell viability

was determined by trypan blue exclusion and only single-cell suspensions with more than 95% viability were used for *in vivo* injections.

Lentiviral shRNA

NFAT1 targeting shRNA 5'- CTGATGAGCGGATCCTTAA -3' or MMP-3 targeting shRNA 5'-TCTGAACAAGGTTCATGCT-3' and Non Targeting (NT) shRNA 5'-TTCTCCGAACGTGTCACGT-3' were designed with a hairpin and inserted into a pSIH-HI-copGFP lentiviral vector. The lentivirus was then produced by transfecting 293FT cells with the pSIH vector containing either the NFAT1/MMP-3 or NT shRNA sequence, the packaging plasmid (MD2G), and the envelop plasmid (PAX2) to produce a viable virus. The NT shRNA has no homology to any known human genes. The supernatant was collected containing a mature virus and was concentrated 10 times. A375SM and WM902B cells were plated at 70% confluence on a six well plate and were transduced with 800ul MEM / 200ul of supernatant containing the virus and were incubated overnight. The cells were then grown in culture and the top 50% GFP expressing cells were cell sorted by FACS.

***Nontargetable NFAT1 Expression Vector*, Empty Vector (EV)**

The NFAT1 gene (Isoform D) was amplified from A375SM cDNA with the following primers; Forward 5'-ATAAGAATGCGGCCGCGCCACCATGCAGAGAGAGGCTGCGTTCAG-3' and Reverse 5'- GCTCTAGATAATATGTTTTGTATCCAGCTAAG-3', cut with NotI and XbaI restriction enzymes, and inserted into the pCDNA3.1(+) vector. The NFAT1 shRNA targeting site was mutated to CAGACGAACGCATACTAAA (the underlined nucleotide are the entitled mutated sites) with the following primers; nfat1-Forward 5'-CTTCAGATCTT CATTGGGACAGCAGACGAACGCATACTAAAGCCGCACGCCTTCTACCAGG-3' and Reverse- 5'-CCTGGTAGAAGGCGTGCGGCTTTAGTATGCGTTCGTCTGCTGTCCCAATGAAGATCTGAAG-3'. This construct was used as a control for both NFAT1 rescue in highly metastatic A375SM and WM902B cells and NFAT1 overexpression in the low metastatic SB2 cells throughout the work.

NFAT1 overexpression Lentiviral Vector

NFAT1 gene (Isoform D) was cloned fromA375 cDNA into pCDNA3.1(+), using the following primers NFAT1XbaIF-5'-GCTCTAGAGCCACCATGCAGAGA-GAGGCTGCGTTCAG-3' and NFAT1NotIR-5'-ATAAGAATGCGGCCGCTCATAATATGTTTTGTATCCAG-3'. NFAT1 gene was cut with the designated restriction enzymes, inserted into a pCDH-CMV-MCS-EF1-puro vector and packaged with MD2G and PAX2 plasmids in a lentiviral virus as previously described. SB2 cell line was plated at 70% confluence on a six well plate and then transduced with

800ul MEM / 200ul supernatant containing virus overnight and were selected with MEM containing 1ug/ml puromycin.

NFAT1 Rescue Lentiviral Vector

NFAT1 rescue gene was generated on NFAT1-pCDNA3.1(+), using the following primers NFAT1-res-F- 5'-CTTCAGATCTTCATTGGGACAGCAGACGAACGCATACTA

AAGCCGCACGCCTTCTACCAGG-3' and NFAT1-res-R- 5'-CCTGGTAGAAGGCGTGC

GGCTTTAGTATGCGTTCGTCTGCTGTCCCAATGAAGATCTGAAG-3'. NFAT1 rescue gene was cut with the designated restriction enzymes, inserted into a pCDH-CMV-MCS-EF1-puro vector and packaged with MD2G and PAX2 plasmids in a lentiviral virus as previously described. A375SM and WM902B cell lines which NFAT1 was silenced using NFAT1 shRNA lentiviral vector were plated at 70% confluence on a six well plate and then transduced with 800ul MEM / 200ul supernatant containing virus overnight and were selected with MEM containing 1ug/ml puromycin.

MMP-3 Overexpression Lentiviral Vector **and Nontargetable MMP-3 vector -EV**

MMP-3 gene overexpression plasmid was purchased from shRNA and ORFeome core facility at MD Anderson. The gene was inserted into a PLOC vector which has RFP and Blasticidin as markers and packaged in a lentiviral virus as previously described.

Nontargetable MMP-3 expression vector (EV) was purchased from shRNA and ORFeome core facility at MD Anderson as well. SB2 cell line was plated at 70% confluence on a six well plate and then transduced with 800ul MEM / 200ul supernatant containing virus overnight and were selected with MEM containing 1ug/ml puromycin.

Fluorescence activated cell sorter and flow cytometry (FACS)

A375SM, WM902B and SB2 cells which were transduced with lentiviral constructs were detached from the flask using Tripsin EDTA 0.05%. Cells were centrifuged at 1400 rpm and the supernatant was removed. Cells were than resuspended in 500ul of PBS and were obtain into FACS.

Protein extraction

Total protein extracts were acquired from 70%-80% confluent cell culture on a six well plate or 10 cm dish. Cells were washed twice with cold PBS. After complete drain of PBS cells were incubated 10 min on ice with 200ul of RIPA "B" lysis buffer (1% Triton x-100, 150mM NaCl, 5mM EDTA, 20mM Sodium Phosphate, pH-7.4) supplemented with 1% protease inhibitor cocktail (Roche). Cells were scraped from the plate using a scraper and lysates were transferred into a 1.5ml clean tube. After 30sec of vortexing, lysets were centrifuged at 14,000 rpm at 4^{o}C for 15 min and supernatant was collected. Protein concentration was measured using Bradford assay (Bio-Rad).

Western Blot Analysis

To detect NFAT1, 20ug of whole cell protein lysate was loaded onto 8% SDS-PAGE and transferred into 0.45um Polyvinylidene Difluoride (PVDF) membranes (Millipore). To detect MMP-3 protein expression, which is a secreted protein therefore expressed best in the medium, one million cells were plated in a 10cm dish and were incubated in serum starvation conditions with 5ml of serum free MEM for 48hrs. The supernatant from cell culture was concentrated to 100ul. A total of 10ug of protein from the supernatant was loaded onto 10% SDS-PAGE. The membranes were blocked with 5% milk for 1 hour. Blots were incubated, usually over-night at 4^{o}C, with primary antibodies with the right dilution. Rabbit polyclonal anti-NFAT1 1:1000 (Santa Cruz Biotechnology); Rabbit polyclonal anti-MMP-3 1:1000 (Abcam), Rabbit polyclonal anti beta-Actin 1:2000 (Santa Cruz). Proteins were detected by ECL detection system (GE Healthcare). To confirm equal loading of the supernatant, the membrane was coomassie blue stained and destained with 40% methanol, 50% water, and 10% acetic acid until protein bands were visible.

Matrigel Invasion Assay

Matrigel invasion assays were performed using Biocoat Matrigel invasion chambers (BD Biosciences). Briefly, $1x10^5$ cells diluted in 500 µl of serum-free MEM and were placed on top of the upper chamber of the Matrigel plate in triplicates. The lower chamber contained MEM supplemented with 20% FBS. Matrigel plates were incubated for 40 h at 37 °C. Hema3 stain set was used to stain the cells which migrated to the lower surface of the Matrigel filter (Fisher Scientific). Filters were glued on a microscope slide. Pictures from

different fields where taken under light microscope and the stained cells were counted and statistically analyzed.

In vitro proliferation assay

One thousand of the cells that were used in this experiment, were plated in each well of a ninety six well plates that were used in this experiment (12 repetitions for each sample). The cells that were plated were the highly metastatic melanoma cell lines A375SM and WM902B NT and NFAT1 shRNA and the low metastatic cells SB2 EV and NFAT1 overexpression. The cells were cultured for 5 days in 10% FBS normal growth MEM medium. Cell growth was analyzed by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay that determines relative number of cells based on the conversion of MTT to formazan (has a purple color) in viable cells. Each day after plating the cells, MTT (Sigma) was added to each well at a 1mg/ml concentration in PBS, 20ul for each well. After addition of the MTT, a 2 hr incubation period was applied at 37°C. Medium and MTT were removed from the wells and were replaced by 100ul of diametyl sulfoxide (DMSO, Sigma). After 1 hr of incubation at room temp with DMSO the plate was read and quantified by measuring absorbance at 570nm using Epoch BioTek plate reader. This procedure was repeated daily over 5 years in order to compare if there are differences between the proliferation rates of cell lines that were NFAT1 manipulated using lentivirous stable transduction.

Reverse transcription-PCR

Regular PCR: RNA isolation was performed with the RNAqueous kit (Ambion). One microgram of total RNA was reverse transcribed (RT) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). One microgram Real time PCR was performed with the Taqman Gene Expression Assay and standardized to GAPDH (Applied Biosystems). NFAT1, IL-8 and MMP-3 Taqman Gene Expression Assays were acquired from Applied Biosystems and qRT-PCR was performed on A375SM WM902B and SB2 melanoma cell lines. Each probe was standardized to one with control sample.

Quantitative Real time PCR (qRT-PCR)

RNA (20 ng/µl) from the A375SM, WM902B and SB2 cell lines was harvested using a mirVana kit (Ambion) according to the manufacturer's instructions. The RNA was then transcribed into cDNA using TaqMan reverse transcriptase reagents for general cDNA. The primers for NFAT1, IL-8 and MMP-3 and fluorescence probes were obtained from Applied Biosystems. Reaction components for reverse transcription-PCR and amplifications were described previously [81]. Amplifications were run in triplicates, and averages were obtained after normalization with 18s (Applied Biosystems). Data was expressed in -fold change.

Chromatin Immunoprecipitation Assay (ChIP)

ChIP Assay was performed using ChIP-IT Express Kit (Active Motif) according to the manufacturer's protocol. 1×10^7 melanoma cells were plated in 15 cm culture dishes. The next day cells were fixed with 37% Formaldehyde containing medium. The cells were then

scraped from plates, and cell suspensions centrifuged for 10 minutes at 2500 rpm at 4 °C. Supernatant was discarded. The cells at the bottom of the tube were then lysed and incubated on ice for 30 minutes. Next, chromatin enzymatic shearing was performed to cut the DNA to fragments sized between 200-1000-bp. Fixed protein DNA complexes were then pulled down using magnetic beads with anti-NFAT1 antibody (Santa Cruz) followed by separation from the magnetic beads and Protein-DNA reverse cross-linking at 65°C for 2.5 hours. Next, the proteins were digested for one hour at 37°C with of Proteinase K and the DNA was prepared for PCR. PCR was performed by surrounding both NFAT1 binding sites with the following primers; NFATF- GCTCAAACTGCCAGCAAAAT and NFATR- CACAGGGTGTTCACAAATCG. The PCR product was run in a 1.5% agarose gel.

Reporter Constructs and Luciferase Activity Analysis

The IL-8 and MMP-3 promoters were cloned from A375SM melanoma cells to encompass 851 (IL-8) or 2682 (MMP-3) base pairs upstream of the transcriptional initiation site with the following primers respectively; IL-8 Forward-5'-GGGGTACCCTGCTCTTATGCCTCCACTG-3' and Reverse-5'-GGAAGATCTCTTGTGTGCTCTGCTGTCTCT-3' and for MMP-3 Forward-5'-GGGGTACCCACTCAGTTGCTCTTTAATTTTACC-3' and Reverse-5'-GGAAGATCTCGCACAGCAACAGTAGGATTG-3'. PGL-3 basic was cut with Kpn1 and BglII restriction enzymes and the IL-8 or MMP-3 promoters were inserted. Direct site mutagenesis of NFAT1 binding sites were carried out using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. Cells were plated in

a 24 well plate with 2.0 x 10^4 cells/ well. After 48 hours, transfection with Fugin 6 (Promega) was performed according to manufacturer's instructions. Briefly, each well was transfected with 0.8 μg of the basic pGL3 expression vector with no promoter sequence or with 0.8 μg of pGL3 with the inserted IL-8 or MMP-3 promoter; mutations were performed in the NFAT1 binding sites. As a control, 2.5 ng of cytomegalovirus (CMV) driven renilla luciferase construct (pRL-CMV, Promega) was included per well. Each group was plated in replicates of six. After 48 hours the cells were lysed and luciferase activity was assayed with the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The ratio of PGL3 firefly to CMV-driven renilla luciferase activity was used to normalize each sample.

Immunohistochemistry (IHC)

Mice were killed and tumors were collected. Half of each tumor was formalin fixed and embedded into paraffin and the other half was frozen at -80°C. Paraffin-embedded tumor specimens were mounted on positively charged superfrost plus slides (Fisher Scientific). Slides were heated at 56°C for 20 min and de-paraffinized in xylene, hydrated in graded ethanol (100%, 95%, and 80% ethanol), and then rehydrated in PBS twice. Antigen retrieval was performed in pepsin (BioCare Medical) at RT for 10 minutes or with citrate buffer 0.1M. Endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS. Samples were exposed to protein block (5% normal horse serum and 1% normal goat serum in PBS) for 20 minutes and incubated with each primary antibody – rabbit anti-human IL-8 (1:100), rabbit anti-human NFAT1 (1:400) and rabbit anti-human MMP-3 (1:100) antibodies overnight at

4°C. Slides were washed, incubated for 10 minutes in protein-blocking solution, and then reacted with goat anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch, West Grove, PA).

CD31 staining was executed by the same protocol as procedures of other antibodies, except that a goat anti-mouse CD31 (1:200) antibody (PharMingen, San Diego, CA) was used. Upon washing with PBS, the signals of specimens were developed with 3,3'-Diaminobenzidine (DAB) for 5-10 minutes at RT. The slides were washed with dH_2O, air-dried and applied with cover slides. The photo images were obtained from the Leica microscope DFC 320 (Wetzlar, Germany) For CD31 staining, frozen sections were fixed for 5 minutes in each of the following solutions: acetone, acetone:chloroform (1:1) and acetone. Slides were then washed with PBS three times and blocked with protein block 4% Fish gelatin in PBS for 20 minutes at room temperature. Slides were incubated at 4°C over night with primary antibody (rat anti-mouse CD31, PharMingen Inc. Cat. #01951A). Alexa 494 [125] was incubated for 1 hour at room temperature. Slides were then washed 3 times for 3 minutes in PBS.

TUNEL assay

TUNEL staining was performed utilizing a TUNEL assay kit (Promega, Promega, Madison, WI). Slides were fixed in 4% Paraformaldehyde for 10 minutes at room temperature followed by two 5 minute washes with PBS. Slides were then incubated in 0.2% Triton X-100 in PBS for 15 minutes and washed twice with PBS. Subsequently, slides were incubated with DNase for 10 minutes at 37ºC and washed four times with PBS. Equalibrium buffer was then used

on slides for 10 minutes at room temperature. Incubation buffer containing 5ul Nucleotide Mix and 1 ul of TDT enzyme was then applied on slides. Slides were then incubated at 4°C over night. The following day, slides were washed with SSC buffer twice for 15 minutes followed by washes with PBS to remove unincorporated Fl-dUTP. Hoechst (Molecular Probes, Carlsbad CA) diluted at 1:10,000 in PBS was used to counterstain slides.

Animals

Female athymic BALB/c nude mice at the age of 8-10 weeks were purchased from Taconic (Hudson, NY). Animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and the NIH. All studies were approved and supervised by The University of Texas MD Anderson Cancer Center, Institutional Animal Care and Use Committee (IACUC).

In Vivo subcutaneous tumor growth

Subcutaneous tumors were produced by injecting 0.5-1 $x10^6$ tumor cells/100 ul PBS into the right frank of each mouse. Between 6 to 8 mice mice were injected for each group. Tumor size was monitored twice a week for 27 days. Mice were then sacrificed and tumors were collected. The tumors were processed for IHC to detect alteration of IL-8, MMP-3, CD31. TUNEL assays also were performed to determine the effects on vessel density and apoptosis of tumors.

Experimental Lung Metastasis Assays

For lung metastasis experiments, 6 to 8 mice per group were injected with 0.5-1x10^6 tumor cells in 100 ul PBS via mouse lateral tail veins as previously described [89]. Tumor metastasis burden was closely monitored. Any mouse that demonstrated a sign of thin or hunched postures was scarified earlier. The mice were killed about 6 weeks after injections; the lungs were removed, and fixed in Bouin's solution for 24 hours. The number of surface tumor nodules were counted using a dissecting microscope.

cDNA Microarray

Total RNA was isolated from A375SM NT and NFAT1 shRNA melanoma cells using mirVana Isolation Kit (Life Technologies). RNA was then converted into cRNA using the Illumina TotalPrep Amplificatin Kit (Life Technologies) and hybridized to HT-12 Illumina chip in triplicates. Gene expression analysis was performed between NFAT1 NT and NFAT1 shRNA samples.

Statistical Analysis

Student's *t* test was used to analyze the statistical significance of differences in the *in vitro* data. In the animal studies Mann-Whitney *U* test was used in the tumor growth and lung

metastasis results. Values for tumor growth are given as a mean volume ± S.E.M. P values that are smaller than 0.05 were considered statistically significant.

TCGA Analysis

Statistical analyses were performed in R (version 3.0.1) (http:///www.r-project.org/) and the statistical significance was defined as a p-value less than 0.05. We downloaded and analyzed clinical and mRNA (Level 3 Illumina RNASeqv2) data publicly available from the Cancer Genome Atlas Project (TCGA; http://tcga-data.nci.nih.gov/) for patients with skin cutaneous melanoma (SKCM).

<u>For NFAT1 Tumor vs. Metastatic comparison</u>

The Shapiro-Wilk test determined that NFAT1 (log2 reads) did not follow a normal distribution in tumor or metastatic samples. The nonparametric test Mann-Whitney-Wilcoxon test was applied to compare NFAT1 expression levels between the two groups and a box-and-whisker plot (Box plot represents first [lower bound] and third [upper bound] quartiles, whiskers represent 1.5 times the interquartile range) was used to visualize the data.

<u>For survival IL-8 and MMP-3:</u>

For each gene of interest, we checked for a relation with the survival as follows. Patients were grouped into percentiles according to mRNA expression. The Log-rank test was employed to determine the association between mRNA expression and overall survival and the Kaplan-Meyer method was used to generate survival curves. Cut-off points (log-rank test

p-value <0.05) to significantly split the samples into low/high mRNA groups were recorded. The cut-off to optimally separate the patients in high/low (min p-value) was chosen.

Dual IHC Staining for Tissue Microarray (TMA)

Paraffin sections in 5 μm thickness were made, and immunohistochemistry was performed using monoclonal antibodies against NFAT1 (clone D43B1 [1:50 dilution]; Cell Signaling, Danvers, MA) and CD8 (Clone C8/144B [1:20 dilution]; LabVision, ThermoFisher). After deparaffinization, antigen retrieval was performed using a citrate buffer (pH 6.0) in a pressure cooker. Slides were then incubated with NFAT1 antibody, followed by Envision+ horseradish peroxidase reagent (Dako, Carpentaria, CA), followed by incubation with diaminobenzidine (DAB). After washing, the slides were incubated with CD8 antibody, followed by an alkaline phosphatase-conjugated secondary antibody (Bond Polymer Refine Red Detection, Leica Biosystems, Buffalo Grove, IL), and then incubated with Fast Red/AP chromogen. CD8 (red chromogen) and NFAT1 (brown chromogen) immunoreactivity were considered positive if moderate to strong staining was identified in even a single cell. The extent of staining was quantified using the following scale: 0 indicated no staining; 1+ indicated positive reactivity in < 25% of tumor cells; 2+ indicated positive reactivity in 25-50% of tumor cells and 3+ indicated positive reactivity in > 50% of tumor cells. Localization to the cytoplasm or the nucleus was recorded for all cases expressing NFAT1. CD8 localized to the cytoplasm & membrane when expressed. The TMA that we have used have total 130 specimens: 59 Nevi, 39 primary and 32 metastatic, each sample is represented twice on the block.

CHAPTER 3: Specific Aim 1

To Determine the Status and Contribution of NFAT1 to Melanoma Progression

1.1 Examine the Status of NFAT1 in Melanoma Progression

1.2 Study the *In Vitro* and *In Vivo* Effects of Silencing NFAT1 in Metastatic Melanoma Cell Lines and the Effects of Overexpression NFAT1 in Low Metastatic Cell Lines

Introduction

Melanoma is the deadliest form of skin cancer in the United States [2]. During the last decade there has been tremendous progress in understanding the genetic changes that are associated with melanoma progression. Those newly discovered target genes can potentially help researchers in finding new therapeutic combinations in melanoma. During the progression of the disease, melanoma cells need to acquire the ability to invade the basement membrane and migrate into the dermis. In our research, we are looking at NFAT1 as a potential gene that may be an important protein that helps melanoma cells acquire the invasive phenotype. NFAT1 expression has also been associated with cancer; in breast cancer patient specimens, higher expression of NFAT1 was recognized with primary tumors and lymph node metastasis compared to normal adjacent tissue [112]. By evaluating past publications and previous evidence we hypothesized that NFAT1 (besides its role in the immune system) can be a key player in the invasive phenotype of melanoma cells. NFAT1 is possibly playing an important role in melanoma progression and metastasis formation. In this first specific aim we want to determine the contribution of NFAT1 to the *In vitro* and *In vivo* phenotype of melanoma cell lines and examine the status of NFAT1 in melanoma patients.

Sub Aim 1.1: Examine the Status of NFAT1 in Melanoma Progression (Cell Lines and Patients)

Results

Analysis of NFAT1 Expression in a Melanoma Cell Line Panel

To examine the status of NFAT1 in melanoma progression, a panel of melanoma lines ranging from low to highly metastatic were utilized. Using western blot analysis, we validated that the more metastatic cell lines they express higher levels of NFAT1, while the less metastatic melanoma cells express significantly lower levels of NFAT1. As shown in figure 4, the highly metastatic melanoma cell lines (Mewo, TXM18, A375SM WM2664 and

WM902B) expressed higher levels of NFAT1 as compared to the less metastatic melanoma cell lines (SB2, and DM4). Our cell panel can be further divided into two groups: those with BRAF mutations (highly metastatic in our case) and those with NRAS mutations (low metastatic in our case). When NFAT1 expression was analyzed by this manner, we found that cells with BRAF mutations have higher expression levels of NFAT1 and cell lines with the NRAS mutations had a lower expression of NFAT1. However, it is possible that this cell line panel is not representative. A larger scale analysis should be performed in order to have a conclusion about the connection between the BRAF mutations with NFAT1 expression levels in melanoma cell lines. Therefore this correlation could be due to the panel lacking highly tumorigenic NRAS and non $BRAF^{V600E}$ mutation cell lines. Later, we also performed patients analysis for NFAT1 to confirm.

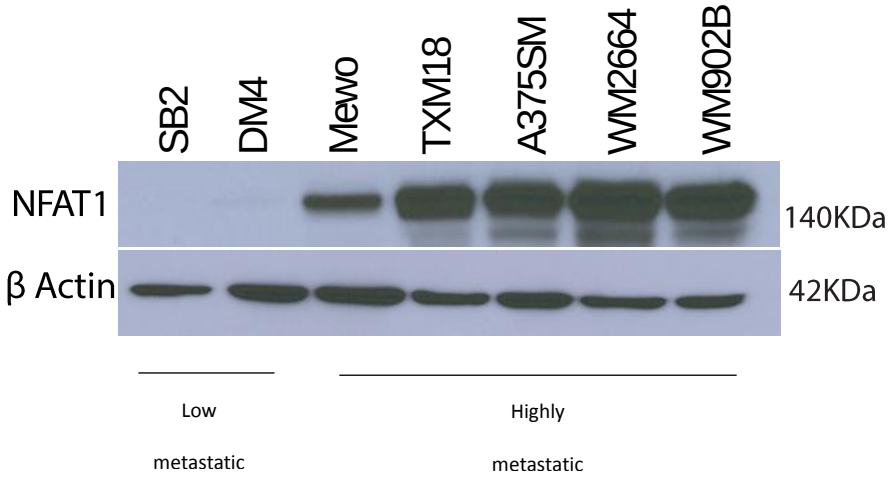


Figure 4: NFAT1 is Expressed at Higher Levels in Metastatic Human Melanoma Cell Lines

Western blot analysis in melanoma cell lines was performed. The less tumorigenic SB2, and DM4 melanoma cell lines express significantly less NFAT1 than the more tumorigenic and metastatic Mewo, TXM-18, A375SM, WM2664 and WM902B cells.

Melanoma TCGA Analysis of NFAT1

In this set of experiments we sought to validate our observations in melanoma cell lines by mining the TCGA (The Cancer Genome Atlas) data for NFAT1 expression. This analysis revealed a significant higher expression of NFAT1 in metastatic lesions compared to primary melanoma lesions ($p=0.0079$) (Figure 5). This analysis validated our observation in melanoma cell lines that, the more metastatic the cell is, the higher NFAT1 expression it has.

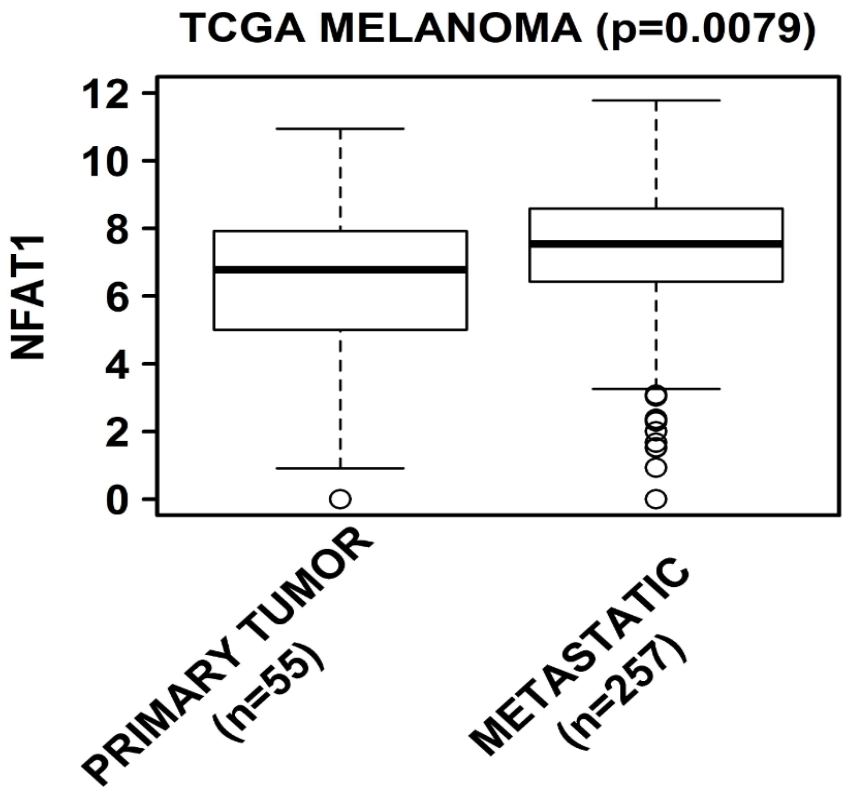


Figure 5: Analysis of TCGA Melanoma Data for the Expression of NFAT1 in Melanoma Patients Analysis of TCGA revealed a significant overexpression of NFAT1 in the metastatic lesions (p=0.0079) compared to primary melanoma lesions. Primary melanoma lesions (n=55), metastatic lesions (n=257).

Analysis of NFAT1 in a Melanoma Patients' Tissue Microarray (TMA)

To further delineate the status of NFAT1 in melanoma progression, the expression of NFAT1 was examined in tissue samples from melanoma patients in different stages (nevi, primary and metastatic). The evaluation was made in a way that the score is given by the expression level of NFAT1 on the tumor cells but not on the immune cells. This scoring was achieved after double staining for NFAT1 and tumor infiltrating lymphocytes (TIL). The expression was evaluated on the tumor cells alone. CD8 (red stain for T cells) and NFAT1 (brown) immunoreactivity were considered positive if moderate to strong staining was identified. For quantification we used the following scale: 0 indicated no staining; 1+ indicated positive reactivity in < 25% of tumor cells; 2+ indicated positive reactivity in 25-50% of tumor cells and 3+ indicated positive reactivity in > 50% of tumor cells. Indeed there was a difference between the expression of NFAT1 in metastatic versus primary melanoma patients. The metastatic tissues expressed more NFAT1. Unfortunately, the scoring is still in progress but from the general trend we can assume that these results support the TCGA analysis and the results from the western blot for the cell panel.

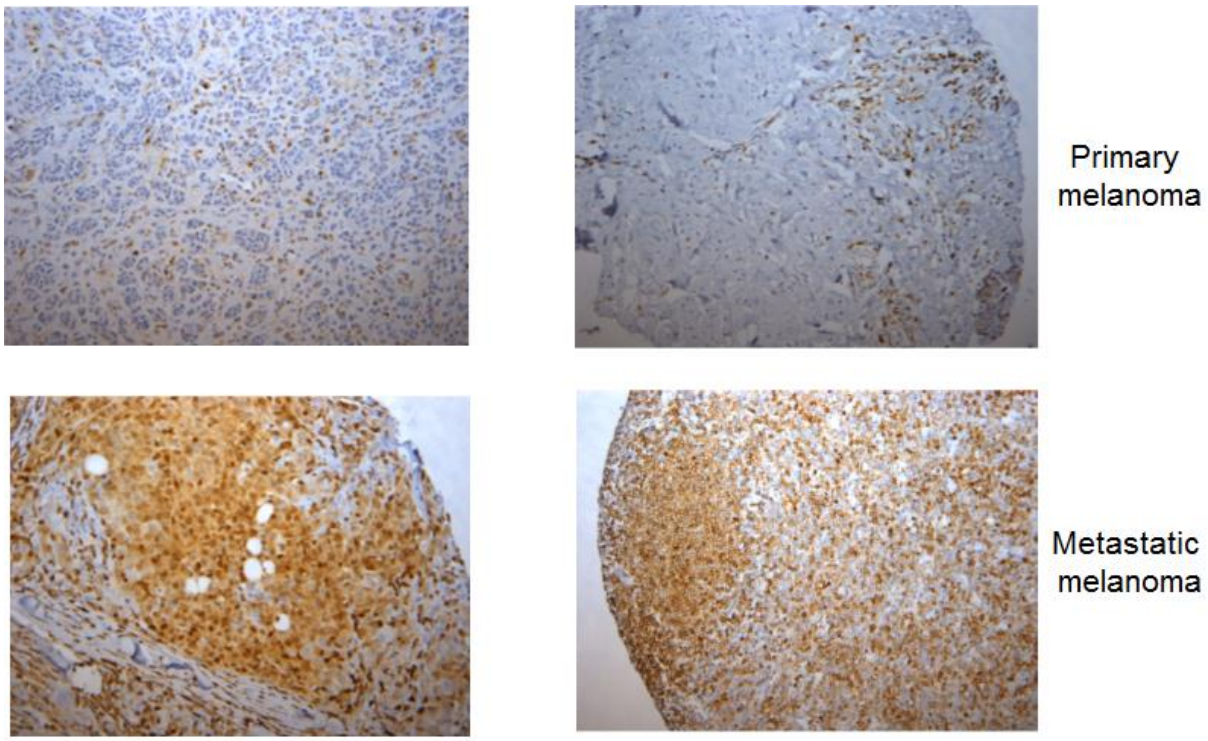


Figure 6: Analysis of TMA for the Expression of NFAT1 in Melanoma Patients. On this array there are 130 human tumor samples. After scoring the expression of NFAT1 on tumor cells we can see a significant difference between metastatic and primary melanoma tumors.

Sub Aim 1.2 Study the *In Vitro* and *In Vivo* Effects of Silencing NFAT1 in Metastatic Melanoma Cell Lines and the Effects of Overexpression NFAT1 in Low Metastatic Cell Lines

Results

The Effect of Silencing NFAT1 in metastatic melanoma and overexpressing it in low metastatic melanoma cell lines.

To establish the role of NFAT1 in melanoma progression and in promoting tumor growth and metastasis, we chose to stably silence NFAT1 in two metastatic melanoma cell lines that have high levels of NFAT1 expression and overexpressing NFAT1 in a low metastatic cell line (with low levels of NFAT1). From our cell line panel, the cells that met these criteria were the WM902B, A375SM and SB2 melanoma cell lines (Figure 4). The two metastatic melanoma cell lines, A375SM and WM902B, were then stably transduced with non-targetable (NT) or NFAT1 shRNA packaged lentivirus. NT shRNA has no known homolog sequence among human genes; it will be used as a control to verify that the transduction by the virus itself did not cause any unexpected changes in the cells. This control vector contains green fluorescent protein (GFP) and it will be used throughout the study. The SB2 cell line was stably transduced with the NFAT1 overexpression construct and for the control we used an empty vector (EV) construct that does not include any inserted

gene. The overexpression transduction has a puromycin selection, and in order to select the positively transduced cells, the culture was grown with 1mg/ml puromycin for 48hr. For A375SM and WM902B, both NT and NFAT1 shRNA melanoma cells were sorted for the top 40% percent of GFP fluorescence, by Fluorescent Activated Cell Sorting (FACS). After cell sorting/puromycin selection, WM902B, A375SM and SB2 melanoma cell lines that were transduced were expanded in culture. We performed western blot analysis to determine the silencing efficiency of NFAT1 shRNA or the overexpression efficiency for NFAT1 over-expressing vector. By using densitometry and normalizing NFAT1 to actin, it was observed that the WM902B and A375SM cell lines have 58% and 75% knock down of NFAT1 respectively as compared to the NT shRNA control (Figure 7). In SB2 cells, NFAT1 overexpression results demonstrated infinite fold of NFAT1 overexpression in that cell line due to no expression in the parental cells. These three cell lines were then used throughout the study.

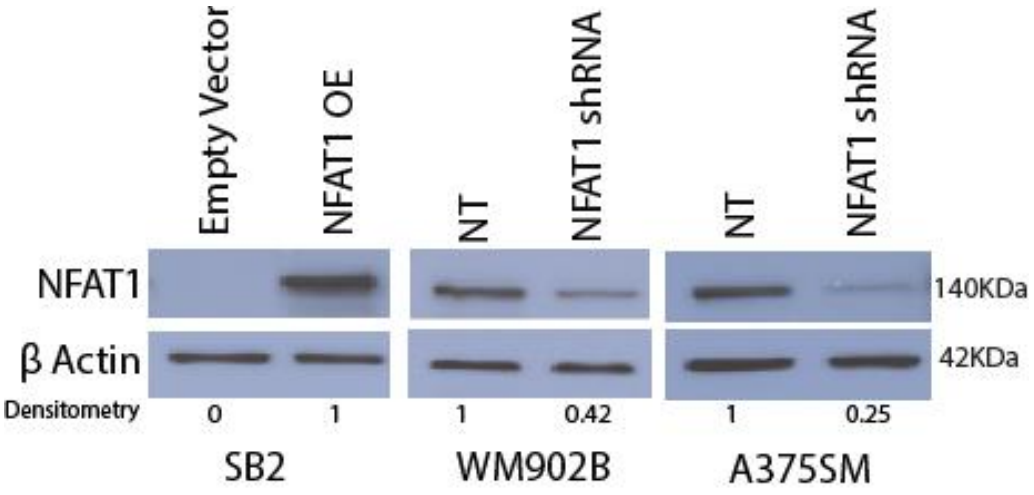


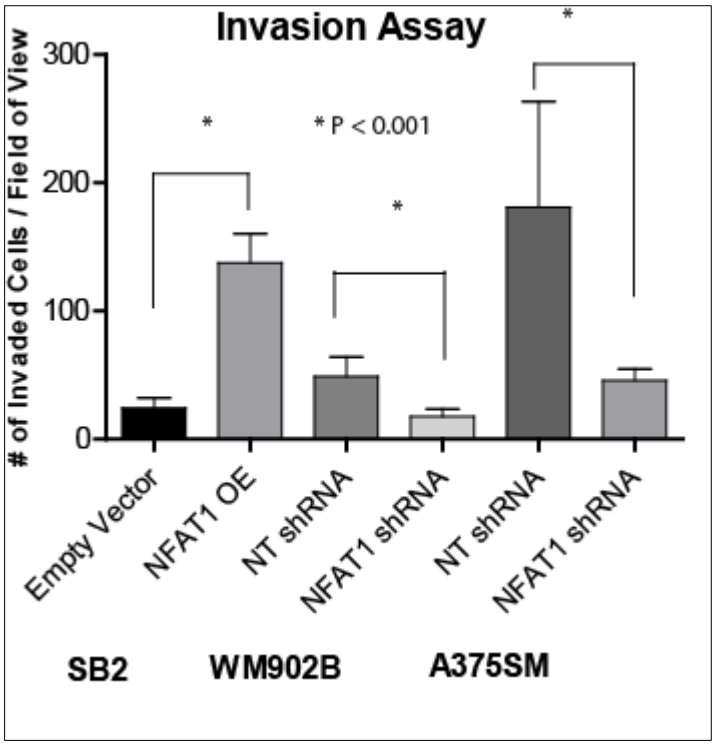
Figure 7: Stable Transduction of NFAT1 shRNA/ Overexpressing Vector Efficiently Reduce NFAT1 Expression in both WM902B and A375SM and Overexpress it in SB2 Melanoma Cell Lines. NFAT1 expression is lost in both melanoma cell lines with the stable lentiviral based transduction of NFAT1 shRNA. Densitometry analysis for WM902B cells confirms that approximately 58% of NFAT1 expression is lost. For A375SM cells, NFAT1 expression is 75% lost. For SB2 cells we have a complete overexpression of NFAT1.

In Vitro Invasive Phenotype of Melanoma Cells Following NFAT1 Silencing/ Overexpression

To validate that NFAT1 increases the invasive phenotype of melanoma cell lines, NFAT1 silenced WM902B and A375SM melanoma cells and SB2 with NFAT1 overexpression were subjected to the matrigel invasion chamber invasion assay. The cells were plated in fetal bovine serum (FBS) free media (MEM) on top of the chamber. The cells were incubated for 24 hours. The bottom chamber contained MEM media with 20% FBS to act as a chemoattractant. The number of invaded cells through the chamber was evaluated by counting equal fields from all membranes. A significant reduction in the number of invaded melanoma cells was observed after silencing NFAT1 in both WM902B and A375SM cell lines, $*p < 0.001$ (Figure 8). A more than 2 fold reduction was observed in WM902B cells and more than 3 fold reduction in A375SM. A significant increase in the number of invaded cells was observed after overexpression of NFAT1 in SB2 cells; more than a 5 fold increase was observed. As seen in figure 8, the invasive capacity of melanoma cells is significantly reduced after silencing NFAT1 in metastatic cell lines $p < 0.001$, with more than a 2 fold reduction in WM902B and 3 fold reduction in A375SM, and on the other hand, the invasive capacity was increased after NFAT1 overexpression in SB2 cells. Therefore, our data support the idea that NFAT1 is critical for the invasive phenotype of melanoma cell lines. To make sure that the invasive phenotype indeed changed as a result of NFAT1 expression in the cell lines and not due to higher doubling time of the tested cells, we performed MTT proliferation assay as presented in figure 9. We found no changes in the doubling time of A375SM and WM902B after NFAT1 silencing compare to the NT. Similar results were observed in this the SB2 cell line after overexpression of NFAT1. Since no significant difference was

detected in the doubling time, we concluded that the changes in the invasion assay were not due to differences in cell division times but due to NFAT1 genetic manipulations which influenced on the invasive and migratory phenotype.

A.


Invasion Assay
# of Invaded Cells / Field of View
300
200
100
0
* P < 0.001
*
*
*
Empty Vector
NFAT1 OE
NT shRNA
NFAT1 shRNA
NT shRNA
NFAT1 shRNA
SB2
WM902B
A375SM

B.

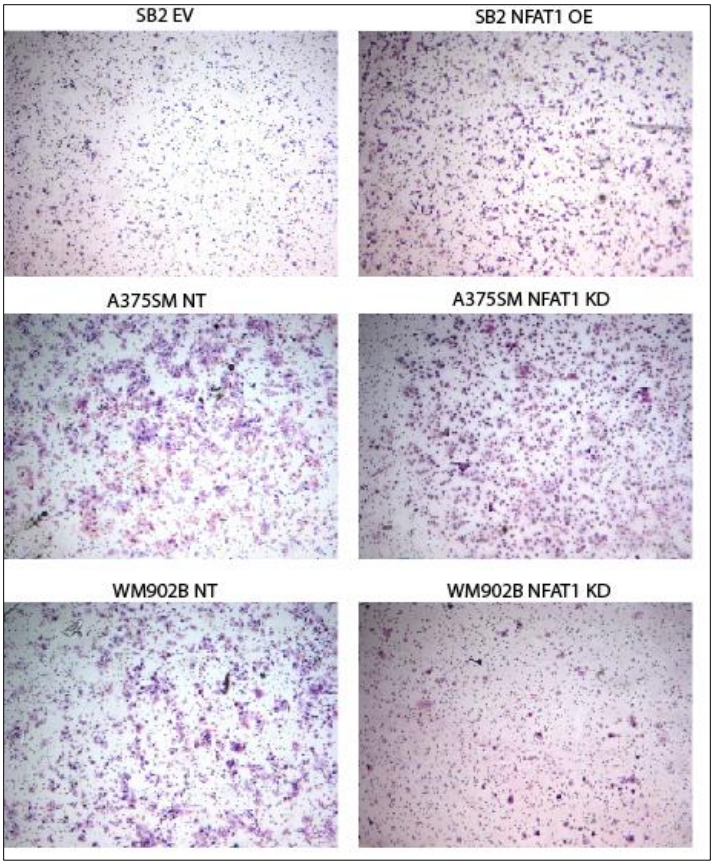
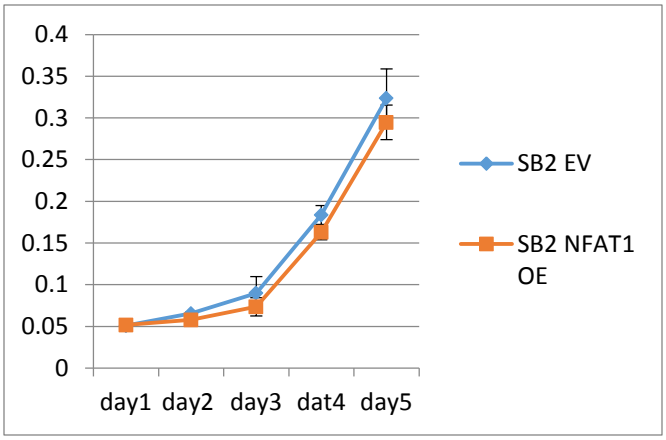

SB2 EV
SB2 NFAT1 OE
A375SM NT
A375SM NFAT1 KD
WM902B NT
WM902B NFAT1 KD

Figure 8: The Invasive Phenotype of Melanoma Cells after Silencing /Overexpressing NFAT1

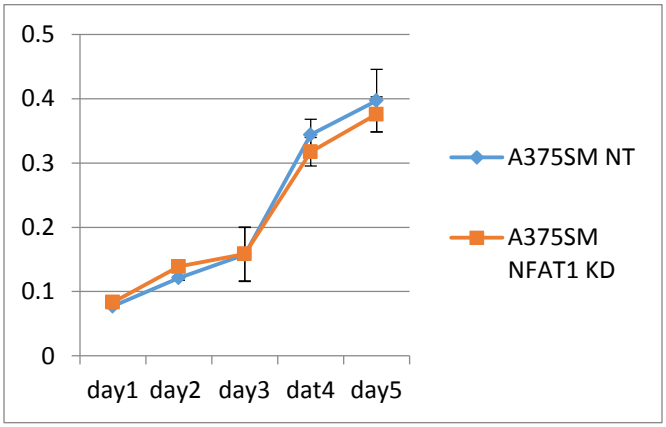
The invasive phenotype of melanoma cells were analyzed by matrigel invasive chambers 8μm (A) The number of invaded cells through the Matrigel invasion chamber is significantly reduced after silencing NFAT1 in both WM902B and A375SM melanoma cells as compared to NT shRNA (*P < 0.001) and on the other side, the number of invaded cells is significantly increased after NFAT1 overexpression in SB2 cells. (B) A representative image for each cell line with either NT or NFAT1 shRNA or NFAT1 overexpression is shown (NT non targeting, OE overexpression).

A.



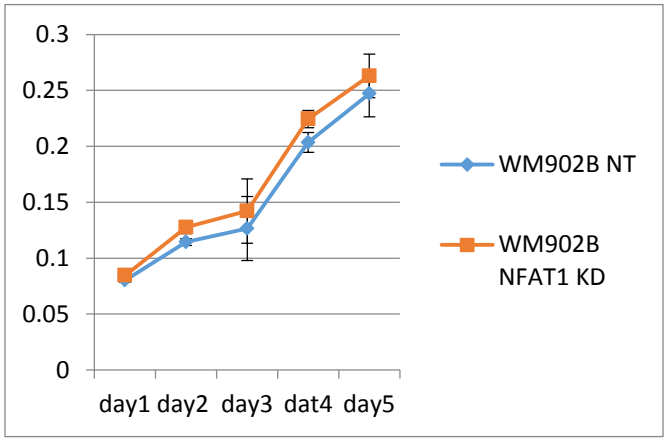
P=0.09736

B.



P=0.11966

C.



P=0.06389

Figure 9: Proliferation Rate of NFAT1 Silenced Metastatic Melanoma Cell Lines or NFAT1 Overexpression in Low Metastasis Cell Lines. MTT assays were performed to determine doubling time of A375SM, WM902B (A,B) cells after transduction with NFAT1 shRNA as compared to NT transduced cells, and SB2 after NFAT1 overexpression compared to empty vector control (C). MTT assays determined relative cell numbers based on the conversion of MTT to formazan in viable cells. Cell growth was measured daily for 5 days period. No significant differences were found in the doubling time in either transduced cell line ($P>0.05$).

Determine the *In Vivo* Effect NFAT1 on Tumor Growth and Experimental Lung Metastases

To determine whether NFAT1 has an effect on melanoma tumor growth and metastasis, an *in vivo* experiment was performed. To that end, we used A375SM highly metastatic cells with NFAT1 shRNA compared to NFAT1 NT shRNA and the low metastatic SB2 after NFAT1 overexpression compared to SB2 EV (empty vector). $5x10^5$ A375SM cells or $1x10^6$ SB2 cells were injected subcutaneously into the right flank of nude mice (n=6 mice per group) for tumor growth, or intravenously into the tail vein of the mice (n=6 mice per group) for experimental lung metastases. Each group had the control group A375SM NT and SB2 EV respectively. The subcutaneous tumors were monitored twice weekly for 31 days for A375SM, and for 41 days for SB2 cell lines. At the end of the measurements the tumor size mean of A375SM NT group was $1557mm^3$ compared to $509mm^3$ in the A375SM with NFAT1 shRNA (Figure 10A). At day 41, SB2 EV cells had a mean tumor volume of $428mm^3$ compared to $1125mm^3$ for SB2 NFAT1 OE cells (Figure 10B). We observed a significant decrease in tumor growth after silencing NFAT1 and significant increase in tumor growth after overexpressing NFAT1 in melanoma cells. Since we already demonstrated that there is no difference in the doubling time of these cell lines, we can conclude that the differences we observed in tumor growth rates are due to NFAT1 expression.

For the experimental lung metastasis, A375SM were injected in two groups NT and NFAT1 shRNA as well as SB2 EV cells and SB2 NFAT1 OE cells. After 6 weeks, the mice were killed and macroscopic lung metastases were counted in all groups. The results showed that the number of lung metastases was significantly decreased after silencing of NFAT1 (mean of 10 mets) in the highly metastatic A375SM cells when compared to the A375SM

NT group (mean of 36.17 mets) (Figure 10C), and conversely, the number of lung metastases was significantly increased in the SB2 NFAT1 OE group (mean of 36 mets) compared to SB2 NFAT1 EV (mean of 11 mets) as presented in figure 10D. The mean number of lung colonies of A375SM groups were 36 for NT and 10 for NFAT1 shRNA $p<0.01$ and 11 for SB2 EV and 36 for SB2 NFAT1 OE $p<0.001$.

Taken together, NFAT1 silencing, which was achieved through lentiviral shRNA, resulted in an inhibition of experimental lung metastasis growth of A375SM metastatic melanoma cells. Furthermore, overexpression of NFAT1 in the low metastatic cell line, SB2, resulted in an increase of lung metastasis growth, thereby strengthening our hypothesis that NFAT1 is essential to melanoma growth and metastasis.

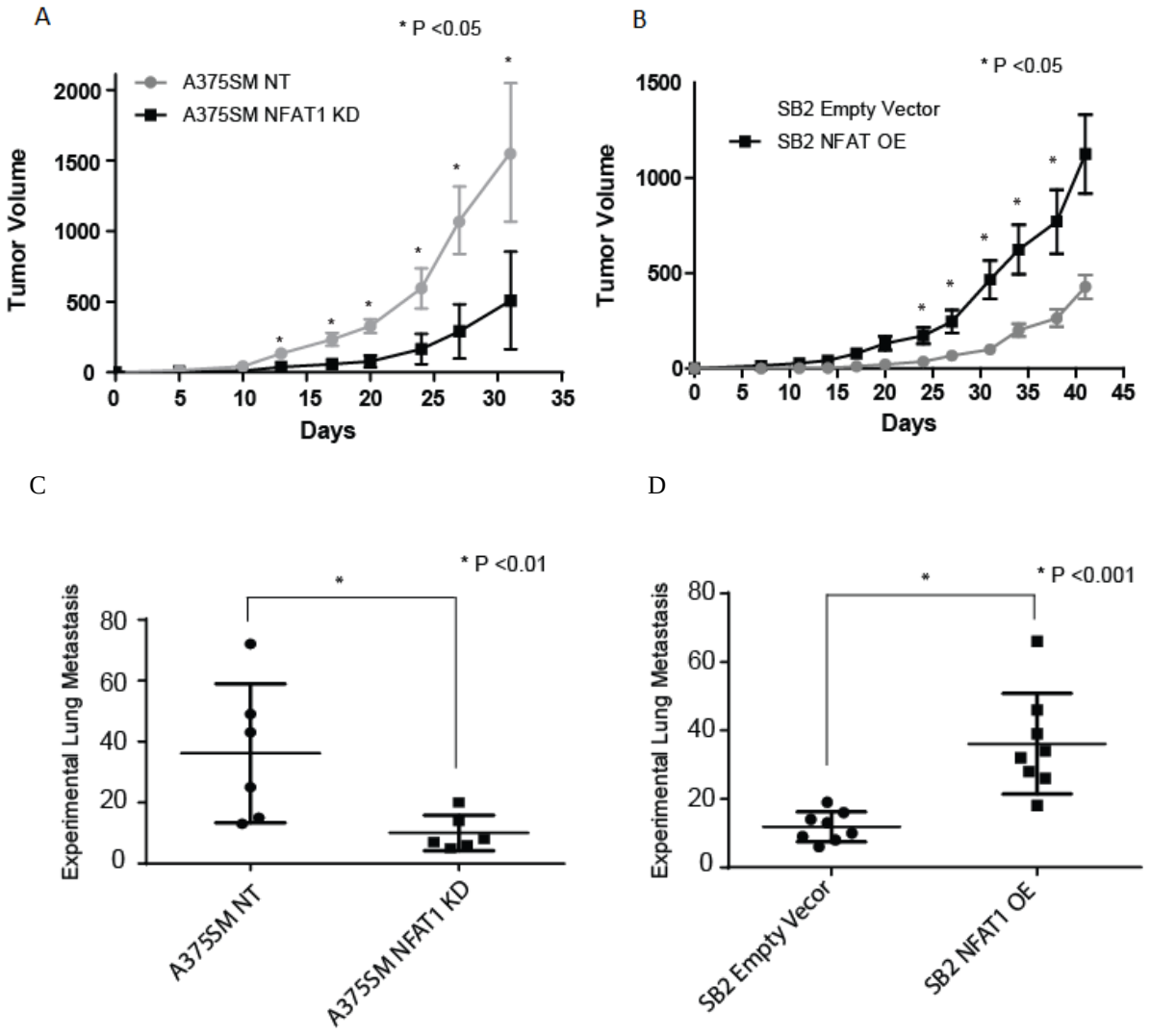


Figure 10: NFAT1 Expression is Required for the Malignant Melanoma Phenotype

Silencing NFAT1 in A375SM melanoma cells reduces tumor growth and experimental lung metastasis in nude mice while overexpression of NFAT1 in SB2 increases both tumor growth and metastasis. (A) A375SM melanoma cells were injected subcutaneously in nude mice and tumor growth was monitored for 31 days. Tumor growth was significantly reduced after silencing NFAT1 at day 31, tumor volume mean of 1557mm^3 compared to 509mm^3 (*P < 0.05). (B) SB2 melanoma cells were injected subcutaneously into nude mice and monitored for 41 days. Tumor growth was significantly increased after overexpressing NFAT1, mean tumor volume of 428 mm^3 compared to 1125 mm^3 (*P<0.05) (C) Six weeks after intravenous injection of A375SM cells, nude mice were sacrificed and the number of lung metastasis were counted. Silencing NFAT1 significantly reduced the number of experimental lung metastasis, mean of 10mets compared to 36 mets (P* < 0.01). (D)

Six weeks after intravenous injection of SB2 cells, nude mice were sacrificed and the number of lung metastasis was counted. Overexpressing NFAT1 significantly increased the number of experimental lung metastasis mean of 36 mets compared to 12 mets ($P^* < 0.001$).

Silencing NFAT1 Changes the Gene Expression Profile of A375SM Melanoma Cells

To identify potential downstream targets of NFAT1, an Illumina microarray was performed (HT-12 Version 3 chip). RNA from three separate 10cm dishes (to confirm reproducibility) was isolated from A375SM NT and NFAT1 shRNA cells. Our data suggest that silencing NFAT1 does deregulate various genes in these melanoma cells. We then mined the data to identify novel genes with significant fold changes in the gene expression array. We focused our attention on genes that were down regulated after silencing NFAT1, since they were likely to be tumor promoter genes. The top identified genes then were mined and sorted by their relevance to cancer are presented in Table 1. Among these genes that were downregulated after NFAT1 silencing, we found follistatin (FST) that has been reported as a contributor to bone metastasis [126] and placenta-specific 8 (PLAC8) whose overexpression is reported to protect cancer cells from apoptosis [127-129]. Also, frizzeld family receptor 4 (FZD4), nicotinamide N-methyltransferase (NNMT). Of the potential genes, we decided to focus our research on interleukin 8 (IL-8/CXCL8) and matrix metallopeptidase 3 (MMP-3). IL-8 is known to play an important role in the progression and metastasis of several different cancers including melanoma. Our laboratory has previously demonstrated that overexpression of IL-8 is associated with increasing tumor stage, disease progression, and recurrence in human melanoma. Furthermore, a direct correlation between high levels of IL-8 and tumor angiogenesis, progression, and metastasis in nude mouse xenograft models of human melanoma were previously shown [130]. Therefore, we hypothesize that NFAT1 may contribute to melanoma tumor growth and metastasis through the regulation of IL-8. The second gene that we decided to study as a downstream target of

NFAT1 is MMP-3. MMP-3 was shown to be associated with Alzheimer's, multiple sclerosis and Parkinson's disease. MMP-3 also contributes to several pathologies such as asthma, rheumatoid arthritis and cancer [131]. MMP-3 transcription can be triggered by reactive oxygen species (ROS), cytokines, growth factors and cell interactions. MMP-3 can activate cell adhesion molecules, growth factors, chemokines, cytokines and receptors. Likewise, MMP-3 is able to activate other MMP family members. In melanoma MMP-3 has not been much studied, and the role of MMP-3 in melanoma progression has yet to be elucidated, therefore we decided to study the role of MMP-3 in the metastatic melanoma phenotype.

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Gene	Fold Decrease	Function in cancer
follistatin (FST)	9.23	Play a role in the malignant progression of prostate cancer, may also contribute to the formation of bone metastasis
placenta-specific 8 (PLAC8)	6.48	PLAC8 overexpression protects some cancer cell lines from apoptosis
fms-related tyrosine kinase 1 (FLT1,VEGFR1)	6.02	Oncogene belongs to the src gene family and is related to oncogene ROS
voltage-dependent anion channel1 (VDAC1)	4.41	Facilitating and promoting the high glycolytic tumor phenotype and increases tumor resistance to chemotherapy
frizzled family receptor 4 (FZD4)	3.48	Transmembrane receptor transducing WNT pathway based on ligand-dependent preferentiality
cytochrome c oxidase (COX6B1)	3.36	In colon cancer two SNPs in COX6B1 were associated with lymph node metastasis
matrix metallopeptidase 3 (MMP-3)	3.22	MMP-3 is potential mediator of melanoma invasion
fibronectin 1(FN1)	2.85	Hypoxia can influence aggressiveness of melanoma by inducing FN1
nicotinamide N-methyltransferase (NNMT)	2.57	Increased of NNMT enzyme activity in non-small cell lung cancer cause normal-looking tissue change toward cancer
Autotaxin (ENPP2)	2.76	Autotaxin is a secreted protein with both oncogenic and pro-metastatic properties. ATX controls the level of LPA which promotes the progression of osteolytic bone metastases
ubiquitin conjugating enzyme (UBE2J1)	2.44	Involved in endoplasmic reticulum (ER)-associated degradation of T cell receptor subunits
interleukin 8 (IL8)	2.40	Targeting IL8 in melanoma reduce tumor growth and metastases

Table 1: Top Potential Genes Down Regulated after Silencing NFAT1

The top potential candidate genes are shown. These genes were downregulated after silencing NFAT1 in A375SM melanoma cells as compared to the non-targeting (NT) control. Note that our gene expression array confirmed that after NFAT1 silencing, autotaxin was reduced by almost 3 fold as was published by our lab before [122]. The two genes of interest IL-8 and MMP-3, are downregulated by 2.4 and 3.22 fold respectively.

Summary Specific Aim 1

Herein, we report that there is a positive correlation between the metastatic potential of the melanoma cell lines and the expression level of NFAT1. The more metastatic the cell is, it correlates with higher NFAT1 expression level. Furthermore, from TCGA data analysis, we found that specimens from metastatic patients demonstrated significantly higher expression of NFAT1 compared to patients with primary tumors. These results support our observations in the cell lines. We validated these results by staining our own in house TMA. Also, we showed that silencing NFAT1 in two metastatic melanoma cell lines, WM902B and A375SM, significantly reduced the invasive potential of these cell lines, while overexpression of NFAT1 increases the invasiveness of SB2 cells; although these manipulations did not change the proliferation ability of the cell lines. The role of NFAT1 in melanoma tumor growth and experimental metastasis was also examined by utilizing an *in vivo* study. The *in vivo* studies confirmed that the expression of NFAT1 in the melanoma cells is important for the progression of the disease. In the presence of NFAT1 the tumor growth and the number of experimental lung metastasis were higher. After confirming that NFAT1 is necessary for the metastatic potential of the cells, we performed gene expression profiling in an attempt to identify potential NFAT1 downstream target genes that might be playing important roles in melanoma progression. In the next chapter we will concentrate on the link between NFAT1 regulation of IL-8 and MMP-3 expressions.

CHAPTER 4: Specific Aim 2

To Identify Novel NFAT1 Downstream Target Genes that Contribute to the Metastatic Melanoma Phenotype

2.1 Study NFAT1 as a Potential Regulator of IL-8 Expression in Melanoma Cells

2.2 Study NFAT1 as a Potential Regulator of MMP-3 Expression in Melanoma Cells

2.3 Determine the Contribution of MMP-3 to Melanoma Growth and Metastasis

Introduction

We have demonstrated that NFAT1 silencing in A375SM significantly reduced tumor growth and experimental metastasis *in vivo* when compared to the NT control group (Figure 10 A,C). We have also demonstrated that overexpressing NFAT1 in the low metastatic SB2 cells increased their invasion *in vitro* as well as incresed tumor growth and their metastastic potential *in vivo* (Figure 10 B,D) . Next we wanted to identified downstream target genes of NFAT1 and determine the mechanism by which NFAT1 regulates these genes. From cDNA microarray studies, we identified that NFAT1 silencing in A375SM resulted in a significant decrease of several downstream gene targets as presented in Table 1. These genes may contribute to the metastatic phenotype of melanoma. Among these genes that were downregulated after NFAT1 silencing we found follistatin (FST), which has been reported as

a contributor to bone metastasis [126], placenta-specific 8 (PLAC8), whose overexpression is reported to protect cancer cells from apoptosis [127-129]. Also frizzeld family receptor 4 (FZD4), nicotinamide N-methyltransferase (NNMT) and others were downregulated. In our research we decided to concentrate on two genes that were downregulated after NFAT1 silencing, interleukin 8 (IL-8) and matrix metallopeptidase 3 (MMP-3). Previous data indicated that NFAT1 can have a major effect on the transcriptional regulation of a variety of genes. In breast cancer it was found that NFAT1 promotes breast cancer cell invasion through the induction of COX-2 [114]. In breast cancer it was also found that there is a negative cross talk between Stat5 and NFAT1 signaling cascades, which may affect breast tumor growth and metastasis [112]. NFAT1 has also been shown to support tumor induced anergy of $CD4^+$ T cells [119]. In this aim, we wanted to identify novel downstream genes regulated by NFAT1 that may contribute to melanoma growth and metastasis. To that end, a gene expression microarray (Illumina) was performed. Our microarray data has identified many potential target genes including IL-8 and MMP-3 and many others. The regulation of IL-8 and MMP-3 by NFAT1 has not been described yet. Therefore, we chose to concentrate on these two target genes and clarify the link between NFAT1, IL-8 and MMP-3 and their contribution to melanoma progression.

Unfortunately, increasing melanoma incidence has led to a growing number of patient deaths due to the aggressive nature of melanoma which commonly metastasizes to the lymph nodes, lung, liver, and brain [132, 133]. Currently, less than 10% of patients presented with malignant melanoma survive longer than 5 years [2]. Therefore, other treatment modalities besides targeting BRAF are needed. Of note, more than 50% of melanoma patients do not harbor the $BRAF^{V600E}$ mutation.

One of these potential molecules is the chemokine IL-8 (CXCL8). It has been shown that IL-8 expression is positively correlated with melanoma progression [134-136]. Chemokines are 8-14 kDa signaling proteins that bind to their seven membrane spanning G protein-coupled receptors. Chemokines are classified by the position of two conserved cys residues at the N-terminal. These families include the CXC, CC, C, and CX3C chemokines [134]. IL-8 is a 8-kDa protein that belongs to the CXC chemokine family. Chemokines were initially identified as chemoattractants for leukocytes, however, their expression and corresponding receptors have been observed in multiple cell types [134]. In melanoma, IL-8 was the first chemokine shown to facilitate cancer cell migration. The overexpression of IL-8 in melanoma cells up-regulates the expression and activity of the matrix metalloproteinase MMP-2, which in turn contributes to a more invasive phenotype [137]. IL-8 binds to its receptors CXCR1 and CXCR2 (located on both melanoma and endothelial cells), thus promoting tumor growth and angiogenesis [137-140]. Due to the pro-tumorigenic and survival- dependent effects of IL-8 in cancer, targeting IL-8 could be therapeutically beneficial for melanoma patients. Previously our lab has shown that neutralizing IL-8 with a fully human antibody, ABX-IL8, can significantly reduce tumor growth and experimental metastasis of A375SM and TXM-13 melanoma cells [141]. IL-8 has been shown to promote the growth, invasiveness, motility, angiogenesis, and metastatic potency of melanoma cells [137, 141-143]. Furthermore, our lab has also demonstrated that neutralizing IL-8 ABX-IL-8 inhibited melanoma growth and metastasis *in vivo*, mostly by inhibiting angiogenesis [141]. Interestingly, targeting IL-8 reduced MMP-2 expression, and incubation of melanoma cells with ABX-IL-8 reduced the invasive potential of melanoma cells through Matrigel coated membranes. Decreased CD31 staining *in vivo* and HUVEC tube formation *in vitro* was also

observed [141]. In a new study in the lab, we utilized a siRNA delivery approach to silence IL-8 to reduce melanoma growth and metastasis *in vivo.* In the present study we want to further understand the regulation mechanism of IL-8 during melanoma progression by the NFAT1 transcription factor.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases. The proteins in this family have the ability to degrade extracellular matrix components such as collagen, gelatin, elastin and casein [144]. Matrix metalloproteinase 3, (MMP-3) which is also known as stromelysin-1, is a member of the MMP family. It is known to cleave extracellular components including different types of collagens (types III, IX, and X) [145]. MMP-3 has been associated with Alzheimer's, multiple sclerosis and Parkinson's disease. MMP-3 also contributes to several pathologies like asthma, rheumatoid arthritis and cancer [131]. MMP-3 transcription can be triggered by reactive oxygen species (ROS), cytokines, growth factors and cell interactions. MMP-3 has many substrates. Besides extracellular matrix (ECM) proteins, it can activate cell adhesion molecules, growth factors, cytokines, chemokines, cytokines and receptors. Likewise, MMP-3 is able to activate other MMP family members. Secreted MMP-3 can be activated extracellularly and then transported back into the cell [131]. In melanoma, MMP-3 was investigated as a potential serum marker and the levels of MMP-3 in the serum were evaluated between healthy patients and malignant melanoma patients, but no significant difference was found [146, 147].

Melanoma progression is a multistep cascade, which requires chemokines, angiogenic factors, adhesion molecules and proteases. Several studies suggested that the metastatic potential of melanoma is influenced by elastin, which is considered to be one of the most important ECM components, especially on the wall of blood vessels and in the lung. MMP-2

and MMP-3 degrade elastin proteins, and overexpression of these two genes is correlated with aggressive melanoma and poor clinical outcome [148, 149]. It was shown that higher stage melanoma is associated with higher levels of elastin, and that the interactions between the elastin peptides and the melanoma cells are key for melanoma progression [148, 150]. Another study in the field reported that Angpt2, MMP-3 and MMP-10 are all upregulated in the lung due to the primary B16F10 tumor in mice [151]. The lung microenvironment could be critical for presenting the pre metastatic niche that resulted from the influence of the primary tumor. Further, it has been shown that Angpt2, MMP-3 and MMP-10 have a synergistic effect on vascular destabilization that promotes the extravasation of melanoma cells and supports lung metastasis [151]. Recently SOX2, an embryonic stem cell transcription factor, was found to be expressed in human melanoma cells [152, 153]. It was also found that after silencing SOX2 in melanoma cells, the expression of MMP-3 was reduced by almost 90% [154]. From the association between SOX2 and MMP-3 it was evident that MMP-3 is regulated by SOX2 and that their co-expression may be used as a functional biomarker for invasive melanoma cells [154].

As per our knowledge, this study is the first to identify that NFAT1 is a positive regulator of MMP-3 and IL-8 expression in melanoma, thus adding a posible mechanism of how NFAT1 contributes to melanoma progression. Furthermore, this is the first study to demonstrate the importance of MMP-3 in melanoma growth and metastasis.

Sub Aim 2.1: Study NFAT1 as a Potential Regulator of IL-8 Expression in Melanoma Cells

Results

IL-8 Expression is Reduced in Melanoma Cells after Silencing NFAT1, and Increased after Overexpressing NFAT1

To validate our gene expression microarray, qRT-PCR and ELISA assays were performed. To identify IL-8 expression, the A375SM and WM902B highly metastatic melanoma cells were transduced with NT or NFAT1 shRNA while the SB2 low metastatic cell line was transduced with EV and NFAT1 overexpression. To check that the downstream targets are not changing due to off target effects after transduction we also performed rescue experiment where the cells containing NFAT1 shRNA were transduced with an NFAT1 rescue vector (Figure 11). The rescue construct does not contain the sequence recognized by the NFAT1 shRNA. As seen in figure 11, stable transduction of A375SM and WM902B NFAT1 KD cells with NFAT1 rescue vector, NFAT1 expression levels were upregulated. Silencing of NFAT1 in both melanoma cell lines, A375SM and WM902B, resulted in ~25% decrease in mRNA expression of IL-8 (Figure 12 A,B). IL-8 mRNA expression was observed to be reverted to initial levels and even more so when the rescue of NFAT1 experiment was performed in both cell lines. In SB2 cells that were overexpressed with NFAT1 we noticed a significant increase in mRNA levels (~100 fold) compared to the EV control group (Figure 12C). Since IL-8 primarily a secreted protein, this can be an explanation for why the whole cell lysate western blots did not detect IL-8. Therefore, the supernatant of melanoma cells

was collected from all three manipulated cell lines after 48hr incubation in serum free media and was subjected to ELISA assay. As shown in Figure 13, silencing NFAT1 significantly reduced the amount of IL-8 within the supernatant by ~ 4 fold in WM902B cells and ~2.5 fold in A375SM cells (Figure 13). Also, IL-8 protein levels were significantly increased in SB2 cells after NFAT1 overexpression compared to the EV control by more than 10 fold. These results from the ELISA assay show that the protein was correlated to the mRNA levels of IL-8 after NFAT1 silencing or NFAT1 overexpression as presented in figure 12. In figure 11, the western blot from the rescue experiment is presented. In both A375SM and WM902B cell lines, we can see clearly more than 5 fold rescue of NFAT1 compare to the actin expression. Taken together we conclude that IL-8 is being regulated by NFAT1 both at the mRNA and protein level and that rescue of NFAT1 in NFAT1 silenced cells also rescues the expression of IL-8.

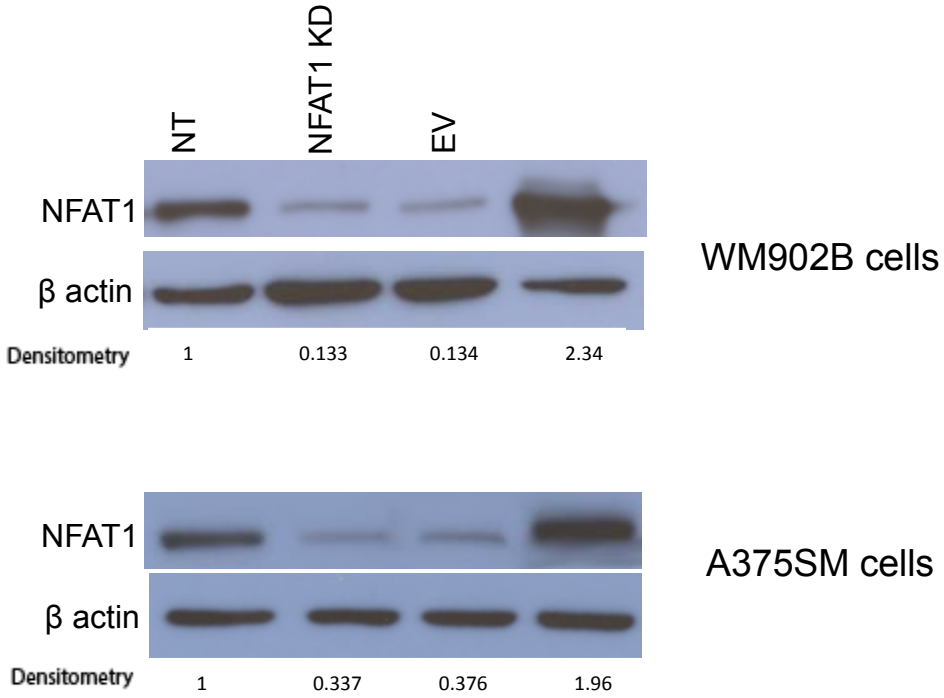
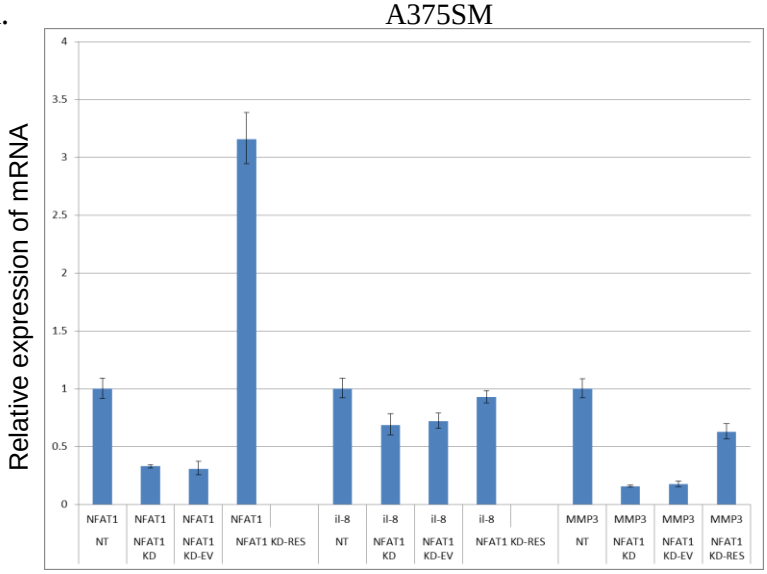


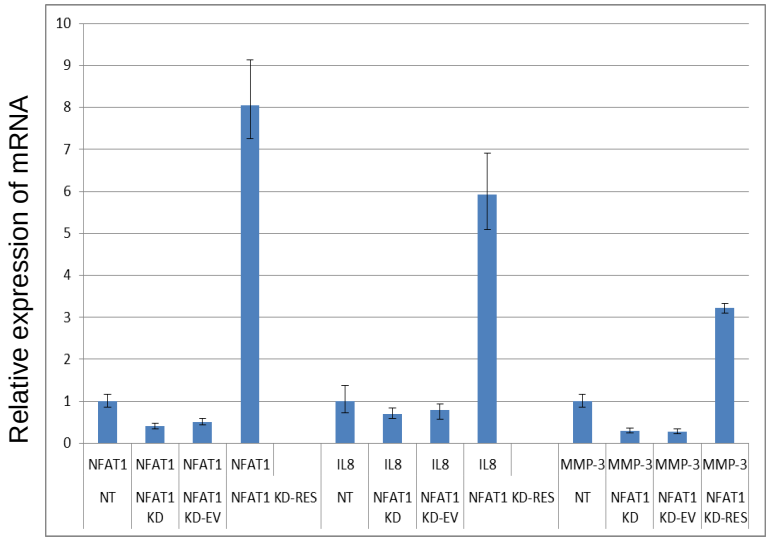
Figure 11: Rescue Experiment of NFAT1

In order to make sure that the effects of NFAT1 silencing is not a result of off target influence due to the virus transduction we utilized rescue cell lines for A375SM and WM902B. These cell lines were used to check downstream targets IL-8 and MMP-3 to see that indeed rescue of NFAT1 is also rescuing the expression of the downstream targets. The western blot demonstrated NT (control), NFAT1 shRNA, then the EV, which represents the control for the overexpression vector that was transduced into the silenced cells and then the rescue of the expression of the NFAT1 and last the NFAT1 RES. In both cell lines we can see clearly more than a 5 fold rescue of NFAT1 compared to the actin expression (NFAT1 140KDa, β actin 42KDa).

A.


A375SM
Relative expression of mRNA
4
3.5
3
2.5
2
1.5
1
0.5
0
NFAT1 NT
NFAT1 KD
NFAT1 KD-EV
NFAT1 NFAT1 KD-RES
il-8 NT
il-8 NFAT1 KD
il-8 NFAT1 KD-EV
il-8 NFAT1 KD-RES
MMP3 NT
MMP3 NFAT1 KD
MMP3 NFAT1 KD-EV
MMP3 NFAT1 KD-RES

B.


WM902B
Relative expression of mRNA
10
9
8
7
6
5
4
3
2
1
0
NFAT1 NT
NFAT1 KD
NFAT1 KD-EV
NFAT1 NFAT1 KD-RES
IL8 NT
IL8 NFAT1 KD
IL8 NFAT1 KD-EV
IL8 NFAT1 KD-RES
MMP-3 NT
MMP-3 NFAT1 KD
MMP-3 NFAT1 KD-EV
MMP-3 NFAT1 KD-RES

C.

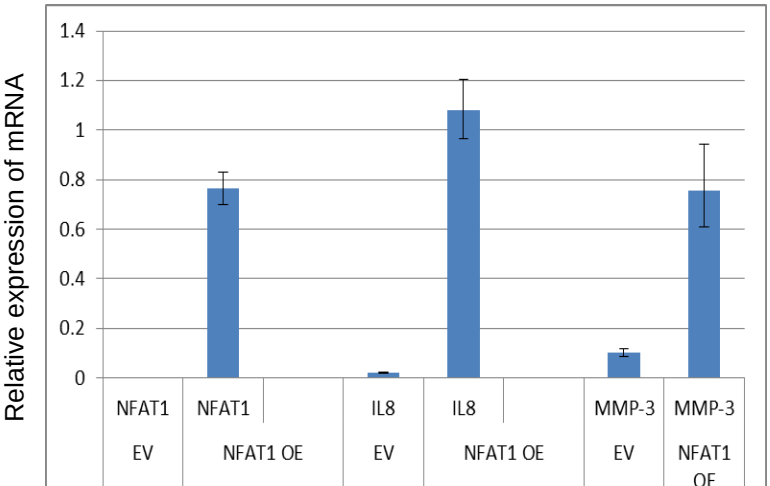

SB2
Relative expression of mRNA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NFAT1 EV
NFAT1 NFAT1 OE
IL8 EV
IL8 NFAT1 OE
MMP-3 EV
MMP-3 NFAT1 OE

Figure 12: Relative mRNA Expression of NFAT1, IL-8 and MMP-3. Real time PCR results showing the relative expression change in the mRNA levels of the downstream targets after manipulating NFAT1, silencing it and rescuing it. Genes that were checked are NFAT1, for manipulation verifications, and both downstream targets IL-8 and MMP-3 (A) A375SM melanoma cell line (B) WM902B melanoma cell lines (C) SB2 cell lines after overexpression of NFAT1 compared to the control.

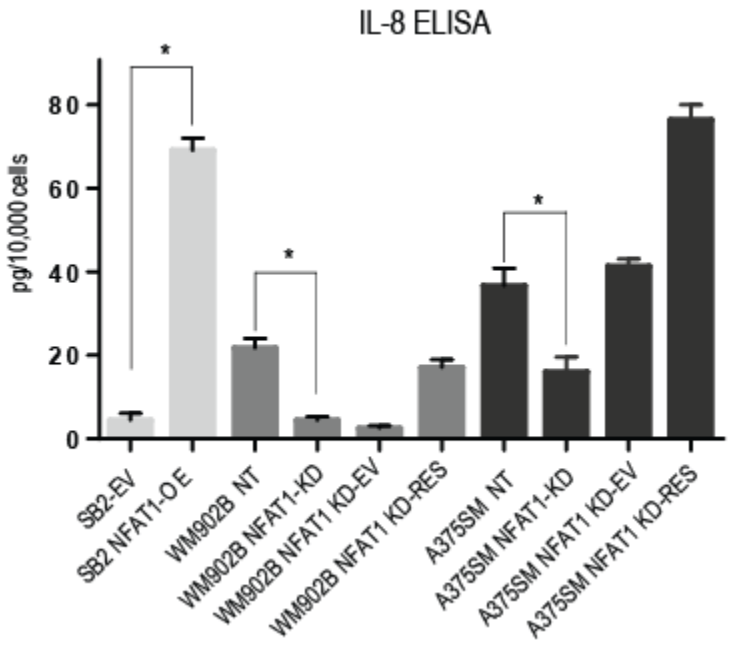


Figure 13: Rescue of NFAT1 Rescues the Protein Levels of Secreted IL-8. ELISA assay for the secreted protein IL-8. Results demonstrate that after NFAT1 silencing; the levels of IL-8 are downregulated and when we rescued the expression of NFAT1 the levels of IL-8 are also rescued in both A375SM and WM902B melanoma cell lines. SB2 overexpressing NFAT1 caused upregulation of IL-8 expression.

NFAT1 Enhances the Promoter Activity and Expression of IL-8

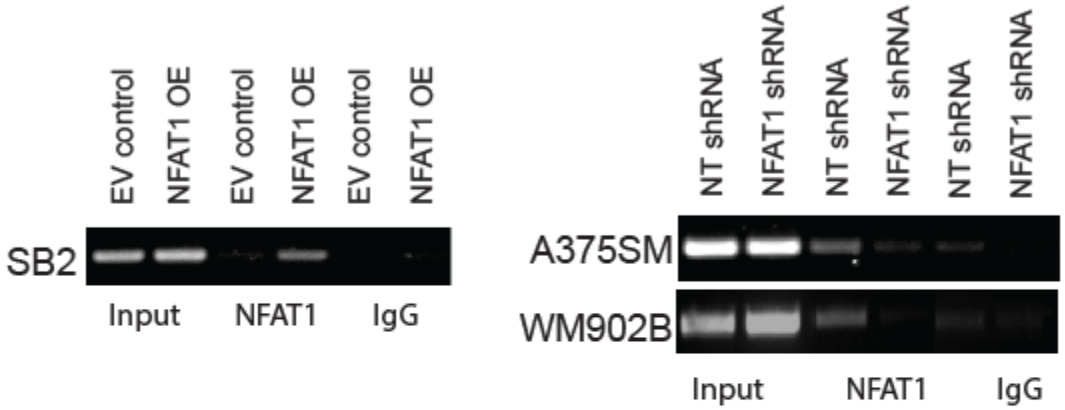
To further understand the mechanism by which NFAT1 is regulating IL-8 we performed ChIP assays to verify the binding of NFAT1 to the promoter of IL-8. First, we identified a binding site of NFAT1 on the IL-8 promoter at 32 base pairs from the transcription initiation site (TIS). We expect that the reduced IL-8 mRNA and protein expression after silencing NFAT1 will be a result of NFAT1 not binding to the IL-8 promoter. To that end, we tested whether NFAT1 binds to the IL-8 promoter and if silencing or overexpressing NFAT1 affects the binding, by utilizing the chromatin immunoprecipitation (ChIP) assay. We used an anti-NFAT1 antibody to IP DNA-NFAT1 complexes in our engineered melanoma cells (A375SM, WM902B NT/NFAT1 shRNA and SB2 NFAT1 EV and OE). End point PCR was performed on the area surrounding the region of NFAT1 binding site on the IL-8 promoter. As shown in Figure 14A, NFAT1 binds to the promoter of IL-8 in both A375SM and WM902B melanoma cell lines. When NFAT1 is silenced, no binding to the IL-8 promoter was detected in both melanoma cell lines. Also, when NFAT1 was overexpressed in SB2, we observed a strong band demonstrating the binding of NFAT1 to the binding site (Figure 14A).

The chromatin immunoprecipitation confirms that NFAT1 indeed binds to the IL-8 promoter. NFAT1 binding is lost after silencing in A375SM and WM902B and a gain in binding was observed in the SB2 cells after overexpressing NFAT1. To elucidate the role of NFAT1 in regulating IL-8 at the transcriptional level, a dual luciferase promoter assay was used. The

assay was designed in a way that the IL-8 promoter was cloned in front of the luciferase. The promoter was designed with and without mutations in the IL-8 promoter binding site at location 32. Silencing NFAT1 resulted in ~40% reduction of luciferase activity with the wild type promoter (Figure 15A,B). When the mutated promoter was inserted into NT shRNA melanoma cells, the luciferase activity was reduced to ~50% of the wild type promoter. The mutation also had an effect on luciferase activity in NFAT1 silenced melanoma cells as compared to the wild type promoter (Figure 15 A,B). Therefore, we concluded that our reduced promoter activity (after silencing NFAT1) is a direct result of reduced binding of NFAT1 protein to the IL-8 promoter. Overexpression of NFAT1 in SB2 resulted with a significant increase in the IL-8 promoter activity (Figure 15 C).

Our initial microarray and qRT-PCR studies confirmed that NFAT1 regulates IL-8 expression at the mRNA level. ELISA assay demonstrated that the secreted protein levels are also regulated by NFAT1. Furthermore, we also demonstrated that NFAT1 is directly binding to the IL-8 promoter by ChIP and Luciferase assays.

A.



B.

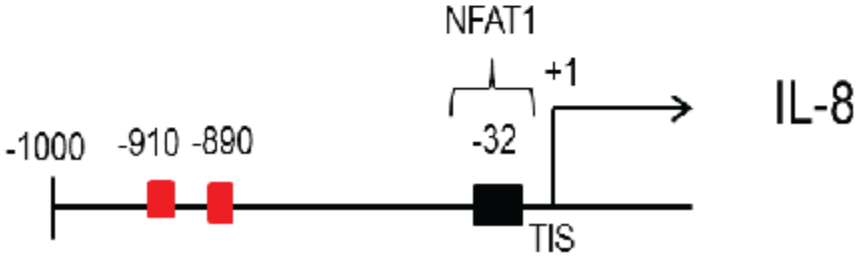


Figure 14: NFAT1 binds to the Promoter Region of IL-8 (A) Chromatin immunoprecipitation of NFAT1 on the IL-8 promoter is lost when NFAT1 is silenced in A375SM and WM902B cells, binding is increased when NFAT1 is overexpressed in SB2 cells (B) One NFAT1 binding site is located on the IL-8 promoter within the first 100bp of the transcription initiation site. (red square represent NFAT5 binding site)

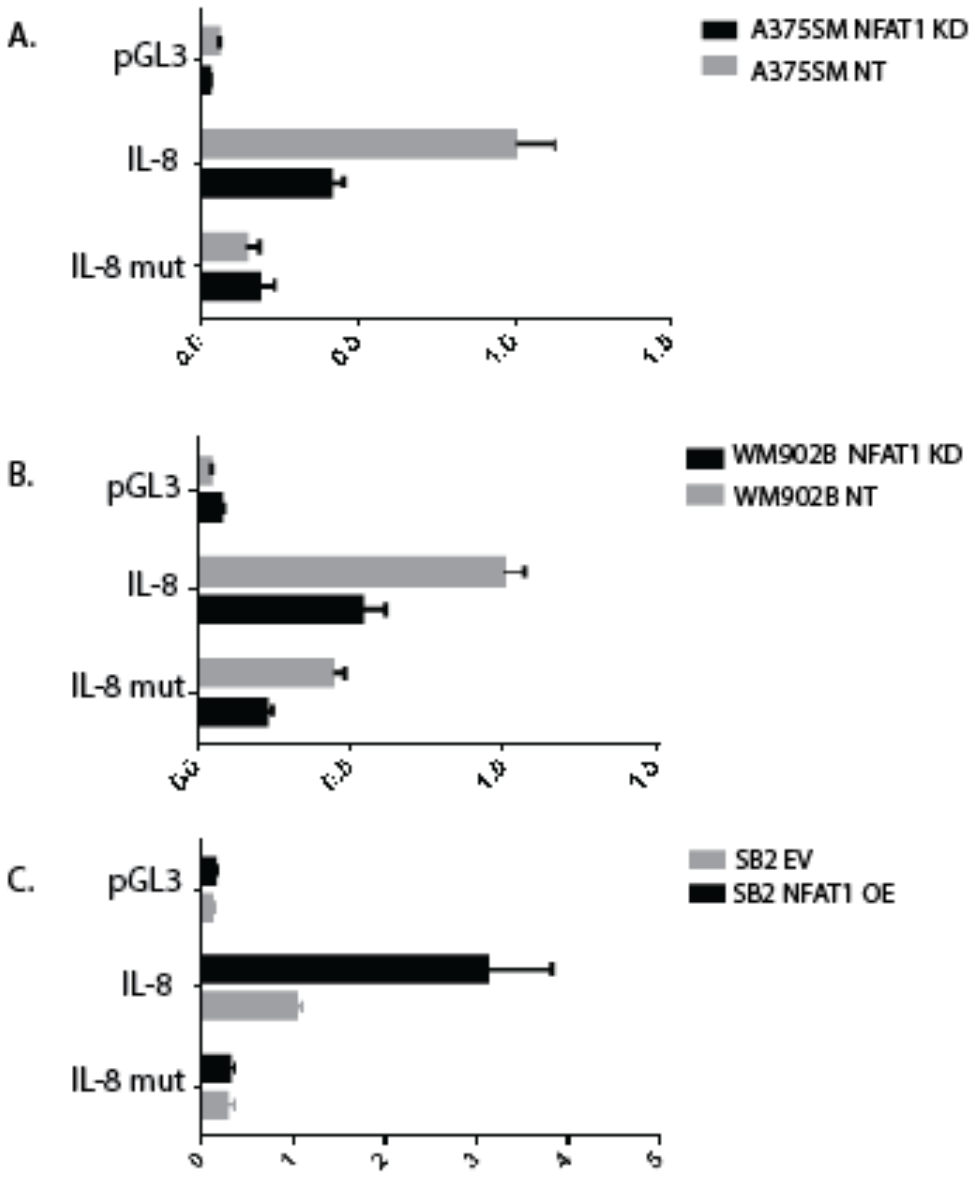


Figure 15: Dual Luciferase Promoter Activity is Reduced in the Presence of NFAT1 Binding Site Mutations. (A,B) Silencing NFAT1 in both A375SM and WM902B significantly reduced the luciferase promoter activity of the wild type promoter by approximately 50% as compared to NT shRNA ; $P < 0.05$. Mutating NFAT1 binding site at location 32 resulted in reduced promoter activity to at least 50% of the wild type promoter; $P < 0.05$. (C) NFAT1 overexpression in SB2 cells increased luciferase promoter activity.

Sub Aim 2.2: Study NFAT1 as a Potential Regulator of MMP-3 Expression in Melanoma Cells

Results

MMP-3 Expression is Reduced in Melanoma Cells after Silencing NFAT1 and Increased after Overexpressing NFAT1

To identify downstream targets of NFAT1, cDNA microarray was performed in A375SM metastatic melanoma cell line after NFAT1 silencing. Among the genes that were downregulated, MMP-3 (also known as stromalysin-1) was downregulated by more than 3 fold after NFAT1 silencing. In order to validate the cDNA results, A375SM and WM902B metastatic melanoma cell lines, which express high levels of NFAT1, were stably transduced with a NFAT1 lentiviral shRNA construct and western blot analysis was performed to demonstrate the NFAT1 silencing. The results demonstrated that NFAT1 expression was reduced by 75% in A375SM and in 58% in WM902B cells. Furthermore, SB2 cells that express low NFAT1 levels, were transduced with an overexpression vector for NFAT1 (Figure 7). The MMP-3 gene was recognized as a downstream target of NFAT1 after a cDNA array was performed on A375SM NFAT1 silenced cells. From the array data we can see more than a 3 fold reduction in MMP-3 expression after NFAT1 silencing (Table 1). For further validation, western blot analysis for MMP-3 was performed after silencing NFAT1 in A375SM and WM903B as well as after overexpressing NFAT1 in SB2 cells. Since MMP-3 is a secreted protein, in addition to a whole cell lysate western blot, we also performed a western blot for the medium of the cells. The western blots were done using protein from

whole cell lysate and from concentrated medium after the cells were serum starved for 48hr. The results of the western blot revealed a 50% decrease in A375SM and a 15% in WM902B. When the protein was extracted from whole cell lysates, a 40% reduction in A375SM and 65% reduction in WM902B was observed when the protein was obtained from conditioned media. After NFAT1 overexpression in SB2 cells, an increase of 48% in the expression of MMP-3 was observed when protein extract was generated from conditional media and 33% in whole cell lysate (Figure 16). The densitometry for the whole cell lysates was analyzed compared to actin and for conditional media, it was compared to a media loading control. Taken together, the secretion of MMP-3 is decreased after silencing NFAT1 and the opposite results were observed in SB2 after NFAT1 overexpression. The secretion of MMP-3 increases after overexpressing NFAT1 in the low metastatic cell lines (Figure 16). Furthermore, since MMP-3 is a secreted protein, an ELISA assay was performed (Figure 17). In the ELISA assay we included A375SM and WM902B cell lines both after NFAT1 silencing and after rescue of NFAT1. The rescue of NFAT1 also rescued the expression of MMP-3, demonstrating that the downregulation of MMP-3 after NFAT1 silencing is not an off target effect but directly regulated by NFAT1. We also included the SB2 low metastatic cells in which NFAT1 was overexpressed. The ELISA results showed a significant reduction of MMP-3 secretion after NFAT1 silencing in both A375SM and WM902B. To further validate that indeed the downregulation of MMP-3 is directly regulated by NFAT1 silencing and not from off target effects, we rescued NFAT1 in both A375SM and WM902B cell lines (Figure 11). After NFAT1 rescue there was a complete rescue of the MMP-3 secretion, where the secretion levels returned back to NT levels in both cell lines. In SB2 cells we can

see a significant increase in MMP-3 secretion levels after NFAT1 overexpression in both western blot and the ELISA assays (Figure 16, Figure 17).

Rescue of MMP-3 in Melanoma Cells Results in the Rescue of NFAT1

Lentiviral based shRNA is a very powerful and useful tool for silencing target genes. However, using this method may cause nonspecific effects. These effects are possible results of antiviral response to the double stranded mRNA or can be due to targeting different genes. To rule out these effects, and to make sure our shRNA construct is on target for NFAT1, NFAT1 was rescued in both A375SM and WM902B melanoma cell lines. We were able to rescue NAFT1 successfully in both cell lines as presented in figure 11. The NFAT1 overexpression resulted in re-expression of MMP-3 in both cell lines while the empty vector (EV) control had no effect on these genes as demonstrated by qPCR in figure 12 and by the ELISA assay presented in figure 17. Therefore, we concluded that the regulation of MMP-3 by NFAT1 is not generated due to an off target effect of the shRNA construct, but occurs through NFAT1.

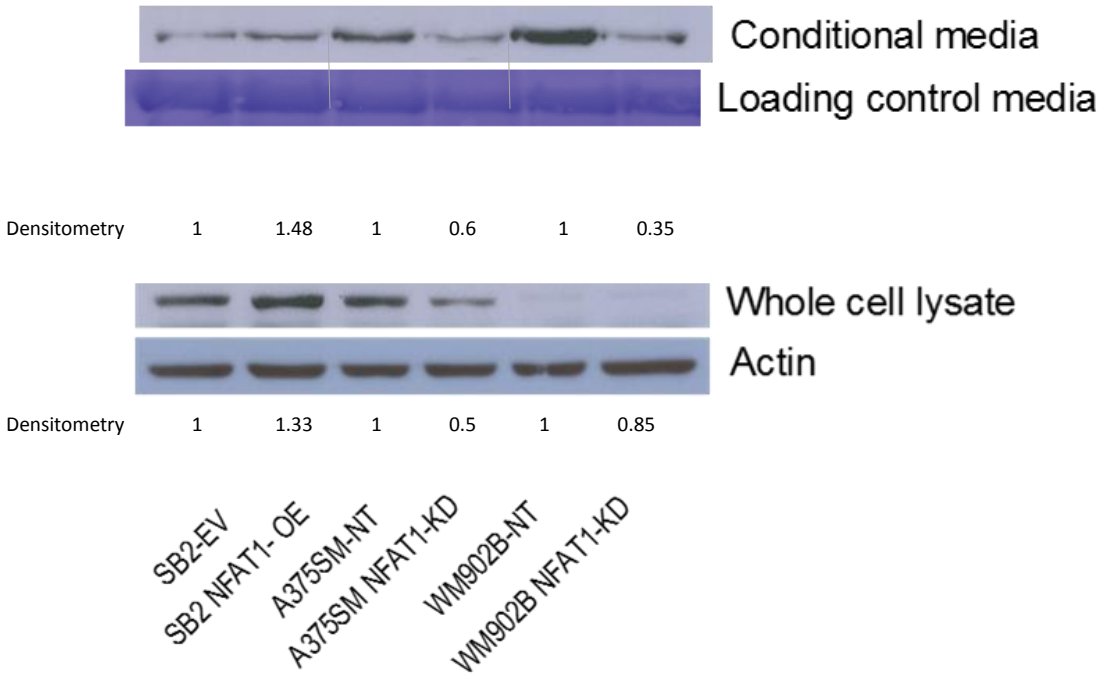


Figure 16: Silencing NFAT1 Decreases MMP-3 Expression in Melanoma Cells. Western blot using MMP-3 antibody to learn about protein levels. The results show approximately a 40-65% reduction of MMP-3 expression within the supernatant in A375SM and WM902B respectively. Coomassie blue staining of the membrane was used to confirm equal loading of the supernatant. Overexpression of NFAT1 in low metastatic SB2 melanoma cells increases the expression of MMP-3 in the supernatant by approximately 50%.

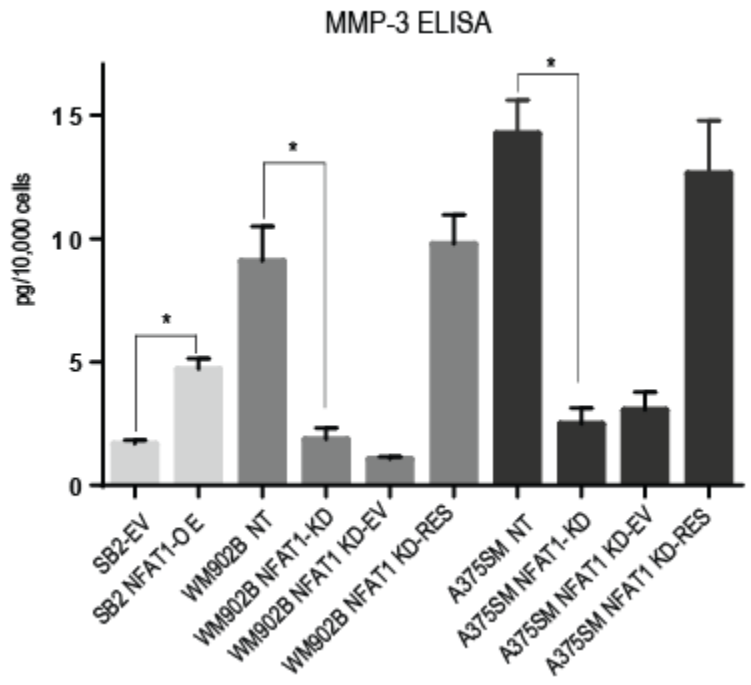


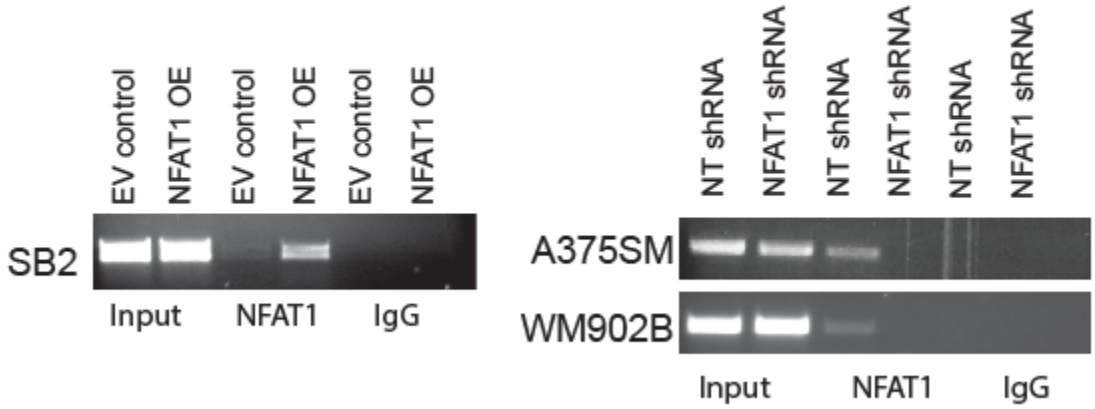
Figure 17: Rescue of NFAT1 Rescues the Protein Levels of Secreted MMP-3. ELISA assay for the secreted protein MMP-3. Results demonstrate that after NFAT1 silencing, the levels of MMP-3 are downregulated and when we rescue the expression of NFAT1, the levels of MMP-3 are also rescued in both A375SM and WM902B melanoma cell lines. SB2 overexpressing NFAT1 caused upregulation of MMP-3 expression.

NFAT1 Enhances the Promoter Activity of MMP-3

Our cDNA microarray results indicated that NFAT1 regulates MMP-3 in the mRNA level (Table 1). However, we had yet to determine whether this is a transcriptional regulation. To answer that question we performed qRT-PCR and as observed the expression of MMP-3 mRNA was decreased by ~5 fold in both NFAT1 silenced A375SM and by ~3 fold in WM902B NFAT1 KD melanoma cells (Figure 12A,B), corroborating the cDNA microarray results. For the SB2 low metastatic cell line in which NFAT1 was overexpressed, an increase of ~7 fold was observed in MMP-3 expression (Figure12 C). These results demonstrate that NFAT1 regulates MMP-3 in the transcriptional level. Since NFAT1 is a transcription factor we reasoned that the reduced MMP-3 protein expression after silencing NFAT1 is due to less binding of NFAT1 to the MMP-3 promoter. To that end, we utilized chromatin immunoprecipitation assay using an anti-NFAT1 antibody to pull down NFAT1-DNA complexes in A375SM, WM902B and SB2 melanoma cells. End point PCR was performed on the surrounding area of the first NFAT1 binding site on the MMP-3 promoter, which is located at -414bp from the transcription initiation site. NFAT1 binds to the promoter of MMP-3 in both A375SM and WM902B melanoma cells. When NFAT1 is silenced (NFAT1 KD), no binding to the MMP-3 promoter in both melanoma cell lines was observed (Figure 18A). In SB2 the binding was observed after overexpressing NFAT1 in the cells and was not observed in the empty vector control cells (Figure 18A). The ChIP assay confirms that

NFAT1 binds to the MMP-3 promoter and that binding is lost after NFAT1 silencing. However, it was not yet clear if NFAT1 directly binds to the MMP-3 promoter, which binding site is more significant and if that binding had an effect on transcriptional activation. To further determine the regulation mechanism, the dual luciferase promoter assay was used. MMP-3 promoter (-1500 to the TIS) was cloned in front of the luciferase reporter gene. The luciferase activity driven by the MMP-3 promoter and was significantly decreased after NFAT1 silencing, ~4 fold in A375SM and ~7 fold in WM902B (Figure 19 A,B). In NFAT1 overexpressing cells, the promoter activity of SB2 was significantly increased (~3 fold), after NFAT1 overexpression (Figure 19 C). These results indicate that NFAT1 regulates MMP-3 at the transcriptional level. To further prove that NFAT1 directly binds to the MMP-3 promoter we generated luciferase constructs with mutations which were inserted into the binding sites. Three constructs were made, the first has a mutation in the first binding site, MMP3-mut1 (site 414), the second construct had a mutation in the second binding site MMP3-mut2 (site 1291) and the third construct had mutations in both binding sites MMP3-ded (‘ded’ stand for double edited, mutations in both sites). When the mutations were inserted, there was a significant decrease in luciferase activity in the control samples (A375SM NT, WM902B NT). Interestingly, both mutations had approximately the same effect (no significant different was observed) on the promoter activity and the dual mutation had no additive effect (Figure 19). These results suggest that both sites are equally important for the transcription activation of MMP-3.

A.



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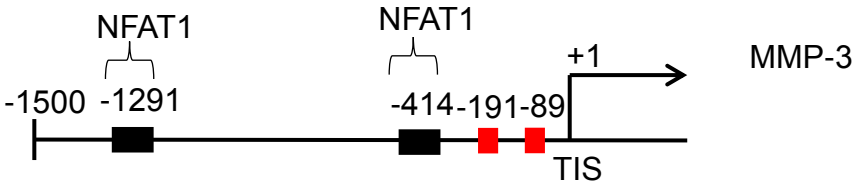

Figure 18: NFAT1 binds to the Promoter Region of MMP-3 (A) Chromatin immunoprecipitation of NFAT1 on the MMP-3 promoter is lost when NFAT1 is silenced in A375SM and WM902B cells, binding is increased when NFAT1 is overexpressed in SB2 cells (B) Two NFAT1 binding sites are located on the mmp-3 promoter within the first 1500bp of the transcription initiation site. First site is at -414bp, second site at -1291bp.

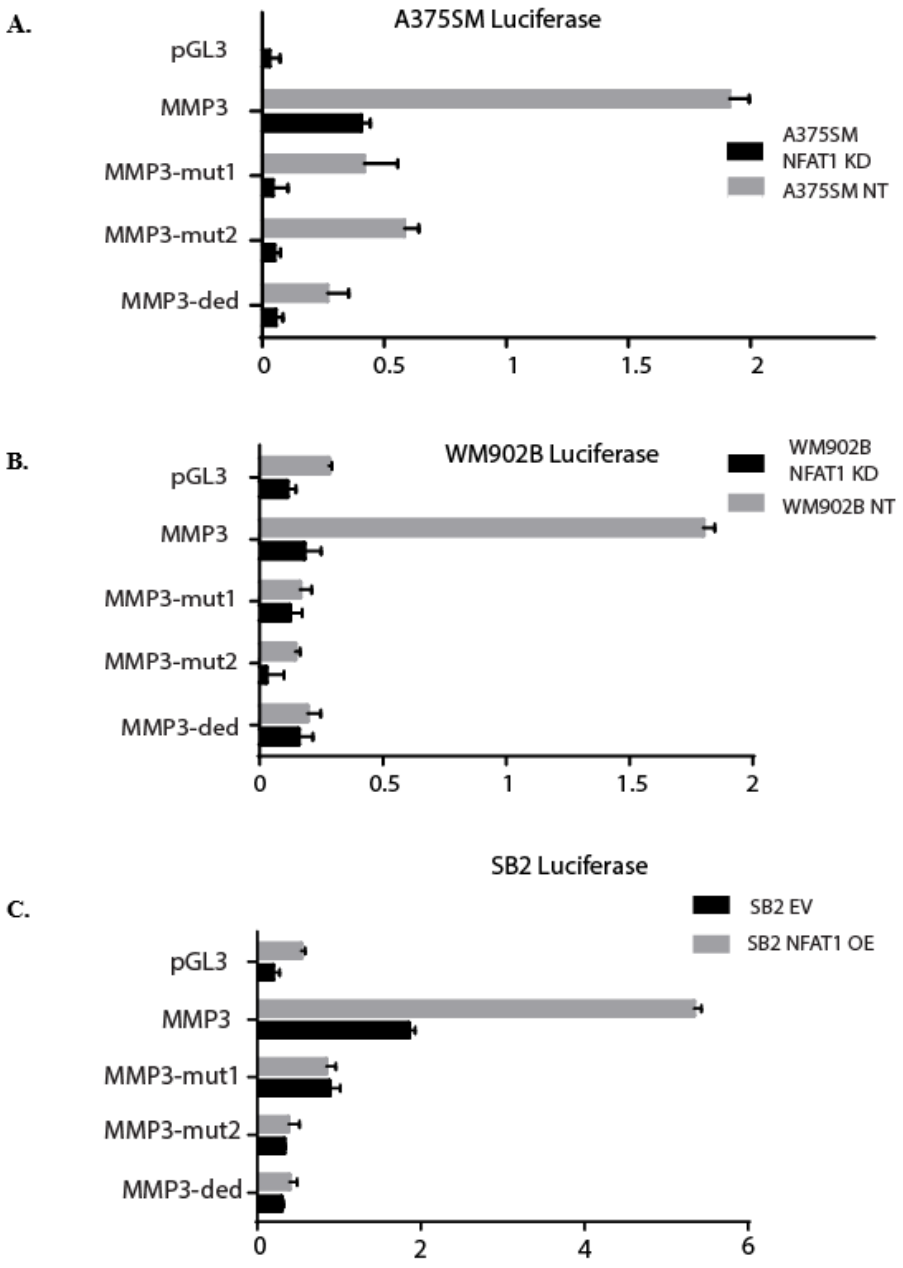

Figure 19: Dual Luciferase MMP-3 Promoter Activity is Reduced in the Presence of NFAT1 Binding Site Mutations. Silencing NFAT1 in both A375SM and WM902B significantly reduced the luciferase promoter activity of the wild type promoter by approximately 75% as compared to NT shRNA. Mutating NFAT1 binding sites (each one separately or both) resulted in reduced promoter activity compared to the wild type promoter; $P < 0.05$. (A) A375SM melanoma cells (B) WM902B melanoma cells after silencing NFAT1 (C) SB2 melanoma cells after overexpressing NFAT1.

Sub Aim 2.3: Determine the Contribution of MMP-3 to Melanoma Growth and Metastasis

Results

MMP-3 Expression is Positively Correlated in Melanoma cell lines

Although we already identified that MMP-3 mRNA and protein levels are reduced after the silencing of NFAT1, we have yet to determine whether the expression levels of MMP-3 has any relevance in melanoma growth and metastasis. To our knowledge, the correlation between MMP-3 and metastatic potential was not yet established for melanoma. To study whether MMP-3 expression is correlated with the metastatic potential of the cell lines we used ELISA assay to measure MMP-3 secretion from a panel of cell lines. The cells that were used in this panel are the same cells that we used in the NFAT1 panel: SB2, DM4, Mewo, TXM18, A375SM, WM2664 and WM902B. The results from the ELISA demonstrated a direct correlation between the metastatic potential of the cell lines and MMP-3 secretion.

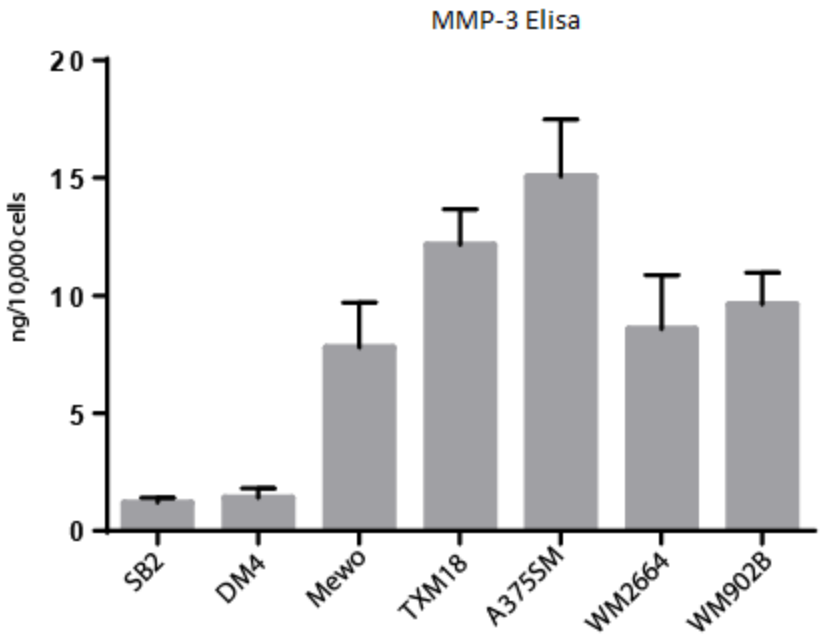

Figure 20: Positive Correlation between the Metastatic Potential of Melanoma Cell Line to MMP-3 protein Expression. ELISA assay for a panel of melanoma cell lines used in our lab. The less tumorigenic SB2, and DM4 melanoma cell lines they secreted significantly less MMP-3 than the more tumorigenic and metastatic Mewo, TXM-18, A375SM, WM2664 and WM902B cells. These results correlate with the western blot analysis for NFAT1 expression in the same panel of cells that is presented in Figure 4.

The Overexpression of MMP-3 Rescues Tumor Growth and Metastasis *In Vivo*

To study the role of MMP-3 in melanoma growth and metastasis, we decided to overexpress MMP-3 in the SB2 low metastatic cell line, which expressed low levels of MMP-3, and silence MMP-3 expression in A375SM, a highly metastatic cell line with high expression of MMP-3. We generated a stable SB2 cell line with high levels of MMP-3, and stable A375SM cell lines with silenced MMP-3. The expression of the MMP-3 was validated using ELISA assay, as presented in figure 21. These cells (A375SM NFAT1 shRNA, SB2 NFAT1 overexpression) were then injected both subcutaneously and intravenously into nude mice to investigate tumor growth and experimental lung metastasis assays respectively. As control groups A375SM NT and SB2 EV cells were injected. Tumor volume was measured for 28 days for the following injected groups; A375SM NFAT1 shRNA vs. A375SM NT and SB2 NFAT1 OE vs. SB2 EV. As seen in figure 22, our *in vivo* studies demonstrate that MMP-3 is an important player in melanoma tumoregenicity. Indeed, 3 weeks from injections, there was a significant difference in the tumor size. In A375SM the control group (A375SM NT) presented a higher tumor volume than A375SM MMP-3 shRNA. At day 28 the tumor size of the control group reached a mean of 1100mm^3 while the tumor size, after silencing MMP-3, had a mean of only 450mm^3 (Figure 22A). Accordingly we found that overexpression of MMP-3 in SB2 cells increased their tumor growth. The MMP-3 overexpression group presented a mean of 700mm^3 at day 28 compared to a mean of 200mm^3 in the empty vector group (Figure 22B). The results for the experimental lung metastasis also supported the hypothesis that high levels of MMP-3 contribute to a higher number of experimental lung metastases. We found that the mean number of lung metastases derived from A375SM NT was significantly higher when compared to the A375SM MMP-3 shRNA group (mean of 56

compared to 25 respectively). The differences in the number of lung metastases were also significant in the SB2 group. The number of lung metastasis after overexpressing MMP-3 was higher than the empty vector control group (mean of 9 compared to 4). Taken together, we conclude that MMP-3 expression is a contributor for the malignant phenotype of melanoma cell lines.

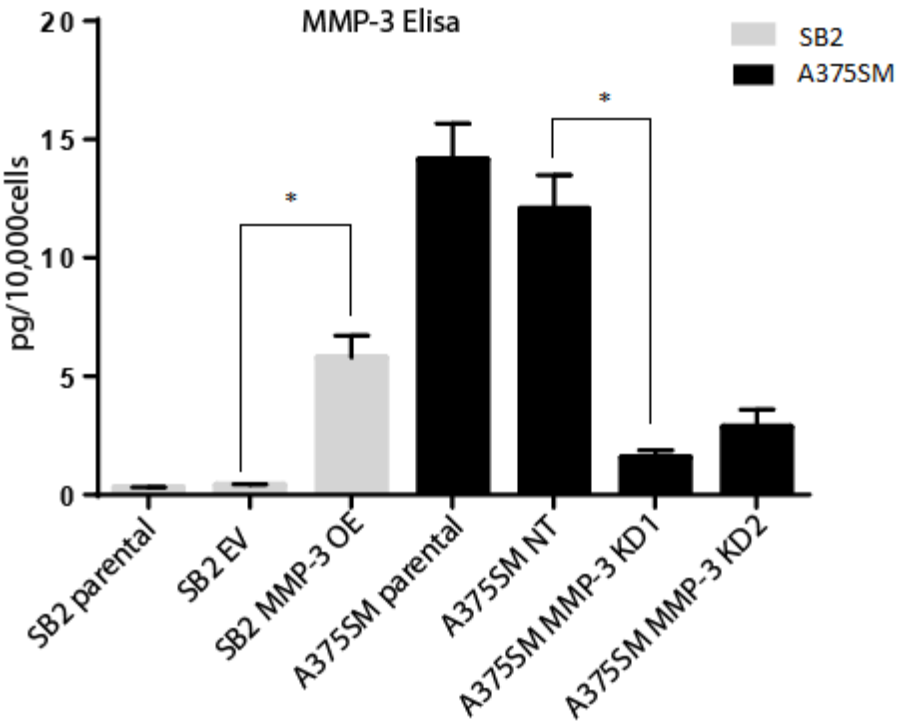

Figure 21: MMP-3 ELISA Demonstrating a Verification of MMP-3 Silencing in A375SM and MMP-3 Overexpression in SB2 Cells. ELISA assay for the secreted protein MMP-3. Results demonstrate a successful silencing of the protein levels of MMP-3 with both targets (KD1,KD2). Results also demonstrate a significant overexpression of MMP-3 in SB2 cells (*P<0.05).

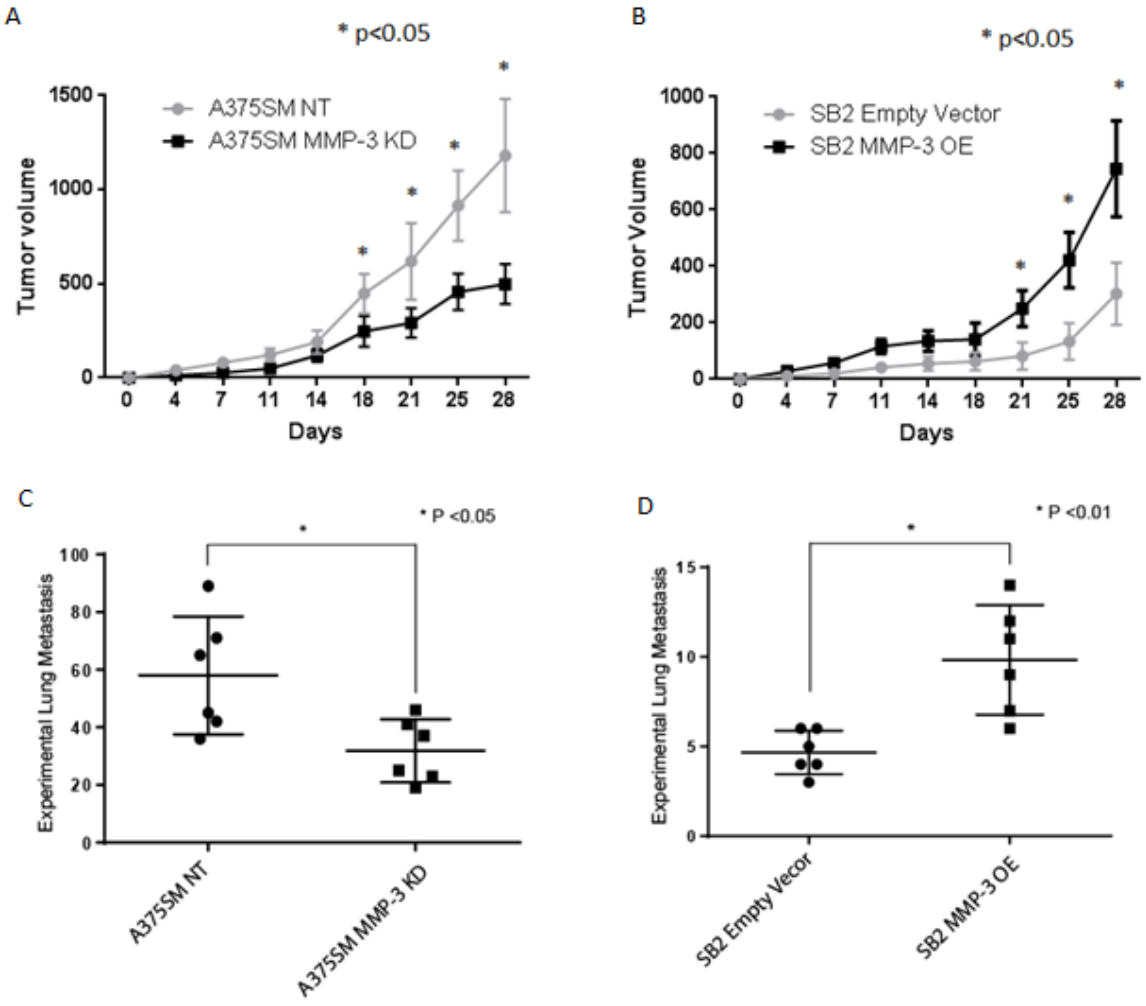

Figure 22: MMP-3 Expression is Required for Melanoma Tumor Growth and Metastasis *In Vivo*

Silencing MMP-3 in A375SM melanoma cells reduces tumor growth and experimental lung metastasis in nude mice while overexpression of MMP-3 in SB2 increases both tumor growth and metastasis. (A) A375SM melanoma cells were injected subcutaneously in nude mice and tumor growth was monitored for 28 days. Tumor growth was significantly reduced after silencing MMP-3 at day 28, tumor volume mean of 1100mm^3 compared to 450mm^3 (*P < 0.05). (B) SB2 melanoma cells were injected subcutaneously into nude mice and monitored for 28 days. Tumor growth was significantly increased after overexpressing MMP-3; mean tumor volume of 200 mm^3 compared to

700 mm^3 (*P<0.05) (C) Six weeks after intravenous injections of A375SM cells, nude mice were sacrificed and the number of lung metastasis were counted. Silencing MMP-3 significantly reduced the number of experimental lung metastasis; mean of 56mets compared to 25 mets (P* < 0.05). (D) Six weeks after intravenous injections of SB2 cells, nude mice were sacrificed and the number of lung metastasis was counted. Overexpressing MMP-3 significantly increased the number of experimental lung metastasis; mean of 4 mets compared to 9 mets (P* < 0.01).

Immunohistochemistry for the Expression of Downstream Targets *In Vivo*

We next investigated the expression of IL-8, MMP-3 and CD31 at the tumor level using immunohistochemistry of the tumor sections from the *in vivo* study (presented in Figure 10). The staining confirmed that indeed IL-8 and MMP-3 expression are decreased *in vivo* in A375SM cells in which NFAT1 was silenced and MMP-3 and IL-8 expression were increased after overexpressing NFAT1 in SB2 cells (Figure 23).

To verify the angiogeneic role of IL-8, further immunohistochemistry staining was performed on both A375SM and SB2 xenograft tumors with anti-CD31, a widely used endothelial marker, detecting blood vessels (angiogenesis). A375SM, NFAT1 NT shRNA melanoma cells present a high number of blood vessels when compared to NFAT1 shRNA. The overexpression of NFAT1 in SB2 cells shows similar phenotype as the highly metastatic line A375SM, and it is clearly noticeable that the number of blood vessels is higher after NFAT1 overexpression compared to the empty vector SB2-EV (Figure 23). Apoptosis was also analyzed in the tumor sections from our xenograft model using the TUNEL assay. The number of positively stained (dead) tumor cells was significantly increased in NFAT1 silenced A375SM tumors. The overexpression of NFAT1 in SB2 cells reduced the number of apoptotic cells (Figure 23).

TCGA Analysis for the Expression of IL-8 and MMP-3 and Survival

To further validate the relevance of our downstream target genes in the clinics, we mined the TCGA data to investigate if there is a correlation between the expression levels of IL-8 and MMP-3 to the survival of patients. The analysis was based on 45 patients that expressed IL-8 or MMP-3. The results demonstrated that patients with higher expressions of these genes had a significantly worst overall survival ($p<0.05$) than patients with low expression of these proteins (Figure 24).

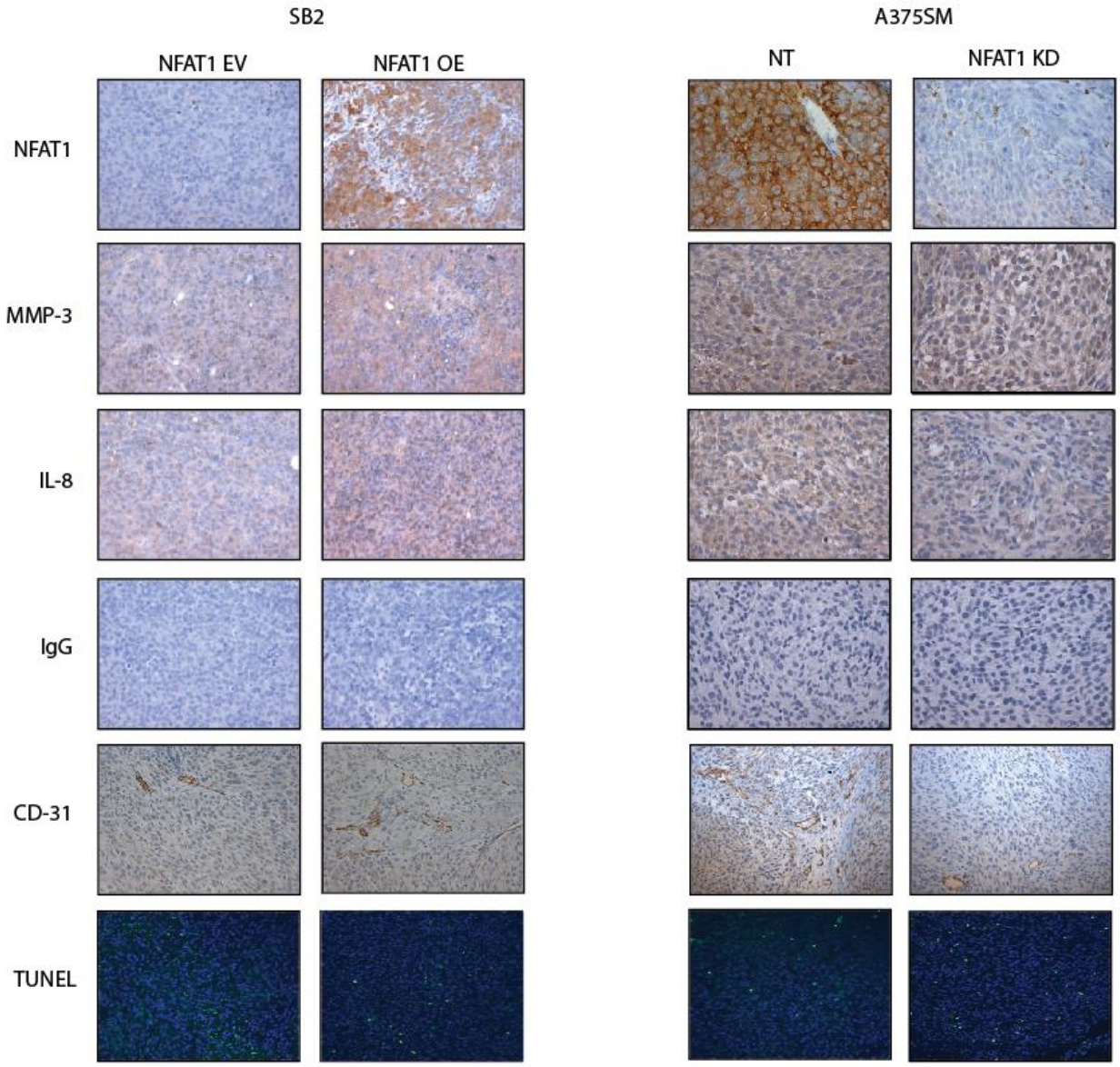

Figure 23: Immunohistochemistry of Tumor Samples after Silencing or Overexpressing NFAT1 in Melanoma Cells

A375SM melanoma cells with NT /NFAT1 shRNA and SB2 cells with Empty vector/NFAT1 OE were injected subcutaneously in nude mice. NFAT1 remains silenced or Overexpressed in the xenograft tumors (upper panel). NFAT1 downstream targets are demonstrating up- and down-regulation as expected (second and third panel). A representative image of the number of CD31 positive endothelial cells is shown from A375SM and SB2 xenograft tumors (fifth panel). Silencing

NFAT1 reduces the number of CD31 stained blood vessels within the tumor, while overexpression of NFAT1 increases the number of blood vessels. Silencing of NFAT1 increases the number of apoptotic TUNEL positive cells within the subcutaneous tumor. Overexpression of NFAT1 in SB2 cells reduces the number of apoptotic cells in the xenograft model.

A. B.

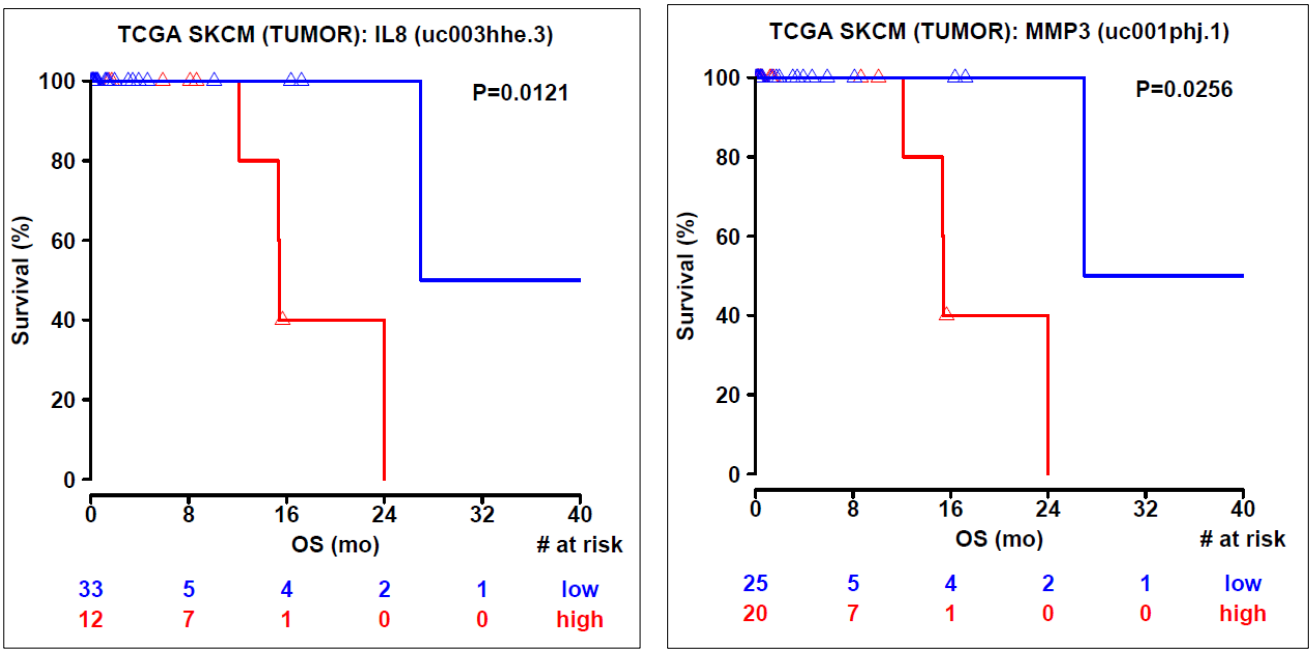

Figure 24: Expression of IL-8 and MMP-3 Correlates with Survival in Patients (based on 45 patients from TCGA data)

Analysis based on TCGA data demonstrating a correlation between survival of patients to levels of the proteins IL-8 and MMP-3 in their tumors. (A) Patients with higher IL-8 expression showed a lower overall survival (in red) compared with patients have low IL-8 expression (in blue) (B) Patients with higher MMP-3 expression showed a lower overall survival (in red) compared with patients that have low MMP-3 expression (in blue)

Summary Specific Aim 2

In this aim we identified two novel downstream target genes that are regulated by NFAT1 and contribute to melanoma progression. We established that there is a positive correlation between NFAT1 expression with IL-8 and MMP-3 protein expression. We first demonstrated that IL-8 is positively regulated at both the transcriptional level and protein levels by NFAT1. Silencing of NFAT1 in A375SM and WM902B metastatic cell lines resulted in downregulation of IL-8 mRNA and secreted protein levels. Rescue of NFAT1 in those two cell lines also rescued the IL-8 expression at both mRNA and protein levels, which emphasizes that IL-8 is directly regulated by NFAT1 expression and not due to an off target effect of the transduction. Moreover, when overexpressing NFAT1 in the low metastatic cell line SB2, IL-8 mRNA and secreted protein levels were increased. Furthermore, we demonstrated that NFAT1 enhances the promoter activity of IL-8 by utilizing ChIP assay. Mutation on the NFAT1 binding site on IL-8 promoter resulted in a significant reduction in luciferase activity, indicating a direct binding of NFAT1 to the IL-8 promoter. At last we demonstrated using immunohistochemistry staining, that the regulation of IL-8 and MMP-3 is also maintained *in vivo* in the tumor tissue from the xenograft model.

The second novel target that is regulated by NFAT1 is MMP-3. Little is known about the contribution of MMP-3 in melanoma. A possible role of MMP-3 in the progression and metastasis was previously suggested as MMP-3 protein levels were reported to be expressed in highly metastatic melanoma cell lines [155, 156], however the role of MMP-3 in melanoma progression was not elucidated. In our study, we validated that indeed the cell lines that are used in our lab also have a similar expression profile when looking at MMP-3. The more metastatic the cell line is, the higher the MMP-3 expression level it has. In this

study we found that silencing NFAT1 in A375SM and WM902B metastatic melanoma cell lines reduced MMP-3 mRNA and protein expression levels. In SB2 cells, overexpression of NFAT1 increased the expression of MMP-3 at both the transcriptional and protein levels. We further demonstrated that NFAT1 enhances the activity of the MMP-3 promoter. MMP-3 promoter has two NFAT1 binding sites and mutation analysis of the promoter showed that both binding sites has the same effect on the promoter activity (no significant difference was observed) and dual mutation had no additive effect. Silencing NFAT1 or mutation in the binding site reduced the luciferase activity of the MMP-3 promoter further establishing the role of NFAT1 in the transcriptional regulation of MMP-3. Finally we aimed to elucidate the role of MMP-3 in melanoma tumor growth and experimental metastasis. We silenced MMP-3 expression in a metastatic melanoma cell line (A375SM) and overexpressed MMP-3 in a low metastatic cell line (SB2). These cell lines were then utilized for *in-vivo* studies, where they were injected subcutaneously and intravenously to investigate the effect of MMP-3 on melanoma tumor growth and metastasis. Our results demonstrate that higher expression of MMP-3 in the cells promoted tumor growth and metastasis, which emphasizes the importance of MMP-3 to the metastatic phenotype.

To validate the clinical relevance of our results, we mine the TCGA data and found a correlation between the expression levels of IL-8 and MMP-3 to the overall survival of patients. Patients with lower levels of IL-8 and MMP-3 show a significantly better survival than patients with high expression of the proteins.

CHAPTER 5: Discussion, Significance and Future Directions

The role of NFAT1 in the immune response as a T cell activator is very well established. Its role in cancer and more so in melanoma is less documented. During this study we revealed several novel findings, which further elucidate the role of NFAT1 in melanoma progression. We demonstrated that NFAT1 expression positively correlated with the metastatic melanoma phenotype in both patient specimens as well as in a panel of cell lines with different metastatic potential. In patient specimens, we demonstrated that NFAT1 expression is significantly higher in metastatic lesions compared to patients with primary tumors. In cell lines, NFAT1 expression increased with the metastatic potential of the cells. To further establish the contribution of NFAT1 in melanoma metastasis, we silenced NFAT1 expression by lentiviral shRNA in two metastatic melanoma cell lines A375SM and WM902B. We found that silencing NFAT1in both cell lines significantly reduced their invasive potential *in vitro.* Overexpression of NFAT1 in the low metastatic melanoma cell line, SB2 significantly increased their invasive potential *in vitro. In vivo*, we demonstrated that silencing of NFAT1 reduced the tumor growth and metastatic potential of A375SM melanoma cells while overexpression of NFAT1 in the low metastatic SB2 cells increased their *in vivo* metastatic properties. To further elucidate the mechanism of action of NFAT1 in melanoma progression we subjected the metastatic melanoma cell line A375SM NFAT1-silenced cells to a cDNA microarray analysis. We found that NFAT1 positively regulates the expression of IL-8 and MMP-3 at the transcriptional level. Chromatin immunoprecipitation assays as well as promoter analysis demonstrated that NFAT1 binds to the promoter of IL-8 and MMP-3 and

promotes their transcription. Rescue of NFAT1 expression in NFAT1-silenced cells restored the protein expression of both IL-8 and MMP-3. This confirms that our results are not an off-target effect of the sh-NFAT1 used in our studies.

The role of IL-8 in melanoma progression and metastasis has been previously established in our laboratory. We therefore decided to concentrate our further studies on elucidating the role of MMP-3 in promoting the metastatic melanoma phenotype. In cell lines we demonstrated that secreted MMP-3 levels increased with the metastatic potential of the cells. To further elucidate the role of MMP-3 in melanoma progression, we stably silenced MMP-3 expression using letiviral shRNA in the highly metastatic A375SM cells and overexpressed MMP-3 in the low metastatic SB2 cells. These cells were injected into nude mice, both subcutaneously and intravenously, to study the role of MMP-3 in tumor growth and metastasis. *In vivo,* we demonstrated that silencing of MMP-3 reduced tumor growth and the metastatic potential of A375SM melanoma cells, while overexpression of MMP-3 in the low metastatic SB2 significantly increased their tumor growth and metastatic potential. Mining the TCGA data, we demonstrated that patients with low expression of IL-8 or MMP-3 have significantly better survival rate when compared to patients with high expression of these proteins.

Taken together my work assigns a previously undescribed role for NFAT1 in regulating the melanoma metastatic phenotype.

The main cause of mortality in the majority of melanoma patients is due to distal metastases. Regardless of the latest advances in the field, such as BRAF and immunocheckpoint modalities, malignant melanoma still presents a major clinical challenge.

During melanoma progression, there is a signature of genetic alterations, but to better promote the field it is critical to understand the regulation and mechanisms associated with metastasis. Therefore, recognizing the events, and genes involved that lead to melanoma metastasis is essential for identifying novel therapeutic molecular targets that will successfully cure this disease.

Earlier reports have described the correlation between NFAT1 and melanoma. One such published work had demonstrated that the absence of NFAT1 expression in the microenvironment caused a significant difference in the ability of the B16F10 melanoma cell line to grow [116]. NFAT1 deficient mice presented less experimental lung metastasis colonization after B16F10 melanoma cell injections when compared to the WT mice [116]. Another related publication presentd NFAT1 as a potential therapeutic target in melanoma, since NFAT1 was found to promote proliferation and inhibits melanoma cell apoptosis [117]. In addition, NFAT1 was found to be an activating transcription factor for the MDM2 oncogene in response to DNA damage signals [118]. NFAT1 also supports tumor induced anergy of $CD4^+$ T cells [119] and regulates a set of genes that are responsible for helper T-cell ($CD8^+$) anergy [120]. Data from a recent publication has demonstrated that NFAT1 increased CTLA-4 promoter activity in $CD4^+$ T cells compared to $CD8^+$ T cells. The expression of CTLA-4 mediated by NFAT1 in $CD4^+$ can potentially be important for anti CTLA-4 therapy [121]. Our laboratory has previously demonstrated that Gal-3 regulates autotaxin through NFAT1, and high levels of Gal-3 supports melanoma growth and metastasis [122]. Taken together, these data indicate that NFAT1 regulates multiple genes during melanoma progression. However, the majority of the downstream genes have yet to be identified. The present study showed that indeed NFAT1 positively correlates with the

metastatic potential of melanoma cell lines although the mechanism by which NFAT1 contributes to melanoma progression remains unknown. In the effort to identify downstream target genes regulated by NFAT1, NFAT1 shRNA was used to silence NFAT1 in A375SM cells and a CDNA microarray was performed. We narrowed our study on two downstream target genes: IL-8 and MMP-3. NFAT1 was also overexpressed in the low metastatic cell line SB2 and both manipulated cell lines (A375SM and SB2) were injected into nude mice both subcutaneously (for tumor growth) and intravenously (for experimental lung metastasis) to clarify the role of NFAT1 in tumor growth and metastasis. Results from the *in vivo* experiment revealed that overexpressing NFAT1 in the low metastatic cells increased tumor growth and metastasis while silencing NFAT1 significantly reduced tumor growth and metastasis. Our data validate the assertion that NFAT1 has an active role in melanoma progression and metastasis formation. Moreover, upon mining available TCGA data we found that NFAT1 is expressed more in metastatic lesions compared to specimens taken from benign nevi (Figure 5) so it is consequently clinically relevant to consider NFAT1 and its downstream regulated genes, IL-8 and MMP-3, as possible targets to melanoma therapy. Imunohistochemistry performed on tumors from the *in vivo* study demonstrated that in the absence of NFAT1, less blood vessels formation and increased apoptosis were observed. Imunohistochemistry staining also demonstrated that the correlation between NFAT1 and its downstream genes is conserved *in vivo*. Silencing NFAT1 reduced IL-8 and MMP-3 expression in the tumor tissue suggesting that its role in regulating crucial downstream targets which are necessary for dimelanoma progression. Thus, the NFAT1 transcription factor may be an important factor to promote melanoma progression.

The chemokine IL-8 is one of the downstream targets identified as being regulated by NFAT1 and its expression is shown to be positively correlated with melanoma progression [134-136]. Overexpression of IL-8 in melanoma cells up-regulates the expression and activity of the matrix metalloproteinase MMP-2, which contributes to a more invasive phenotype [137]. Targeting IL-8 could be therapeutically beneficial for melanoma patients, due to the pro-tumorigenic and survival dependent effects of IL-8 in cancer. Using a fully human antibody against IL-8 (ABX-1L8) we demonstrated its effect on melanoma growth and metastasis. ABX-IL8 significantly reduced tumor growth and experimental lung metastasis of A375SM and TXM-13 melanoma cells *in vivo* mostly by inhibiting angiogenesis [141]. IL-8 has been shown to promote the growth, invasiveness, motility, angiogenesis, and metastatic potential of melanoma cells [137, 141-143]. Interestingly, targeting IL-8 reduced MMP-2 expression, and incubating melanoma cells with ABX-IL-8 reduced the invasive potential of melanoma cells through Matrigel coated membranes. Decreased CD31 staining *in vivo* and HUVEC tube formation *in vitro* was also observed [141]. In a new ongoing study in the lab, a siRNA delivery approach is being utilized to silence IL-8 to reduce melanoma growth and metastasis *in vivo.*

The second downstream target, MMP-3, is less investigated in melanoma. MMP-3 was investigated as a potential serum marker and the levels of MMP-3 in the serum were evaluated between healthy patients and malignant melanoma patients but no significant differences observed [146, 147]. Another *in vivo* study reported that Angpt2, MMP-3 and MMP-10 are all upregulated in the lung by a mice bearing the B16F10 tumors [151]. The lung microenvironment is therefore presenting the pre-metastatic niche that resulted through the influence of the primary tumor. Recently, SOX2 (an embryonic stem cell transcription

factor) was found to be expressed in human melanoma cells [152, 153]. It was also shown that after silencing SOX2 in melanoma cells, the expression of MMP-3 was reduced by almost 90% [154]. From the association between SOX2 and MMP-3, it was suggested that MMP-3 is regulated by SOX2 and that their co-expression may be used as a functional biomarker for invasive melanoma cells [154]. Data from our current study showed that NFAT1 binds to the MMP-3 promoter and regulates its expression. Also, we validated that there is a direct correlation between MMP-3 protein levels with the metastatic potential of melanoma cell lines. To further clarify the role of MMP-3 in melanoma progression we utilized A375SM cells with MMP-3 shRNA and SB2 cells with MMP-3 overexpression. Both manipulated cell lines were injected subcutaneously and intravenously to study the role of MMP-3 in tumor growth and metastasis. In SB2 cells, higher MMP-3 expression resulted in an increase of tumor growth and experimental lung metastasis. In contrast, silenced MMP-3 in A375SM cells resulted with a significant reduction in both the number of metastases and tumor size (Figure 22). Evidence from this investigation led to the conclusion that NFAT1 is a regulator of a number of important genes that support melanoma growth and metastasis including IL-8 and MMP-3. Hence, NFAT1 expression is essential for melanoma progression. Our findings identify a previously unknown mechanism by which NFAT1 promotes melanoma growth and metastasis through its positive regulation of IL-8 and MMP-3 protein expression.

Therapies for the treatment of metastatic melanoma that target NFAT1, IL-8 or MMP-3 have not yet to been studied. Throughout our study, we identify a novel mechanism for melanoma progression in which NFAT1 regulates IL-8 and MMP-3 and promotes the malignant phenotype. Targeting NFAT1 could be a potential therapeutic tool. Therapy

directed at NFAT1 is clinically feasible as shown with cyclosporine A to inhibit T-cell mediated organ transplant rejection [157]. However, in melanoma, immunotherapy and immunesurveillance promoted by T-cells are considered methods for melanoma treatment. Therefore, systemic therapy directed towards NFAT1 in melanoma could be counterintuitive by reducing T-cell activity. Yet, not all T-cells have anti-tumor function. Regulatory T-cells (Treg) can reduce the immune response towards tumors, and it's been shown that NFAT1 enhances Treg activity as well (Reviewed in [158, 159]). The multiple roles of NFAT1 within the tumor microenvironment create a "double edged sword" in regards to its therapeutic potential. Therefore, directing therapy towards the downstream targets IL-8 and MMP-3 instead of NFAT1 could be a better choice.

The impact of these findings can translate to the clinic by improving treatment options for metastatic melanoma. Results suggest that targeting IL-8, MMP-3, or both as therapy for melanoma is possible either alone or in combination with immunotherapy or chemotherapy. Data from the survival curves (Kaplan Meier) reveal a potential connection between IL-8 and MMP-3 expression and patient survival highlighting the likely success of utilizing these two targets for therapeutic purposes. Treatment options focused on targeting IL-8 and MMP-3 can be taken towards several directions including finding the connection between IL-8 expression and BRAF resistance in melanoma cells. Further analysis may examine the change in IL-8 levels due to resistance and whether the use of IL-8 inhibiting antibody will regress cellular resistance to BRAF inhibitors.

Based on our results, it can be established that after silencing NFAT1, reduction in tumor size can be partially due to a reduction in angiogenesis resulted from a decrease in IL-

8 expression. These data suggest that NFAT1 may promote melanoma by functioning as a regulator of downstream targets that in turn can be affecting interactions with the microenvironment.

Our previous data present a novel mechanism in which autotaxin is regulated by Gal-3 through NFAT1 [122]. Along with our current data, this implicates that NFAT1 plays major roles in modulating the tumor microenvironment to support melanoma growth and metastasis (Figure 25). Taken together, our findings establish a novel mechanism by which NFAT1 contributes to melanoma growth and metastasis through the regulation of IL-8 and MMP-3. This is the first report of a mechanistic role of NFAT1 in melanoma progression and serves as foundation for future studies in this area.

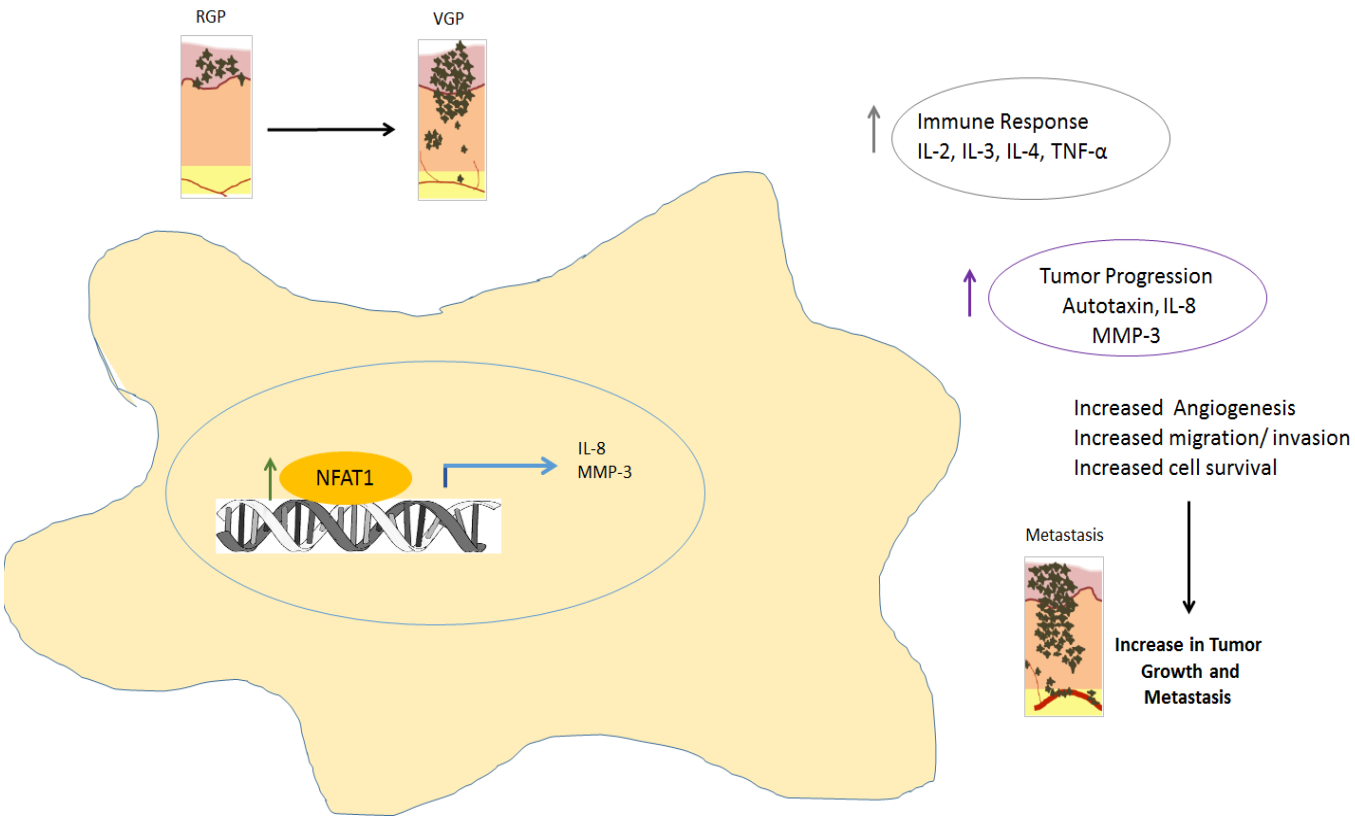

Figure 25: Proposed Mechanism for the Role of NFAT1 in Melanoma Progression

Increased of NFAT1 expression in metastatic melanoma cells promotes its activation as a transcription factor in the nucleus. Therefore, promotes its downstream target genes. IL-8 and MMP-3 are supporting angiogenesis, invasion and cell survival, and result increase in tumor growth and metastasis.

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Vita

Einav Shoshan was born and raised in Beer Sheva, Israel on January 30, 1979 and is the daughter of Frida and Moshe Oron. After graduating from Mekif Gimel High School in 1997, Einav joined the Israeli Defense Force (IDF) where she served 3 years as an officer. In 2000 she began his undergraduate studies at the Ben Gurion University of the Negev at Beer Sheva, Israel. Einav received her Bachelor of Science degree in Biotechnology engineering in Jun 2004. In October 2004 Einav joined Weizmann institute of Science and got her Master of Science Degree in brain research in November 2006. In May of 2010, she entered the Ph.D. graduate program at the University of Texas Health Sciences Center at Houston Graduate School of Biomedical Sciences. In January 2011, she joined the lab of Dr. Menashe Bar-Eli at the university of Texas M.D. Anderson Cancer Center to study melanoma and the molecular changes that lead to the metastatic phenotype.

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