The role of ATF-3 in physiologic and neoplastic cellular proliferation

HARVARD UNIVERSITY
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"The Role of ATF-3 in Physiologic and Neoplastic Cellular Proliferation"

A Thesis Presented by

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DISCLOSURES

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TABLE OF CONTENTS

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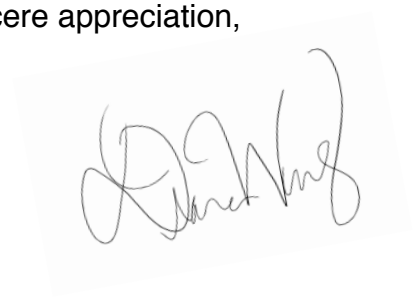
With sincere appreciation,



Abstract:

Activating transcription factor 3 (ATF-3), a cyclic AMP-dependent transcription factor that is encoded by the *ATF-3* gene, plays a potential regulatory role in physiologic and pathologic cell proliferation including wound healing and cancer. However, data is limited that compares and contrasts its mechanistic role in the regulation of regenerative cell proliferation in healing responses seen in oral mucosal reparative cell proliferation or scar formation affecting traumatized skin, and in the unregulated cell behavior as exemplified in the standard cancer progression models such as human melanoma. To this end, we evaluated ATF-3 expression during re-epithelialization at wound edges in oral mucosa and skin as well in a more in-depth mechanistic manner in the context of melanoma progression models. We found no significant differences by immunofluorescence microscopy in ATF-3 immunoreactivity between wound edges of ulcerated human skin and oral tissue types as they both exhibited similarly increased ATF-3 expression in comparison to its normal counterparts. In contrast, ATF-3 staining declined in metastatic versus primary melanoma cell lines and with progression from patient nevi to primary to metastatic melanomas. Analysis of TCGA melanoma database exhibited lower ATF-3 expression which correlated with poor prognosis. Metastatic melanoma cell lines with retrovirally-induced ATF-3 overexpression (A2058 and C8161 ATF-3 OE) exhibited decreased proliferation, migration and invasion in vitro. GO and KEGG analyses of the RNA *seq* data showed downregulation of phosphorylated ERK and AKT in A2058 ATF-3 OE cells that was further validated by Western Blot. In vivo, xenografted A2058 ATF-3 OE cells formed smaller and less

abundant tumors in murine subcutis than did control cells, and Ki-67 staining confirmed lower labeling indices in ATF-3 overexpressing lesions. Taken collectively, ATF-3 appears to play a dichotomous role in reparative versus neoplastic cell proliferations, with increased expression associated with the former and decreased expression with the latter. Aside from its potential use as a biomarker to define cell subpopulations most actively involved in tissue repair, it also may serve as a target for novel therapeutic approaches to inhibiting the virulence of cancers that affect skin and oral mucosa.

Introduction:

Tumors have been historically described as wounds that do not heal,[1] and wound healing and tumorigenesis are two processes that rely on similar mechanisms. Some of parallel processes involved in both include avoiding immune destruction, resisting cell death, activating invasion and migration and inducing angiogenesis (Fig. 1).[2] But, in particular, cellular proliferation involving host-environmental interfaces such as skin and mucosae is fundamental to both reparative/regenerative processes as well as neoplasia. Although multiple pathways have been implicated in the initiation and perpetuation of cellular proliferation events that drive these seemingly disparate tissue reactions, there is limited knowledge with respect to molecular events that represent common denominators common to both.

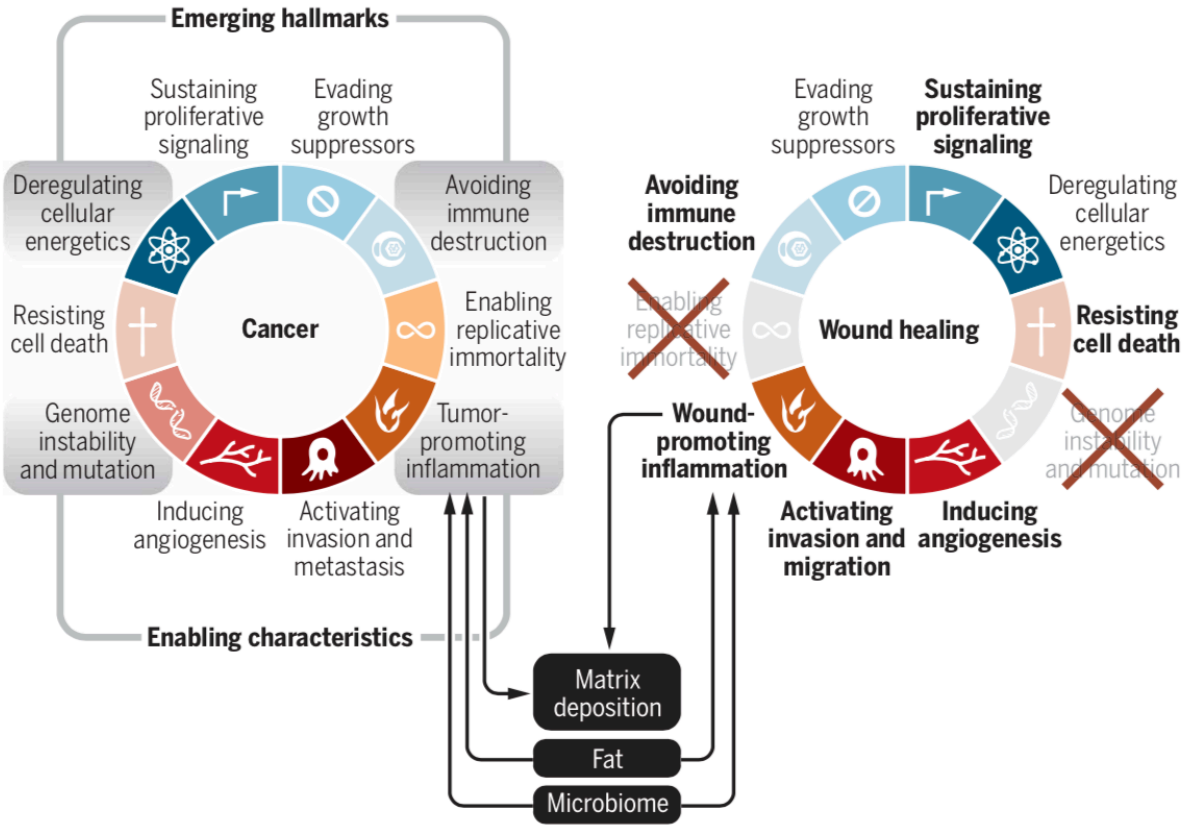


Figure 1: Parallel features of cancer and wound healing. Adapted from Hanahan and Weinberg (2011). MacCarthy-Morrogh & Martin. Sci Signal. 2020 Sep 8;13(648):eaay8690

Recently, stem cells have been recognized to play a crucial role in the complex cellular and molecular underpinnings of oral mucosal and skin wound healing and neoplasia. Oral traumatic ulcers are common pathological lesions observed in clinical dentistry, where most resolve and heal within 7-10 days. However, studies have demonstrated that delayed wound healing of oral and cutaneous ulcers is associated with chronologic aging, diabetes, or medication usage.[3-6] Nonetheless, one remarkable difference between cutaneous and oral mucosal wound healing is the tendency for the latter to involve a less cicatricial, more regenerative healing response. Recent data implicates tissue stem cells in regenerative tissue repair, and stem cell activation and plasticity are integral to true regeneration. Stem cell behavior involved in the dysregulation of signaling pathways during wound healing also has been shown to promote tumorigenesis by enhancing cancer stem cell (CSC) populations.[7] Similar to physiological stem cells, CSCs can self-renew, differentiate and proliferate indefinitely, mechanisms when unregulated cause inexorable tumor growth.[8] A skin and mucosal cancer that has been a particular focus of recent CSC research is melanoma, where stem cell behavior during melanomagenesis is becoming increasingly recognized to involve transcription factors as well as epigenetic events, such as DNA methylation and histone modifications.[9]

Among pathways that potentially play a key role in wound healing and neoplasia is activating transcription factor 3 (ATF-3), a cyclic AMP-dependent transcription factor that is encoded by stress-inducible gene, *ATF-3*.[10] It plays a critical role in DNA damage repair and regulates the expression, activation, or repression of cell-cycle regulators. Similar to other transcription factors in the cAMP response element-binding (CREB)

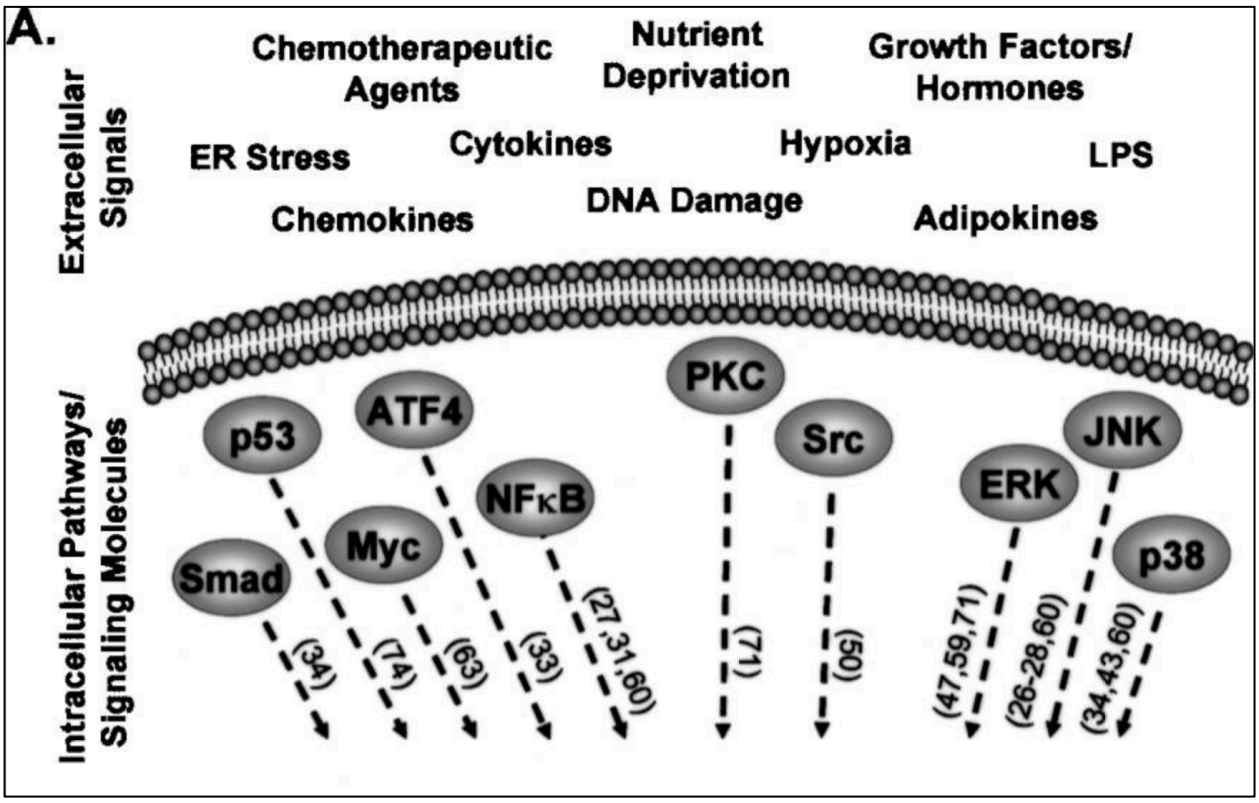


Figure 2: ATF-3, a hub of cellular adaptive-response network. Hai et al. Gene Expr. 2010;15(1):1-11

family, the expression of ATF-3 is associated with various pathophysiological responses, such as inflammation and apoptosis.[11-13] It is considered to be the hub of a cellular adaptive-response network for modulating inflammatory responses and has been linked to pathways involved in wound healing as well as neoplastic processes (Fig. 2).[10-13] Increased stress-induced levels of ATF-3 results in an upregulation of genes involved in tissue remodeling, inflammation, and re-epithelialization.[14] Similarly, ATF-3 has been well studied and implicated as a critical transcriptional repressor of p53 and other senescence-associated genes in mucosal and cutaneous squamous cell carcinoma.[12] Recently, ATF-3 has been detected in melanocytes with a potential oncogenic effect enhanced by calcineurin inhibitors.[13] However, other studies have also shown that ATF-3 expression either promotes or inhibits tumorigenesis, suggesting the regulatory role of ATF-3 to likely

be tumor-specific and signal pathway-dependent.[15,16] Despite the emerging data addressing the role of ATF-3 in the field of medicine and biology, very little is known about its role in physiological proliferation pertaining to oral mucosal regenerative wound healing and cutaneous scar formation, as well as its role in melanoma progression.

Accordingly, in this study, we hope to address the following questions: (1) What is the expression pattern of ATF-3 in healing cutaneous and mucosal wounds, and is the pattern different or similar between the two sites which exhibit differences in regenerative healing? (2) Because epithelial stems cells are critical to proliferative responses during tissue repair, how does ATF-3 expression relate to these subpopulations? (3) In view of the role of stem cells in proliferative response that characterize cancer, what are the characteristics of ATF-3 expression in a well-defined neoplasm where stem cells have been documented to play a critical role (e.g. melanoma), and how do they compare to those documented in proliferative responses due to healing/regenerative phenomena? Thus, the purpose of this study is to investigate the role of ATF-3 in scar formation and regenerative wound healing as well as cutaneous melanomagenesis. *Our **hypothesis** based on preliminary findings already accrued is two-fold: (1) ATF-3 expression is involved in scar formation, regenerative wound healing, and melanomagenesis; and (2) ATF-3 expression, although functionally significant, may be different between regulated (reparative) and unregulated (neoplastic) processes.* We will address these two hypotheses in the following specific aims:

Specific Aim 1: To characterize ATF-3 expression during early re-epithelialization at wound edges in (1) normal human skin and (2) tongue mucosa:

- We will evaluate tissues using multiplex immunofluorescence for a defined panel of relevant epitopes.
- We predict that ulcerated human skin and oral mucosa show differences in ATF-3 and the stem cell marker, CK15, expression in comparison to their normal counterparts and to each other.

Specific Aim 2: To characterize ATF-3 expression in (1) normal human melanocytes, (2) nevi, (3) primary and (4) metastatic melanoma:

- We will evaluate tissues using multiplex immunofluorescence for a defined panel of relevant epitopes.
- We predict that the expression profiles in normal human melanocytes, nevi, primary and metastatic melanoma for ATF-3 expression are different.

Specific Aim 3: To retrovirally transfect melanoma cell lines (A2058 and C8161) with ATF-3 overexpressing vector to determine the mechanistic role of ATF-3 in melanoma proliferation, migration and invasion *in vitro and in vivo*.

- We will validate increased expression of ATF-3 in retrovirally transfected melanoma cell lines by immunofluorescence, Western Blotting and qRT-PCR analysis.

- We will assess the proliferation, migration and invasion properties of melanoma cell lines with ATF-3 overexpression (ATF-3 OE) using EdU kit, Cell Counting Kit-8, colony formation assay, cell migration assay and Matrigel Invasion Assay, respectively.
- We will utilize a xenograft mouse model to confirm and quantify characteristic properties of melanoma cell lines *in vitro* by documenting the size, weight and rate of tumor growth of melanomas with ATF-3 OE versus its control.
- We will investigate potential molecular mechanisms responsible for ATF-3 mediated inhibition of metastatic melanoma cell growth, migration, and invasion in vitro by performing RNA-sequencing (RNA-seq) on A2058 and C8161 ATF-3 OE and NEO cell lines
- Principal component analysis (PCA) and Gene Ontology (GO) Enrichment Analysis will be performed on all genes detected by RNA-seq to identify specific signaling pathways and downregulated and upregulated genes.
- Western blot analysis will be used to confirm representative molecules involved in RNA-seq identified signaling pathways.
- We predict that melanoma proliferation, migration and invasion in melanoma cell lines, A2058 and C8161 ATF-3 OE, will decrease, confirming the tumor suppressive role of ATF-3 in melanomagenesis.
- We also predict that ATF-3 OE significantly inhibits melanoma growth and tumor formation in xenograft mouse model in vivo.

- We predict that signaling pathways involved include ERK and AKT pathways, which are commonly involved in melanoma progression.

Chapter 1: The Role of ATF-3 in Wound Healing

Background:

Cutaneous wound healing is a complex biologic process that involves the spatial interaction between epithelial stem cells and multiple cell types. These interactions are categorized into three stages: inflammatory, proliferative, and remodeling. The inflammatory phase involves platelet accumulation, coagulation and migration of immune cells to contain and destroy pathogens. This process may take up to 4-6 days. The release of cytokines and growth factors initiates the proliferative phase, where keratinocytes at the edge of the wound break down their hemi-desmosomes and transition to a non-proliferative and migratory state in order to move and fill in the damaged epidermis. The keratinocytes further away from the wound, on the other hand, proliferate in order to replace any depleted cells. Re-epithelialization, angiogenesis, granulation tissue formation and collagen deposition take place from day 4 to day 14 after initial injury.[17] However, the earliest events in the migration of epithelial cells commence by 24 hours. Lastly, the remodeling phase is characterized by the restructuring of the extracellular matrix by contractile myofibroblasts, fibroblasts and mesenchymal stem cells to re-establish the protective dermal layer. However, skin postnatally fails to regenerate normal dermis during this phase, resulting in the formation of dysfunctional scar tissue. This process is initiated by day 8 and may last up to a year.[17, 18] Histologically, scar tissue exhibits an overlying atrophic epithelium, altered collagen deposition and depletion of tissue stem cells.[19] Although scar formation is commonly seen in the skin, this process is less commonly encountered in the oral mucosa, which tends to demonstrate a more

regenerative type of wound healing response. After wounding, oral epithelium may become fully regenerated, and histological sections exhibit epithelial architecture and submucosal collagen deposition akin to normal tissue.[20]

Recent studies have shown differences between oral mucosal and skin wounds. Oral mucosal keratinocytes and fibroblasts have a higher proliferation and migration rate than those seen in the skin.[21] In addition, oral mucosal wounds exhibit a lower inflammatory response[22], decreased production of vascular endothelial growth factors (e.g. VEGF) and a blunted angiogenic response.[23] Studies have also shown that oral wounds have a significantly lower level of expression of TGF-ß1, which is a pro-inflammatory and pro-fibrotic cytokine commonly associated with hypertrophic scars.[24] Microarray analysis of overall gene expression in mucosal and skin wounds suggest that wound healing in these two types of tissues exhibits different apoptosis-related gene signatures, where apoptosis in mucosal wound healing predominantly occurs through the intrinsic pathway.[22, 25]

Of interest, studies have also shown that stem cells play a critical role in wound healing. Interfollicular epidermis, sebaceous glands and hair follicles are capable of self-renewal. However, full thickness wounds (where the hair follicle is obliterated) results in a slower rate of re-epithelialization and migration of cells to the injury site.[17] During epithelial injury, stem cells located in these three major compartments of the epidermis participate in re-epithelialization and the orchestration of these cellular responses is due to transcription factors, which play a critical role in wound repair. For instance, stem cells located at the bulge or rete tips of the epidermis are defined by the expression of GLI1,

which is a transcription factor that is activated by sonic hedgehog (SHH).[26] It is well known that transcription factors are key molecules that regulate gene expression in response to injury. Recently, activating transcription factor 3 (ATF-3) has been associated with wound healing. However, the detailed role of ATF-3 in scarless regenerative mucosal wound healing remains incompletely understood and not well characterized.

ATF-3 is a member of the mammalian activating transcription factor/cAMP responsive element binding protein (ATF/CREB) family of transcription factors that is encoded by stress-inducible gene, *ATF-3*.[10] The expression of ATF-3 can be induced by a spectrum of stimuli, including anticancer drugs, endoplasmic reticulum stress, toxic chemicals, oxidative stress, and oncogenic signaling that ultimately triggers cell cycle arrest and apoptosis.[14, 27-29] The transcriptional target of ATF-3 differs in numerous cell types and results in varied effects on cell survival, proliferation and death.[11] Studies have shown that a microarray analysis of human fibroblasts exposed to serum induced ATF-3 and exhibited gene expression profiles that are commonly involved in wound healing: specifically cellular proliferation and tissue remodeling (e.g. inflammation and re-epithelialization).[30] ATF-3 also acts as a transcriptional repressor in immune cells, which are commonly involved in wound healing (Fig. 3).[31] Previous studies have shown that the activation of various toll-like receptors (TLRs) may elevate the

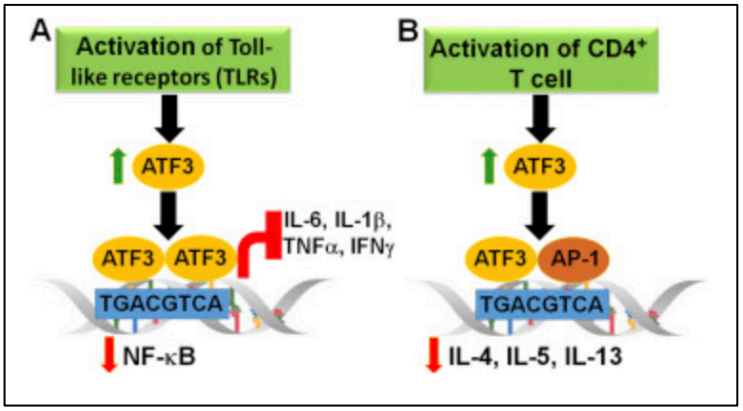


Figure 3: ATF-3 acts as a transcriptional repressor in immune cells. Jadhav & Zhang. Liver Res. 2017 Sep;1(2):96-102.

expression level of ATF-3 in bone marrow-derived macrophages.[32] Similarly, the upregulation of ATF-3 in response to *Streptococcus pneumoniae* infection via TLR2 and TLR4 in vitro suggests that ATF-3 plays a role in TLR signaling.[33] ATF-3 is not only associated with macrophage migration via the Wnt/β-catenin pathway, a signaling pathway that partakes in wound healing, but is also found to be elevated in leading keratinocytes at cutaneous wound edges.[34, 35] However, the precise role of ATF-3 in regenerative wound healing is yet to be determined.

The longstanding interest of the Murphy Lab in contractile/scarring versus regenerative wound healing dates back to studies by Yannas, Orgill, Burke, and Murphy when they developed and characterized a biodegradable scaffold that promoted true dermal regeneration as opposed to the formation of contractile scar (the scaffold is now known as Integra®).[36] Although it was suspected that a stem-like cell was responsible, there existed no biomarkers at the time to identify such cells that ostensibly had sufficient self-renewing and plasticity capacities to result in authentic tissue regeneration. During this period, the Murphy lab also developed a model to study human skin wound healing via xenotransplantation to the backs of SCID mice.[37] A breakthrough in biomarker identification of skin stem cells came when keratinocyte stem cells were identified in the murine hair follicle “bulge” region to express cytokeratin 15. [38] Subsequently the Murphy Lab showed that human and murine (dorsal tongue) epithelial ridges (retia) also expressed this biomarker within their stem cell niches.[39] These observations have opened up avenues of investigation that now seek to explore the role of skin stem cells in the

context of the spatially-defined geometry of biodegradable scaffolds that promote skin regeneration.

Based on these and other aggregate observations, it can be concluded that cellular proliferation seen in wound healing may be subject to similar transcriptional regulation. Although skin and oral mucosal healing involves dissimilar pathways; one mediated by the formation of contractile scar tissue, the other by a more regenerative response, we posit that the key cellular pathways accounting for these differences most likely occur during the earliest biosynthetic and secretory phases of the wound healing response. Accordingly, the subsequent proposal leverages the availability of a panel of relevant skin and mucosal biomarkers and a human bioarchive for comparative characterization of ATF-3 during the healing response following opportunities for a novel and innovative approach to documenting the potential involvement of ATF-3 in scarless, regenerative wound healing.

Significance:

Transcription factors serve as integrative centers for various cellular signaling pathways and oftentimes work together to regulate regions of genes. It is well known that transcription factors respond to external stimuli to coordinate regulation of target genes to generate an appropriate physiological response.[40] Similarly, transcriptional networks are frequently dependent on cell type.[41] As such, the same transcription factor may elicit different cellular responses when induced, as seen with ATF-3.

By exploring the role of ATF-3 in wound healing in skin and oral mucosa, we will gain a better understanding of its regulatory role of regenerative cell proliferation in healing responses seen in oral mucosal reparative cell proliferation or scar formation affecting traumatized skin. Theoretically, by understanding the key comparative differences in ATF-3 expression of epithelial stem cells in oral mucosa and skin, we can potentially target ATF-3-induced signaling pathways in epithelial stem cells in the skin to behave in a similar regenerative manner seen in oral mucosa wound healing, and such a therapeutic breakthrough would be both translationally significant and transformative.

Innovation:

This project is innovative because:

1. It allows us to expand our knowledge on ATF-3 by looking at the physiological conditions and comparing and contrasting cellular proliferation (regulated vs. nonregulated) in the context of physiological stem cells.
2. It utilizes a unique and correlative panel of multiplex stem cell biomarkers.

Materials and Methods:

Specific Aim 1: To characterize ATF-3 expression during early re-epithelialization at wound edges in (1) normal human skin and (2) tongue mucosa:

1.1. ATF-3 and CK15 (epithelial stem cell marker) expression during early re-epithelialization at wound edges in (1) normal human skin and (2) tongue mucosa will be evaluated using immunofluorescence microscopy

Human tissue samples: The biospecimen archives of the Pathology Department of the Brigham and Women's Hospital will provide all tissue samples (3 patient examples per category) with full IRB approval (2019P002747). Histological sections will be mounted on glass slides from formalin-fixed, paraffin-embedded (FFPE) sections of human normal oral mucosa, normal epidermis and oral mucosal and epidermal ulcers. Sections with ulcers will be taken from resected specimens with a previous biopsy diagnosis of squamous cell carcinoma but now diagnosed as "squamous mucosa with ulceration, granulation tissue and acute and chronic inflammation" with a note that "no residual carcinoma or dysplasia is detected". The days between date of biopsy and date of resection will be recorded (**Table 1**).

Hematoxylin and eosin (H&E) staining will be performed for histological evaluation.

Immunofluorescence studies: Immunofluorescence studies for antigens of interest will be performed on 5-μm sections of formalin-fixed, paraffin-embedded (FFPE) human tissue

samples. The tissue sections will be placed on a slide warmer at 58°C for 20 minutes and deparaffinized in xylene, rehydrated through a series of alcohols (from 100% ethanol to 50% ethanol). The tissue sections will be placed in a pressure cooker for 40 min (Dako, 125 °C for 30 min, 90 °C for 10 min) with Target Antigen Retrieval solution (Dako, Cat: S1699, Carpenteria, CA, USA). After this heat-induced antigen retrieval process is completed, sections will be incubated with primary antibodies at 4 °C overnight, followed by incubation with fluorophore-conjugated secondary antibodies for 30 min at room temperature. Isotype-matched irrelevant antibodies were used in place of primary antibodies as controls. The following primary and secondary antibodies will be used for immunofluorescence studies on human wound tissues: Rabbit anti-ATF-3 antibody (1:300, Abcam, Cat: ab216569, Cambridge, MA), Mouse anti-CK15 antibody (1:200, Thermofisher Scientific, Cat: MS-1068-P1, Waltham, MA), DyLight 488 goat anti-mouse IgG(H+L) (1:2000, Invitrogen, Cat: A21131, Carlsbad, CA) and DyLight 647 goat anti-rabbit IgG(H+L) (1:1000, Thermofisher Scientific, Cat: A21245, Waltham, MA). Immunofluorescence studies were evaluated by two independent investigators (G.F.M., C.G.L.).

Scoring system for antigen intensity: Evaluation of antigen intensity was analyzed using the following scoring system: 0 (no staining), 1+ (weak and incomplete nuclear staining), 2+ (strong, complete nuclear staining in less than 30% of the targeted cells or weak/moderate heterogeneous nuclear staining in more than 10% of the targeted cells), 3+ (strong complete homogenous nuclear staining in more than 30% of the targeted lineage cells).

Results:

ATF-3 expression in wound edges of ulcerated skin and oral mucosa were upregulated but there were no significant differences between the two tissue types

We evaluated ATF-3 and CK15 expression in wound edges of ulcerated skin and oral mucosa and compared its level of expression to its normal counterpart. There were no significance differences between the two groups as both tissue types exhibited increased nuclear and cytoplasmic ATF-3 expression (**Fig. 4**). Similarly, CK15, a biomarker for epithelial stem cells, did not exhibit different levels of protein immunoreactivity at the tissue edges from both groups. Interestingly, there were apparent differences between normal epidermis and oral mucosa in comparison to the wound edges. In the normal oral mucosa and epidermis, ATF-3 expression is muted while CK15+ epithelial stem cells were strongly expressed at the rete ridges (**Fig.5**).

Discussion:

Our study suggests that ATF-3 plays a role in physiological proliferation. Previous studies have shown the relationship between ATF-3 induction and its potential regulatory role in wound healing.[30] Upon injury to the epidermis, quiescent keratinocytes transition to leading keratinocytes at wound edges by activating p38.[35] In turn, the activation of p38 leads to keratinocyte migration and increased ATF-3 expression.[35] ATF-3 also plays a negative regulatory role in signaling pathways involved with immune response.[32, 33] Since oral mucosal keratinocytes and fibroblasts have a higher proliferation and migration rate and exhibit a lower inflammatory response than those seen in the cutaneous wound healing,[21, 22] we expected keratinocytes at oral wound edges to exhibit a higher level of ATF-3 expression in comparison to cutaneous wound edges. Although there were no disparate expression levels of ATF-3 in oral mucosal and cutaneous wound healing in our study by immunofluorescence microscopy **(Fig. 4)**, ATF-3 likely plays a role in physiological cellular proliferation (e.g. re-epithelialization) during wound healing. Moreover, it is possible that more precise and quantitative proteomic or genomic (mRNA) analyses may reveal subtle differences between squamous mucosa and skin not readily discernable by immunofluorescence microscopy alone. Previous studies have shown that ATF-3 is critical for niche maintenance of epithelial stem cells and ATF-3 deficiency may compromise stem cell regeneration,[42] suggesting the complex relationship between ATF-3-induced signaling pathways and epithelial stem cells during wound healing. Our data further supported the notion that epithelial stem cells located at the rete ridges most likely differentiated to short-lived

transient amplifying cells that migrate towards the wound bed during the healing response, do so in part in association with upregulation of ATF-3 expression during re-epithelization near the ulcer bed **(Fig. 4 and 5).**

There are some limitations to this study in which we had limited access to human oral and cutaneous wound bed specimens and epithelial stem cell cultures. Based on our search criteria, the average times between biopsy and resection of healing wound beds in oral mucosa and skin were 30 and 37 days, respectively. In theory, re-epithelialization, angiogenesis, granulation tissue formation and collagen deposition take place within two weeks after initial injury, with the earliest events of epithelial migration occurring by 24 hours.[17] It is possible that these representative oral and cutaneous wound bed specimens may not reflect early proliferative stages but perhaps the initiation of the remodeling stage. Likewise, our sample size (n=3) is quite small and may not be statistically significant. Although our data exhibited increased ATF-3 expression in epithelial cells located at the oral and cutaneous wound beds, we did not reconfirm this data with in vitro studies. Unfortunately, our lab does not have access to oral and cutaneous epithelial stem cells, which are needed to further understand the comparative interactions between ATF-3 and epithelial stem cells. As such, additional functional studies are needed to truly understand the effects of ATF-3 in wound healing and how it potentially impacts scar formation versus true regeneration.

While this study did not shed insight into why mucosal epithelium exhibits a greater degree of regenerative healing than does skin, it is consistent with a regulatory role for ATF-3 in healing responses that involve both squamous mucosa and skin. ATF-3

expression is upregulated in cutaneous and mucosal wound edges in comparison to their normal counterparts. Accordingly, functional studies are now indicated to determine how inhibition of ATF-3 during epithelial tissue repair affects the overall integrity and efficacy of wound healing and how it potentially impacts scar formation versus true regeneration.

Chapter 2: The Role of ATF-3 in Melanomagenesis

Background:

Cancer is also associated with stem cell activation, proliferation, and exposure to changes in the microenvironment. In recent years, cancer stem cells (CSCs) have been considered a new subclass of neoplastic cells that exhibit self-renewing and differentiation capabilities and have been implicated in the pathogenesis of breast carcinomas, neuroectodermal tumors and hematopoietic malignancies.[43-46] Similarly, transcription factors and stem cells play a critical role in the development, progression, drug resistance and metastasis of melanoma, in particular.[47, 48]

Melanoma is a common and exceptionally aggressive form of skin cancer that originates from melanocytes.[49] It is considered one of the top five most common cancers that show an increasing incidence every year. Even though it represents less than 5% of all skin cancer cases, melanoma accounts for 60-80% of deaths from skin cancer, with a poorer prognosis with regional lymph node involvement or metastasis.[50] Studies have shown that mutations within the mitogen-activated protein kinase (MAPK/ERK) and phosphatidylinositol 3-kinase (PI3K/AKT) pathway play a role in the progression of melanoma and affects its cell proliferation, growth, and survival.[51, 52] Transcription factors have also been implicated in melanoma drug resistance and are considered critical convergence factors in multiple dysregulated receptors and signaling pathways.[47] These biological findings sparked the evolution of various treatments including new targeted therapies and immunotherapies to treat metastatic melanoma. However, novel molecular

mechanisms that mediate melanoma behavior require more comprehensive understanding in order to inform more effective therapeutic strategies.

Recently, activating transcription factor 3 (ATF-3) has been associated with melanomagenesis and been known to play a role in promoting or suppressing gene transcription associated with tumorigenesis. However, the detailed role of ATF-3 in melanomagenesis remains incompletely understood and not well characterized.

ATF-3 can also act as a transcriptional activator and partakes in a number of cellular signal transduction pathways.[53] ATF-3 is shown to positively regulate tumor suppressor gene, *p53*, in response to DNA damage and *ATF3*-/- mice exhibit genomic instability and spontaneous tumorigenesis.[54] In the past decade, the dysregulation of ATF-3 seems to play different roles in various tumors. Studies have shown that ATF-3 expression is downregulated in hepatocellular carcinoma, prostate cancer, and colon cancer[55-57]. Similarly, ATF-3 inhibits tumorigenesis and progression of esophageal squamous cell carcinoma by downregulating inhibitor of DNA-binding 1 (ID-1).[58] In epithelial cells, ATF-3 appears to bind to ID-1 promoter and mitigates its expression level in response to cellular stress signals.[59] Analogous to these studies, ATF-3 inhibits ID-1 transcription and protein expression in metastatic melanoma and is partly mediated by melanoma cell adhesion molecules (MCAM/MUC18)[60]. However, the role of ATF-3 in signaling pathways for cancer progression is quite complex. For example, ATF-3 plays a dichotomous role in melanoma progression by either preventing transcription by stabilizing inhibitory co-factors at the promoter site or activating transcription of target genes via the C-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK)

stress-inducible signaling pathway.[61, 62] Similarly, ATF-3 is negatively regulated by transient receptor potential vanilloid 1 (TRPV1)-induced calcineurin activation in melanoma, with its oncogenic effect potentiated by calcineurin inhibitors (Fig. 6).[13] However, in another study, ATF-3 suppresses human melanoma growth through induction of expression of ATF-3 in human dermal fibroblasts.[63] Accordingly, the precise role of ATF-3 in melanomagenesis and its function in melanoma progression remains to be explored.

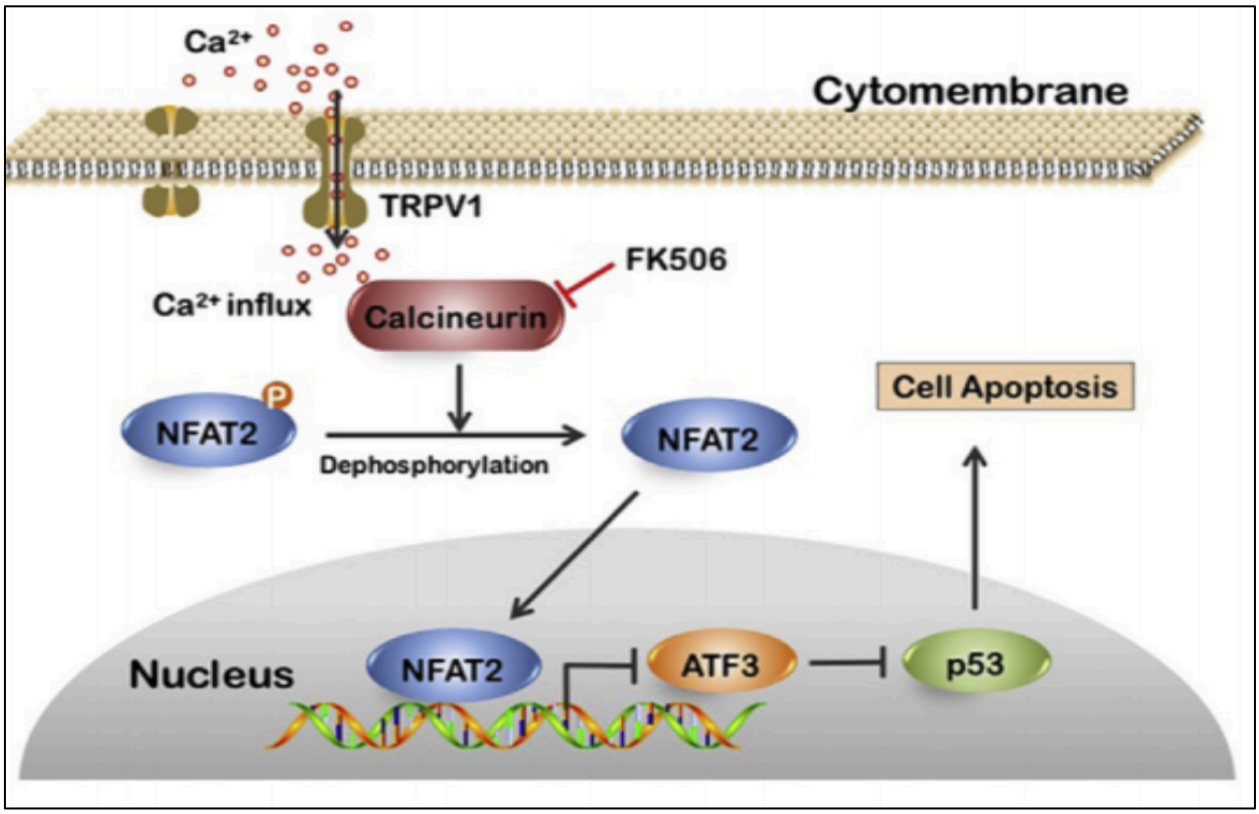


Figure 6: Downregulated TRPV1 Expression contributes to melanoma growth via calcineurin-ATF-3-p53 pathway. Yang et al. J Invest Dermatol. 2018 Oct;138(10):2205-2215.

The Murphy Lab collaboratively discovered a melanoma stem cell biomarker, ABCB5. This biomarker was originally identified in a dermal cell population,[64] and also labeled mesenchymal stem cells important in corneal regeneration and repair.[64] They

learned that ABCB5+ dermal mesenchymal stem cells improve wound healing in a mouse model of chronic venous ulcers.[65] Moreover, in collaboration with Dr. Lian, they noted that the epigenetic mark, loss of 5-hmC, which had been shown to be important in melanomagenesis[9,66], may also be intimately related to ABCB5+ expression by physiologic (non-neoplastic) stem cells.

Based on these observations, it can be concluded that cellular proliferation seen in two distinct processes, wound healing and tumorigenesis, may be subject to similar transcriptional regulation, although important differences are also likely to exist. Accordingly, the subsequent proposal leverages the following opportunities for a novel and innovative approach to documenting the potential involvement of ATF-3 in melanomagenesis; (1) the availability of a panel of relevant biomarkers and a human bioarchive for comparative characterization of ATF-3 during melanomagenesis; (2) the establishment of an *in vitro* cell culture and *in vivo* xenograft mouse model that permits exploration of the role of ATF-3 in melanoma progression; (3) the deployment of RNA-seq analysis to interrogate the functional role ATF-3 in regulating gene expression and cell behavior in melanoma.

Significance:

By understanding the role of ATF-3 in melanomagenesis and its function in melanoma progression, we can possibly use this transcription factor as a prognostic biomarker as well as open new avenues of targeted therapy to combat metastatic melanoma.

Innovation:

This project is innovative because:

3. It allows us to expand our knowledge on ATF-3 by looking at its potential role in melanoma progression.
4. It utilizes a unique and correlative panel of multiplex stem cell biomarkers, in vitro cell cultures, in vivo xenograft mouse models, and high throughput sequencing to look at cell behavior in melanoma progression.
5. It combines descriptive and functional analysis in order to interrogate the dynamic role of ATF-3 in melanomagenesis and melanoma progression.

Methods and Materials:

Specific Aim 2: To characterize ATF-3 expression in (1) normal human melanocytes, (2) nevi, (3) primary and (4) metastatic melanoma:

2.1. ATF-3 expression in different human cutaneous melanocytic lesions (nevi, primary melanoma and metastatic melanoma) will be evaluated using immunofluorescence microscopy

Human tissue samples: The biospecimen archives of the Pathology Department of the Brigham and Women's Hospital will provide tissue samples of benign nevi (n=10), primary cutaneous melanoma (n=38) and metastatic melanomas (n=24) with full IRB approval. The hematoxylin and eosin stained sections, prior diagnoses, and reported prognostic attributes were independently reviewed and confirmed by two dermatopathologists (G.F.M., C.G.L.).

Immunofluorescence studies: Hematoxylin and Eosin (H&E) staining will be performed for histological observation, as described above. The following primary and secondary antibodies will be used for melanoma cell lines and human tissue specimens: Rabbit anti-ATF-3 antibody (1:300, Abcam, Cat: ab216569, Cambridge, MA), Mouse anti-MART-1 antibody (1:1, BioLegend, Cat: 917902, San Diego, CA), DyLight 594 goat anti-rabbit IgG(H+L) (1:500, Multiscience, Cat: GAR5942, Hangzhou, China) and DyLight 488 goat anti-mouse IgG(H+L) (1:500, Multiscience, Cat: GAM4882, Hangzhou, China).

2.2. ATF-3 expression in different human melanoma cell lines in vitro evaluated using immunofluorescence microscopy and western blot analysis

Melanoma cell lines: Melanoma cell lines derived from human primary melanoma (Meljuso, WM115, M14) and metastatic melanoma (A375, SK-MEL-28, SK-MEL-30, UACC62, MeWo) will be provided by Prof. David E. Fisher (Cutaneous Biology Research Center, Massachusetts General Hospital, Boston, USA). A2058 and C8161 melanoma cell lines will be provided by Dr. Tobias Schatton (Dermatology Department, Boston, MA). The cells will be grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibio, Cat:C1199500BT, USA) and will be supplemented with 10% fetal bovine serum (Biological Industry, Cat: 04-001-1ACS, Israel) and10 units/ml penicillin and 100 µg/ml streptomycin (Gibco, REF:15140-122, USA). All cells are maintained in 10 cm tissue culture dishes and stored in humidified incubators at 37°C, 5% CO_2 atmosphere. The media culture will be changed every 2-3 days and cells will be passaged when the cell density reaches 80-90% confluency.

Immunofluorescence studies: Immunofluorescence studies for ATF-3 will be performed for histological observation, as described above.

Whole cell protein lysis (from cell lines): Cultured cells will be harvested, rinsed with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer (Thermofisher Scientific,

Cat: 89900, Waltham, MA) containing Protease Inhibitor Cocktail (Thermofisher Scientific, Cat: 78410, Waltham, MA) and 100 mM Phenylmethylsulfonyl fluoride (Sigma Aldrich, Cat. 10837091001, St. Louis, MO). Whole cell lysate will be collected with a cell scraper, incubated on ice for 15 minutes, and centrifuged at 14000 rpm for 15 minutes at 4ºC. The supernatants will be removed and collected in eppendorf tubes then stored at -20ºC.

Protein assay: Protein content will be determined by Pierce BCA protein assay kit (Thermofisher Scientific, Cat: 23227, Waltham, MA). Per the manufacturer's protocol, concentrations of whole cell lysates will be determined by comparing them to serial dilutions of a control protein, BSA (Bovine serum albumin, 2 mg/ml) (Thermofisher Scientific, Cat:23209, Waltham, MA), using a 96-well round bottom plate (Thermofisher Scientific). 100ul of BCA Protein Assay Reagent A and 4ul of BCA Protein Assay Reagent B will be added to 100ul of sample. The plate will be read in a spectrophotometer plate-reader at 590nm using UV WinLab Software (Perkin Elmer). BSA optical densities will be graphed in Excel as a function of protein concentration. The unknown protein lysate concentrations will be calculated using the line of best-fit formula obtained from this graph.

Western Blotting: Whole cell lysates will be used for western blotting. 50 ug of total protein from cell lysate will be adjusted to the same concentration and volume when loaded into the wells of a 12% SDS-PAGE gel (Thermofisher Scientific, Cat: NP0321PK2, Waltham, MA). The gel will be immersed in 1X Running Buffer (10 X Running Buffer; 250mM Tris

base, 1.92 M glycine, 1% SDS, pH 8.3, Boston BioProduct, Cat:BP-150) and ran at 120 volts for 1.5-2 hours. The resolved proteins will then be transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Cat: IPVH00010, Burlington, MA) using 1X 1X Transfer Buffer (10 X Running Buffer; 250mM Tris base, 1.92 M glycine, 1% SDS, pH 8.4, Boston BioProduct, Cat:BP-190) with 20% methanol for 2 hours overnight. The membranes will then be blocked with 5% non-fat milk in TBS/0.1% Tween 20 (TBST) at room temperature for 1 hour and then incubated with primary antibodies at 4°C overnight. After incubating with primary antibodies, the membrane will be washed with TBS-T (5 min, 3x). Then the membrane will be incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam, Cat: ab6721, Cambridge, MA) at room temperature for 1 hour and washed with TBS-T (5 min, 3x). Immunoreactive bands will be detected by chemiluminescent HRP substrate (Millipore, Cat: WBKLS0500, Billerica, MA) per manufacturer's protocol. MiniChem 610 Image System (Sagereation, Beijing) will be used to visualize chemiluminescence. The density of immunoblot bands will be quantified by Image J and normalized to GAPDH. Antibodies that will be used for western blot analysis are the following: Rabbit anti-ATF3 (1:2000, Abcam, #ab207434).

Specific Aim 3: To retrovirally transfect melanoma cell lines (A2058 and C8161) with ATF-3 overexpressing vector to determine the mechanistic role of ATF-3 in melanoma proliferation, migration and invasion *in vitro and in vivo*.

3.1. To retrovirally transfect melanoma cell lines (A2058 and C8161) with ATF-3 overexpressing vector to determine the role of ATF-3 in melanoma proliferation, migration and invasion in vitro

Melanoma cell lines: Melanoma cell lines, C8161 and A2058, exhibited lower ATF-3 expression in comparison to other melanoma cell lines, and therefore, will be selected for further studies. C8161 and A2058 melanoma cell lines will be cultured as previously mentioned.

Virus preparation and melanoma cell line transfection: Retrovirus preparation and cell line transfection will be performed as previously described.[12] HEK 293T cells (permanent line established from primary embryonic human kidney) will be transfected with ATF-3 overexpressing vector, neomycin control vector and packaging vector by using Lipofectamine 3000 kit (Invitrogen, REF: L3000-015, Carlsbad, CA) according to manufacturer's protocol. Virus particles will be collected at 24h, 48h and 72h after transfection and stored in -80°C. Melanoma cells will be seeded in 10 cm culture dishes and cultured until 60% confluency. Cells will then be incubated with 6ml virus plus 5µl polybrene solution (10ng/ml, Sigma-Aldrich, Cat: TR-1003, St. Louis, MO) for 6h. The virus will be removed, and cells will be washed with PBS and cultured with normal DMEM medium. The transfected melanoma cells will be stored, passaged or tested with related experiments. Retrovirally infected melanoma cell lines, A2058 and C8161, will be

validated for ATF-3 OE by immunofluorescence and qRT-PCR and termed 'C8161ATF-3 OE or A2058 ATF-3 OE'.

Immunofluorescence studies: Immunofluorescence studies for ATF-3 will be performed for histological observation, as described above.

RNA extraction, Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR): Total RNA will be extracted by using RNAeasy kit (QIAGEN, Cat: 74104, Germantown, Maryland) and the cDNAs were synthesized by SuperScript III first strand kit (Bio-Rad, Cat: 1708891, Hercules, CA). The human melanoma cDNA arrays were obtained from Origen (Cat: MERT301). Relative gene expression will be normalized to HPRT. The gene-specific primers and antibodies used for immunoblotting are provided in Table 2. Conditions for RNA extraction and quantitative RT-PCR analysis will be implemented as previously documented: Amplification condition will be set to an initial step of 95°C for 30 sec followed by 50 cycles of 95°C for 5 sec, 60°C for 35 sec, 72°C for 60 sec and a final step of 40°C for 30 sec. All RNA samples will be analyzed in triplicate with gene specific primers.

Table 2: Gene-specific primers for RT-PCR

Gene	Species	Probe length (bp)	Primers (5' – 3')
ATF-3	Human	118	For: GTCCATCACAAAAGCCGAGG

			Rev: GCACTCCGTCTTCTCCTTCT
H36B4	Human	142	For: GCAATGTTGCCAGTGTCTGT Rev: GCCTTGACCTTTTCAGCAAG

5-Ethynyl-2'-deoxyuridine (EdU) incorporation assay: EdU incorporation assay will be performed using an EdU kit purchased from ThermoFisher Scientific (Cat: A10044, Waltham, MA) per manufacturer's protocol. Melanoma cell lines C8161 ATF-3 OE and A2058 ATF-3 OE will each be seeded into a 24-well plate at a density of 50,000 cells and grown overnight. On the second day, the cells will be incubated with EdU solution A (supplied by the kit) for 2h and washed with PBS 2 times for 5 minutes and fixed with 4% paraformaldehyde for 30 mins. The solution will be discarded, and cells will be neutralized with 2mg/ml glycine for 5 minutes then washed with PBS 2 times for 5 minutes. The cells will be incubated with 0.5% Triton X-100 for 10 minutes and washed once with PBS for 5 minutes. Afterwards, the cells will be incubated with 1X Apollo reacting solution for 30 minutes in the dark and the nuclei will be stained with 4',6-diamidino-2-phenylindole (DAPI, Abcam, Cat: ab104139, Cambridge, MA). An immunofluorescence microscope (Olympus BX53-DP80, Tokyo, Japan) will be used for the analysis of staining.

Cell proliferation analysis: Melanoma cell lines C8161 ATF-3 OE and A2058 ATF-3 OE

will be seeded on 96-well tissue culture plates at a density of 2000 cells/well in six replicates. At every indicated time point, 10µl Cell Counting Kit-8 (CCK-8) (Abcam, Cambridge, MA) working solution will be added to each well and cultured for 2h at 37°C in the dark. The plate reader (Spectrostar Nano, BMG Labtech) will measure the absorbance at 450 nm. The experiments will be repeated at least three times.

Cell colony formation assay: Melanoma cell lines C8161 ATF-3 OE and A2058 ATF-3 OE will be seeded in 6-well plates at a density of 1500 cells/well and cultured for 14 days. At the end of the test, cells will be washed with PBS, fixed with freshly made 4% paraformaldehyde solution and stained with 0.1% crystal violet (Sigma Aldrich, Cat: C6158, St. Louis, MO). Cell colonies with more than 50 cells will be counted with an inverted microscope (Olympus CKX41, Tokyo, Japan). The experiments will be performed in triplicate and repeated at least three times.

Wound healing assay: Melanoma cell lines C8161 ATF-3 OE and A2058 ATF-3 OE will be seeded in 6-well plates. Cells will be cultured to reach approximately 90% confluency. The monolayer will be scratched with a sterile 10µl pipette tip (time 0), washed with PBS to remove the cells within the linear scratch and refreshed with DMEM culture medium. At each time pint (0, 16h, 24h), the cell monolayer will be photographed using an inverted microscope (Olympus CKX41, Tokyo, Japan). The wound healing areas will be measured with Image J software. The experiments will be performed in triplicate and repeated at least three times.

Matrigel invasion assay: The Matrigel invasion assay will involve the following components: Corning Matrigel Basement Membrane Matrix (Cat.356234, Bedford, MA, USA), 8µm pore diameter membranes cell culture chamber (Falcon, RFE: 353097, USA) and 24-well plates. 15µl of diluted Matrigel will be added to the growth surface of the chamber 30 minutes in advance according to the manufacturer's protocol. Melanoma cell lines C8161 ATF-3 OE or A2058 ATF-3 OE (60,000 cells) or neomycin control (NEO) melanoma cells in 0.2ml 0.1% FBS DMEM medium will be loaded onto the surface of the gel and 0.75ml of DMEM containing 10% FBS will added to the outer well and incubated at 37°C, 5% CO2 for 24h for A2058 and 16h for C8161. After incubation, non-migrating cells will be removed from the upper surface of the insert membranes by scrubbing them with cotton swabs. The migrating cells on the lower surface of the insert membranes will be fixed with 4% paraformaldehyde solution for 30 minutes and stained with 0.1% crystal violet (Sigma Aldrich, Cat: C6158, St. Louis, MO). Images of 5 random fields of the fixed and stained cells will be captured and counted. The experiments will be performed in triplicate and repeated at least three times.

3.2 To utilize a xenograft mouse model to investigate the effect of ATF-3 on melanoma growth and tumor formation *in vivo*

Virus preparation and melanoma cell line transfection: C8161 and A2058 melanoma cell lines will be retrovirally infected with ATF-3 overexpression vector as previously mentioned and labeled as “ATF-3 OE”.

Melanoma cell lines: C8161 and A2058 NEO and ATF-3 OE melanoma cell lines will be cultured as previously mentioned.

Tumor xenograft mouse model: 6-week-old female nude/nude mice (Charles River Laboratory, MA, USA) will be assigned to two groups: C8161 ATF-3 overexpressing (ATF-3 OE) and C8161 neomycin (NEO) control melanoma cells. Each group will be comprised of six mice and will subcutaneously be injected with either 1.0×10^6 C8161 ATF3 OE melanoma cells or 1.0×10^6 C8161neomycin control (NEO) cells in the left and right anterior and posterior flanks. After 3 weeks, the mice will be euthanized, and the melanoma tumor volumes and weights will be measured. Tumors will be then fixed in formalin and embedded in paraffin for immunofluorescence analysis. The in vivo experiments will be repeated 3 times. The same experiments will also be performed using A2058 ATF-3 OE and A2058 NEO melanoma cells.

Immunofluorescence studies: Immunofluorescence studies for Ki-67 will be performed for histological observation, as described above. The following antibodies will be used: rabbit anti-Ki-67 antibody (1:500, Abcam, Cat: ab15580, Cambridge, MA) and DyLight 488 goat anti-rabbit IgG(H+L) (1:2000, Invitrogen, Cat: A32731, Carlsbad, CA).

3.3 To perform RNA-sequencing (RNA-seq) of A2058 and C8161 ATF-3 OE and NEO cell lines to investigate potential molecular mechanisms responsible for ATF-3 mediated inhibition of metastatic melanoma cell growth, migration, and invasion in vitro.

Melanoma cell lines: C8161 and A2058 NEO and ATF-3 OE melanoma cell lines will be cultured as previously mentioned.

RNA-sequencing: RNA-sequencing of the melanoma cell lines A2058 and C8161 OE ATF-3 and NEO will be performed at the Beijing Genomics Institute (Shenzhen, China) by our collaborators. Three technical replicates of C8161 will be sequenced. Bowtie2 will be used to align the reads and RSEM to quantify gene expression.[67] Gene expression values for the three C8161 technical replicates will be averaged. RPKM values ≤ 1 will be assigned a value of 1 before $\log_2$ transformation. Genes expressed at < 5 RPKM in all samples (row sums) will be excluded from further analysis. To determine genes differentially expressed between A2058 and C8161 ATF-3 OE and NEO cell lines, $\log_2(0.2)$ fold change will be used as a cut-off. Genes meeting this cut-off will be used as input for Gene Ontology (GO) Analysis.

Western blotting: C8161 and A2058 NEO and ATF-3 OE melanoma cell lines will be evaluated for the following antibodies: rabbit anti-ERK (1:1000, Cell Signaling, cat:4695), rabbit anti-phospho-ERK (1:2000, Cell Signaling, cat:4370), rabbit anti-AKT (1:1000, Cell

Signaling, cat:4691), rabbit anti-phospho-AKT (1:2000, Cell Signaling, cat:4060), mouse anti-GAPDH (1:10000, Biolegend, cat:649203).

Statistical analysis: Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). Student's unpaired *t* test was used to compare two groups while one-way ANOVA was used for multiple pairwise comparisons. The data were summarized as mean ± standard error (SEM) for at least three different experiments and a p-value <0.05 was considered statistically significant.

Data Availability: The RNA-sequencing data have been deposited to the NCBI Gene Expression Omnibus under accession number GSE152460.

Results:

Expression of ATF-3 is significantly decreased in metastatic melanoma of human melanoma

Our initial evaluation of ATF-3 expression (protein immunoreactivity) in human melanocytic lesions which included normal melanocytes of skin, benign nevi and primary melanoma further suggests that ATF-3 may play a tumor suppressive role. Strong nuclear positivity was present in normal melanocytes and benign nevi, while primary and metastatic melanoma showed partial and complete loss, respectively **(Fig. 7A)**. Therefore, we expect our cohort of 72 patient cases as well as melanoma cell lines derived from primary malignant melanoma and metastatic melanoma to show similar findings, confirming that ATF-3 may play a potential tumor suppressive role in melanoma progression. Next, we validated these findings with a tissue microarray of 72 patient cases of melanocytic lesions, which included benign nevi (n=10), primary cutaneous melanomas (n=38) and metastatic melanomas (n=24) by IF **(Fig. 7B)**. Multiplex IF revealed that ATF-3 expression decreased significantly with progression from nevi to melanoma ($p<0.01$), with an apparent qualitative diminution in nuclear staining seen in metastatic melanoma when compared to primary melanomas ($p<0.05$) (**Fig. 7C**). Multiplexing IF studies show a significant decrease of nuclear positivity of ATF-3 in the melanoma component in comparison to adjacent dermal nevus in the specimens of melanoma arising in dermal nevus **(Fig 8).** Moreover, using the TCGA skin cutaneous melanoma database, a Kaplan-Meier analysis was performed on patients with ATF-3 mRNA level partitioned from the lowest 30% (n=135) and highest 30% (n=135) from a

total pool of 458 patients. We found that lower ATF-3 mRNA level group exhibited a worse survival over time than those in the higher ATF-3 mRNA level group (p=0.0337) **(Fig. 7D).**

ATF-3 expression decreased in metastatic melanoma cell lines

We compared ATF-3 expression by Western Blot in a panel of melanoma cell lines. While some melanoma cell lines (mel-juso and wm115) expressed high levels of ATF-3, the protein levels of ATF-3 declined from primary to metastatic melanoma, with markedly lower expression of ATF-3 in melanoma cell lines derived from human metastatic melanomas **(Fig. 9A).** These results were further confirmed by immunofluorescence studies of melanoma cell lines **(Fig. 9B).**

Over-expression of ATF-3 inhibits melanoma cell proliferation, migration and invasion

Given our preliminary results of ATF-3 as a potential tumor suppressor for melanoma progression, we expect melanoma cell lines that are retrovirally infected with ATF-3 overexpression vectors to inhibit melanoma cell proliferation, migration and invasion. As such, we investigated the effect of induced expression of ATF-3 in melanoma cells. We first overexpressed ATF-3 in melanoma cell lines derived from human metastatic melanomas, A2058 and C8161. Increased expression of ATF-3 was confirmed by immunofluorescence, Western Blotting and qRT-PCR analysis **(**not shown here**).** Immunofluorescence analysis revealed that the number of EdU incorporating cells was significantly reduced in melanoma cell lines that overexpressed ATF-3, consistent with

suppression of proliferation **(Fig.10A-D).** Cell Counting Kit-8 and colonogenic assays indicated that the proliferation and survival, respectively, of melanoma cells were significantly decreased in ATF-3 OE melanoma cell lines **(Fig. 10E-F)** Moreover, overexpression of ATF-3 in melanoma cell lines also attenuated cell migration (**Fig 10G-H)** and invasion **(Fig. 10I-J)**. Taken together, induced ATF-3 expression inhibited human metastatic melanoma cell growth, migration and invasion in vitro.

Over-expression ATF-3 significantly inhibits melanoma growth and tumor formation in a xenograft mouse model in vivo

Based on the preliminary results of ATF-3 potentially playing a tumor suppressor role in melanoma progression, we expect melanoma cell lines with ATF-3 OE to form small melanoma tumors and at a lower rate of tumor formation. Likewise, the tumors will exhibit a lower proliferative index when compared to their NEO melanoma counterparts.

In order to confirm our in vitro findings, next we investigated the effect of ATF-3 on melanoma growth in vivo. We deployed a xenograft model by subcutaneously injecting ATF-3 OE or control NEO C8161 melanoma cells into the left and right, anterior and posterior flanks of 6-week-old female nude mice. During 3 weeks of observation, the size, weight and rate of tumor growth of melanomas with ATF-3 overexpression were significantly less than that of the control group **(Fig. 11A-C).** These tumors were subjected to IF staining for proliferation marker Ki-67, which revealed a lower proliferative index in melanomas with ATF-3 overexpression **(Fig. 11G-H).** We repeated the same xenograft mouse model using ATF-3 OE or control

NEO A2058 melanoma cells. The results were similar to those seen using ATF-3 OE and control C8161 melanoma cells **(Fig. 11D-F, I-J).** Therefore, the data altogether supported our in vitro findings, suggesting ATF-3 as a tumor suppressor in metastatic melanomas.

Enhanced expression of ATF-3 inhibits melanoma tumor growth by increasing apoptosis and downregulating ERK and AKT phosphorylation

The development and progression of melanoma have been linked to genetic alterations in a variety of pathways.[13, 50, 68] To investigate potential molecular mechanisms responsible for ATF-3-mediated inhibition of metastatic melanoma cell growth, migration, and invasion in vitro, we performed RNA-sequencing (RNA-seq) of A2058 and C8161 ATF-3 OE and NEO cell lines. Principal component analysis (PCA) performed on all genes detected by RNA-seq showed separation specific to the individual cell lines on PC1 while PC2 and PC3 separated the ATF-3 OE and NEO cells **(Fig. 11A).** Prominent genes associated with apoptosis and ERK and AKT signaling pathways were identified using Homo sapiens Gene Summary from NCBI. In particular, genes found to be upregulated in both A2058 and C8161 ATF3-OE cells include, in addition to ATF-3, were the tumor suppressor *ING4*, and genes involved in promoting apoptosis and cell death such as *CDKN1A* and *FOS.* Gene Ontology (GO) analysis revealed pathways such as GO:0034976 'response to endoplasmic reticulum stress' and GO:0008625 'extrinsic apoptotic signaling pathway via death domain receptors' to

be upregulated in C8161 ATF3-OE cells compared to NEO cells (FDR-adjusted p-value = 4.79×10^{-8} and 5.4×10^{-4}, respectively) (**Fig. 12B**).

Downregulated genes, such as GDNF and TNFRSF6B involved with proliferation were also identified, suggesting a possible link to ERK and AKT signaling pathways. Western-Blotting analysis confirmed that levels of phosphorylated ERK and AKT were significantly downregulated in melanoma cell lines in which ATF-3 was overexpression (**Fig. 12C-D**), suggesting that ATF-3 plays a role in negatively regulating ERK and AKT pathways in melanoma cells.

Discussion:

In comparison to the potential of ATF-3 to mediate cellular proliferation during wound repair, ATF-3 expression was inhibitory with respect to cellular proliferation that characterizes melanomagenesis. Previous studies have shown that the migration and proliferation rate of bladder cancer cells is diminished in metastatic cells with ATF-3 overexpression, suggesting that ATF-3 suppresses metastasis through the upregulation of actin filament severing protein gelsolin (GSN)[69]. Similar to these data, we found ATF-3 expression to be significantly decreased with melanoma progression **(Fig. 7A-B, Fig. 8).** It is worth noting, though, that several other reports have found elevated ATF-3 expression in melanoma and lung cancer[13, 70]. Since ATF-3 induction has been associated with oncogenic stress[14], early stages of tumorigenesis may induce high levels of ATF-3 expression, which we also observed in this study **(Fig. 9).** Nevertheless, the contradictory role of ATF-3 in various malignancies, and even among various melanoma cell lines, as observed in our study, may relate to intrinsic heterogeneity among various melanoma primary sources. However, the ability to stratify melanoma patients based on ATF-3 expression based on survival outcomes is consistent with an important role for the ATF-3 pathway in generalized mediation of tumor virulence **(Fig. 7D).**

ATF-3 plays a dynamic role in DNA damage repair and its regulation in activating or repressing cell-cycle regulators in various types of tissues. Previous studies have also shown that elevated ATF-3 expression either promotes or inhibits tumorigenesis[16, 70]. In melanoma, one study shows that TRPV1-induced calcineurin activation regulates p53 via ATF-3, where elevated ATF-3 expression in melanoma cell lines

transcriptionally represses apoptotic-related genes[13]. However, the effect of ATF-3 on apoptosis in melanoma cells in this particular study involved treatment with TRPV1, capsaicin, and a calcineurin inhibitor, creating a completely different microenvironment than was the case in our study. It is well known that tumor microenvironments in melanoma are heterogenous and may be associated with distinctive distributions of immune cells which can potentially affect specific biological processes, e.g. apoptotic signaling pathways[71, 72]. Moreover, another study further demonstrates that ATF-3 suppresses human melanoma growth by inducing the expression of ATF-3 in human dermal fibroblasts[63].

An intriguing finding from this study is the interrelationship among ATF-3, ERK and AKT signaling pathways and p53-dependent apoptosis. Our experiments involving melanoma cell lines with retrovirally-induced ATF-3 overexpression revealed that ATF-3 suppressed the proliferation, migration, and invasion of melanoma cells **(Fig. 10A-J)** presumably by upregulating apoptotic-related genes in part by downregulating ERK and AKT pathways **(Fig. 13).** It is well known that the risk of many forms of cutaneous melanoma is increased with excessive UV light exposure. Studies have shown that UV-related DNA damage leads to a prompt response of ATF-3-induction which triggers p53-dependent apoptosis[27, 29]. In response to UV radiation, ATF-3 can either protect p53 wild-type cells by enhancing DNA repair or promote programmed cell death of p53-defective cells by binding to histone acetyltransferase,Tip60[29]. Our RNA seq analysis of C8161 and A2058 ATF-3 OE melanoma cells exhibited an upregulation of genes, such as *ING4* and *CDKN1A,* which are involved in regulating p53-mediated apoptosis **(Fig.**

13A-B). ING4 is a member of the inhibitor of growth (ING) family and acts as a tumor suppressor protein by increasing p53 acetylation and promoting apoptosis[73]. An elevated level of p53 expression, in turn, leads to an increased expression in cyclin-dependent kinase inhibitor p21 (CDKN1A), which negatively regulates p53 transcription[74]. Our study shows that ATF-3 plays inhibits melanoma tumor progression by enhancing apoptosis. Whether similar mechanisms are at play for oral mucosal melanoma which is not elicited by UV-related pathways now requires further study.

Interestingly, MAPK signaling pathway is also involved with ATF-3 activation in response to genotoxic stress[27]. Mitogen-activated protein kinase (MAPK)/ERK and AKT pathways have been extensively studied and heavily targeted by drugs to treat metastatic melanoma. The Raf/MEK/ERK signaling pathway mediates cell proliferation, cell-cycle arrest, terminal differentiation and apoptosis[75]. Activating mutations within the ERK or AKT signaling pathway can lead to progression of melanoma and affect its cell proliferation, growth, and survival[76]. A recent study has further shown that deficiency in ATF-3 expression leads to a dramatic increase in phosphorylated AKT expression levels, indicating that ATF-3 may act as a tumor suppressor within the AKT signaling pathway in prostate cancer[57]. Similarly, our study demonstrates that the tumor suppressive role of ATF-3 is rescued retrovirally by deactivating AKT and ERK pathways **(Fig 13C-D).** However, a study involving nerve damage has shown that the overexpression of ATF-3 does not affect ERK activity while the combination of ATF-3 overexpression and activation of C-Jun N-Terminal Kinase (JNK) upregulates expression levels of the heat shock protein 27 (Hsp27), which plays a direct or indirect

role in activating AKT pathways[77]. These studies reflect the multifaceted role of ATF-3 in various signaling pathways, suggesting that more work is needed to truly understand the precise mechanistic role of ATF-3 in ERK and AKT pathways. The ability to validate our in vitro findings in a xenograft mouse model further suggests that ATF-3 acts as a tumor suppressor in melanoma in vivo and paves the way for translational approaches focused on manipulation of ATF-3/ERK/AKT signaling pathways as a novel therapeutic means of controlling human melanomagenesis **(Fig.12).**

One significant finding of our study is that ATF-3 may now serve as a novel biomarker for prediction of melanoma virulence. In addition, the emergence of targeted therapy and immune check point inhibitors to treat metastatic melanoma suggests that ATF-3 may also be deployed as a biomarker to assist in calibrating treatment response in the setting of personalized medicine. Indeed, studies already have shown that the ATF-3 screening may be used to determine the efficacy of PD-1 therapy in melanoma.[78] Thus, the potential role of ATF-3 in tumor response to immune checkpoint inhibitors needs to be further explored.

**The research in regard to the role of ATF-3 in melanomagenesis has been accepted and currently in press by Laboratory Investigation.*

Conclusion:

In conclusion, our study clearly depicts a juxtaposition of ATF-3 and its functional role in physiological versus pathological proliferation. While ATF-3 expression is upregulated in oral and cutaneous wound healing, its overexpression is associated with inhibitory effects with respect to cellular proliferation that characterizes melanomagenesis and can be potentially used as a prognostic biomarker for melanoma. The ability to deploy models that permit examination of the potentially divergent role of ATF-3 in the settings of physiologic healing/regenerative responses and cancer, both involving stem cell activation/proliferation, holds future promise for a more complete understanding of the complex role of this transcription factor in human health and disease, as well as how its therapeutic manipulation may benefit patients with deficient wound healing and skin/mucosal cancer.

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Tables

Table 1: Different human tissues provided for immunofluorescence study:

Location	Control	Type of ulcer	Average Time between biopsy and resection (in Days)
Tongue	Normal oral mucosa	Healing wound bed from resection	30
Epidermis	Normal cutaneous epithelium	Healing wound bed from resection	37

Figure Legends

Figure 4. ATF3 expression increased but CK15 expression was muted in cutaneous and mucosal wound edges.

Representative IF images of double staining of ATF-3 (red) and CK15 (green) in human cutaneous and oral wound edges. Strong nuclear and cytoplasmic positivity of ATF-3 (red) present in epithelial cells (*200x*), while rete ridges showed muted CK15 expression (*200x*) in the representative images.

Figure 5. Normal cutaneous and oral mucosa exhibited increased CK15 expression in rete ridges and muted ATF-3 expression.

Representative IF images of double staining of ATF-3 (red) and CK15 (green) in human normal cutaneous and oral mucosa. Muted nuclear and cytoplasmic positivity of ATF-3 (red) present in epithelial cells (*400x*), while rete ridges expressed cytoplasmic positivity for CK15 (*400x*) in the representative images.

Figure 7. ATF3 expression decreased in melanoma.

(A and B) Representative IF images of double staining of ATF-3 (red) and MART-1 (green) in human melanocytic specimens and tissue microarray, respectively. (A) Strong nuclear positivity of ATF-3 (red) present in normal melanocytes (*400x*) and benign nevi (*200x*), while melanoma showed marked decrease (*200x*) in the representative images. (B-C) Representative IF images of tissue microarray of 72 patient cases of melanocytic

lesions, which included benign nevi (n=10), primary cutaneous melanomas (n=38) and metastatic melanomas (n=24) by IF are shown (*100x*) and revealed significant decrease of ATF-3 nuclear positivity (red) with progression from nevi to primary melanoma ($p<0.01$), with an apparent qualitative diminution in nuclear staining seen in metastatic melanoma when compared to primary melanomas ($p<0.05$). (D) Kaplan-Meier survival curves on the ATF3 level in the TCGA skin cutaneous melanoma database. Data are presented as the mean ± standard deviation, *$P < 0.05$; **$P < 0.01$.

Figure 8. Significant decrease of ATF-3 immunoreactivity in the melanoma component in comparison to the adjacent dermal nevus

Representative IF images of double staining of ATF-3 (red) and MART-1 (green) in human melanocytic specimens of melanoma arising from nevi are shown. Strong nuclear positivity of ATF-3 (red) present in the nevus component in comparison to the adjacent melanoma.

Figure 9. ATF-3 expression decreased in melanoma cell lines

(A) Immunoblotting analysis of ATF-3 expression in melanoma cell lines. The column summarized the relative ATF-3 protein expression in melanoma cell lines normalized to GAPDH. (B) Representative images of ATF-3 immunofluorescence double staining in melanoma cell lines. Scale bar represent 20μm. Data are presented as mean + standard deviation, *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Figure 10. ATF3 over-expression inhibited melanoma cell growth, migration and invasion.

(A), (B) and (C) EdU incorporation assay in A2058 and C8161 ATF3 overexpression cell lines. The nuclei of EdU-positive cells were labeled red and cell nuclei were labeled blue with DAPI. The column summarized the staining scores of EdU-positive cells. Scale bar represent 100μm. (D) CCK-8 assay in A2058 and C8161 ATF3 overexpression cell lines. (E) and (F) Cell colony assay in A2058 and C8161 ATF3 overexpression cell lines. The column summarized the colonies of the cells. (G) and (H) Wound healing assay in A2058 and C8161 ATF3 overexpression cell lines. The column summarized the healing percentages compared with NEO control group. Scale bar represent 100μm. (I) and (J) Transwell assay for A2058 and C8161 ATF3 overexpression cell lines invasion tests. The column summarized the invaded cells. Scale bar represent 100μm. Data are presented as the mean ± standard deviation, $^{**}P < 0.01$.

Figure 11. ATF3 over-expression inhibit melanoma tumor formation and growth in vivo

(A) The representative of images of mice at 3 wk. after subcutaneously injection of either C8161 NEO and C8161 ATF3 OE cells, and each tumor was collected shown in the lower panel. (B) The percentage of C8161 NEO and C8161ATF3 OE tumor formation and (C) average weight of each tumor was quantified. (D) Representative images of mice at 3 wk. after subcutaneously injection of either A2058 NEO and A2058 ATF3 OE cells, and each tumor was collected shown in the lower panel. (E) The percentage of A2058 NEO and

A2058 OE tumor formation and (F) average weight of each tumor was quantified. (G) Representative images of Ki67 immunofluorescence staining in C8161 NEO and C8161 ATF3 OE melanoma tumors and (H) summarized staining score of Ki67 expression. (I) Representative images of Ki67 immunofluorescence staining in A2058 NEO and A2058 ATF3 OE melanoma tumors and (J) summarized staining score of Ki67 expression. Data are presented as the mean ± standard deviation, *P<0.05; **P < 0.01.

Figure 12. ATF3 over-expression inhibits melanoma cell growth, migration and invasion by down regulating ERK and AKT phosphorylation.

(A) PCA of all genes detected by RNA-seq of A2058 and C8161 ATF-3 overexpressing (OE) and neomycin control (NEO) cell lines. (*n*=2). (B) Dot plot of enriched GO terms. The 25 GO processes with the largest gene ratios are shown in order of gene ratio. Dot size represents the number of genes with increased expression in C8161 ATF3 OE cells compared to NEO associated with that GO term. Dot color indicates the FDR-adjusted p-value. (C) and (D) Total and phosphorylation level of ERK, and AKT changes in A2058 and C8161 NEO control and ATF3 overexpressed cell lines. The column summarized the protein phosphorylation level in melanoma cells, which normalized to total protein respectively. Data are presented as the mean ± standard deviation, **P < 0.01.

Figures

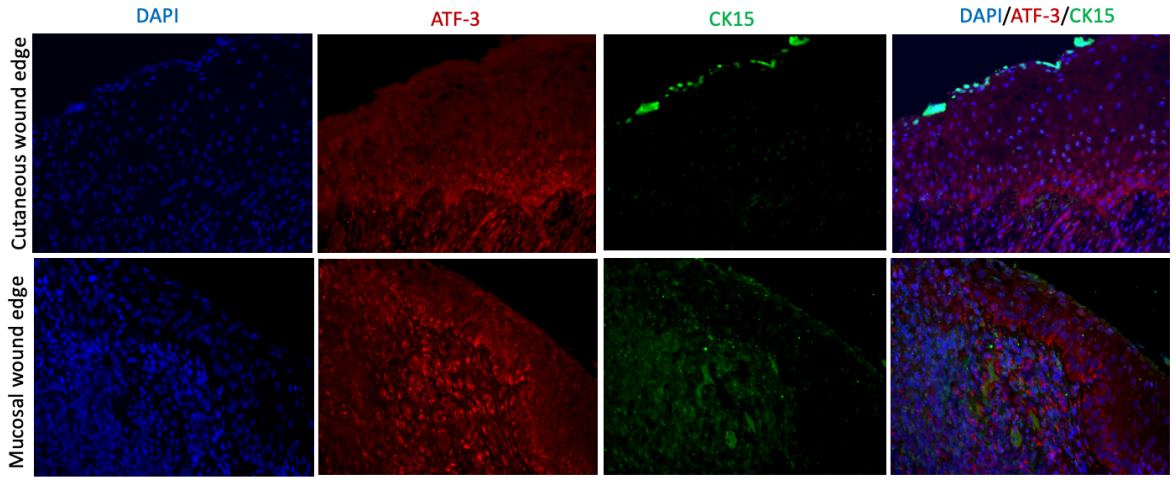
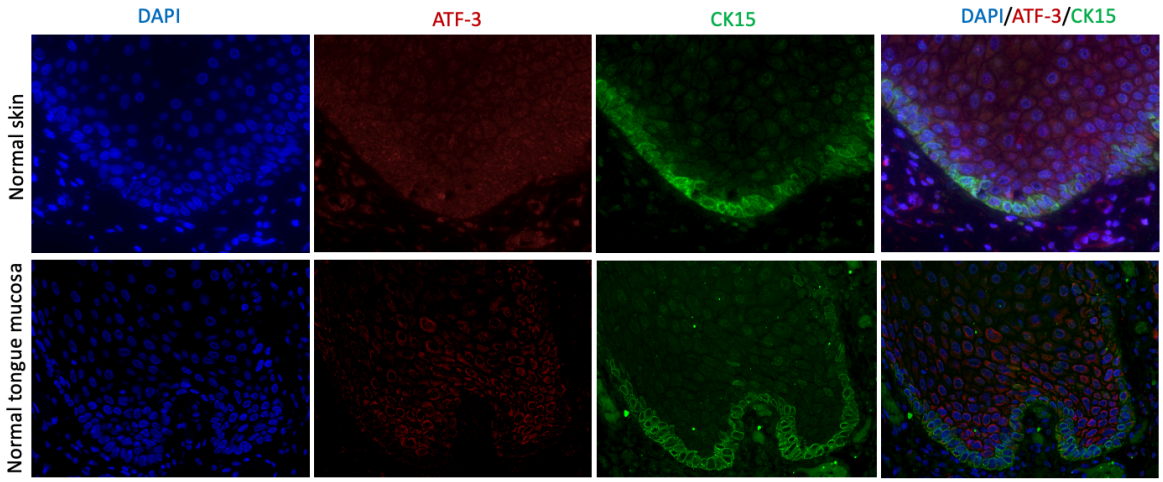


Figure 4. ATF3 expression increased but CK15 expression was muted in cutaneous and mucosal wound edges.



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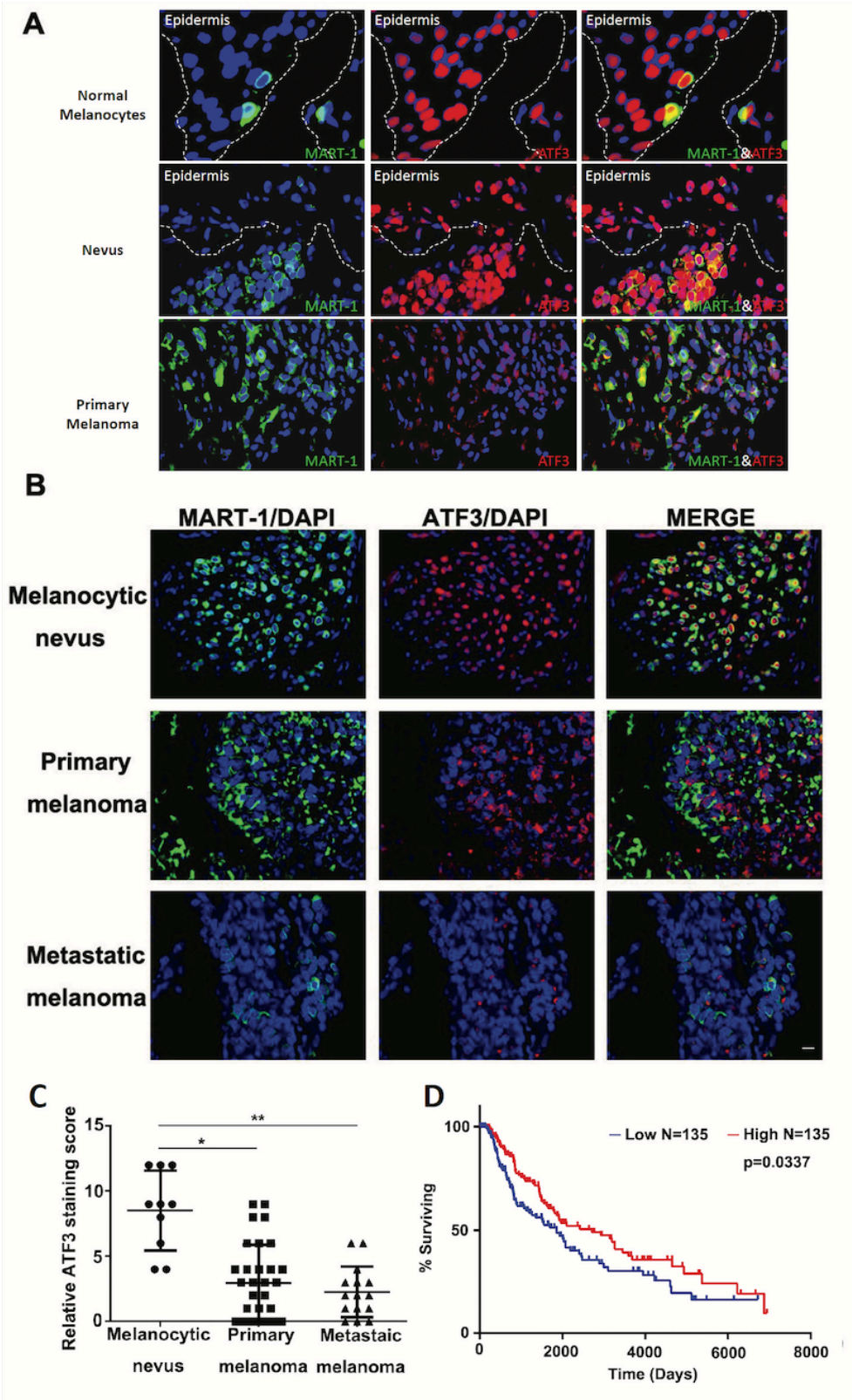

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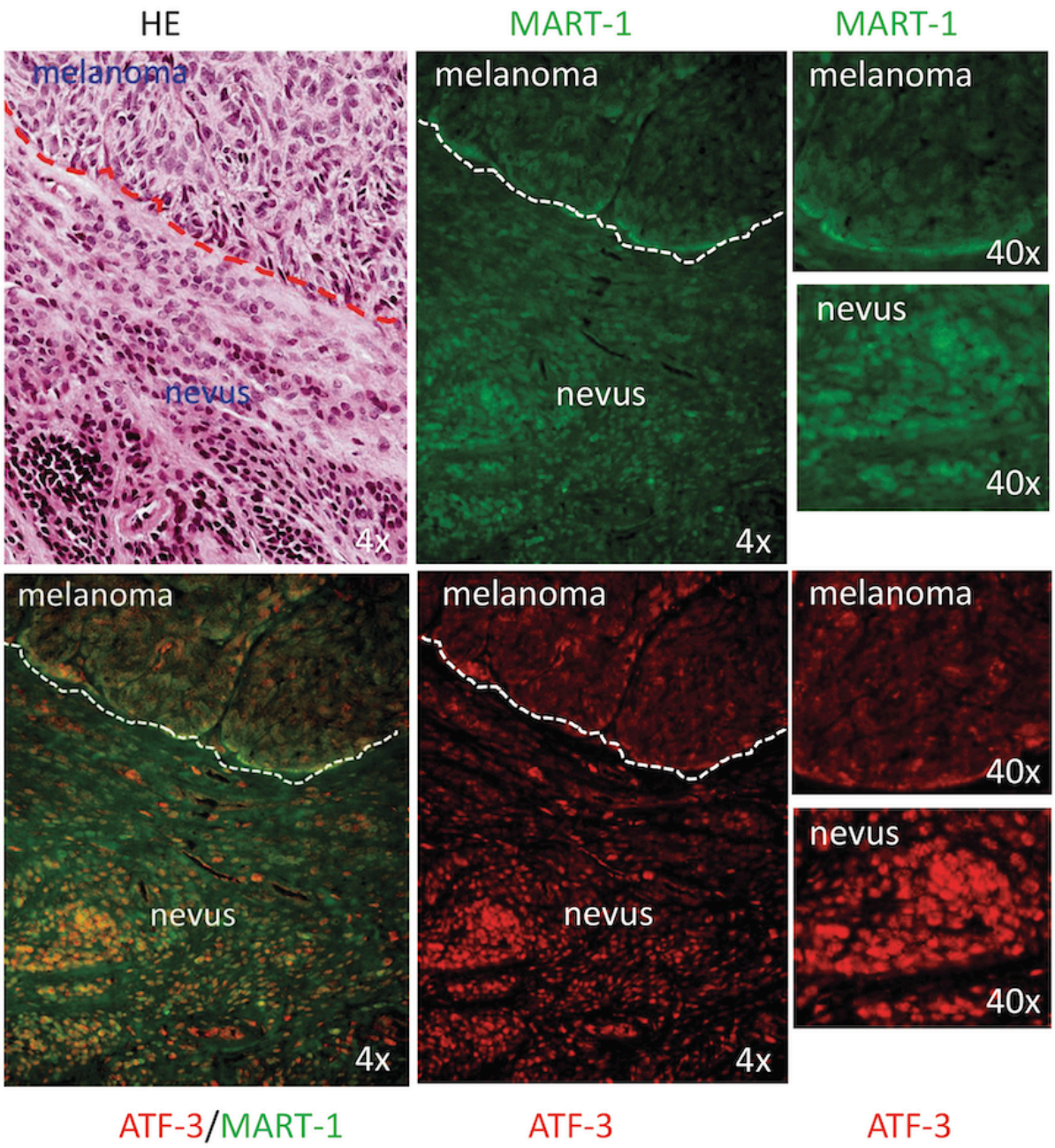

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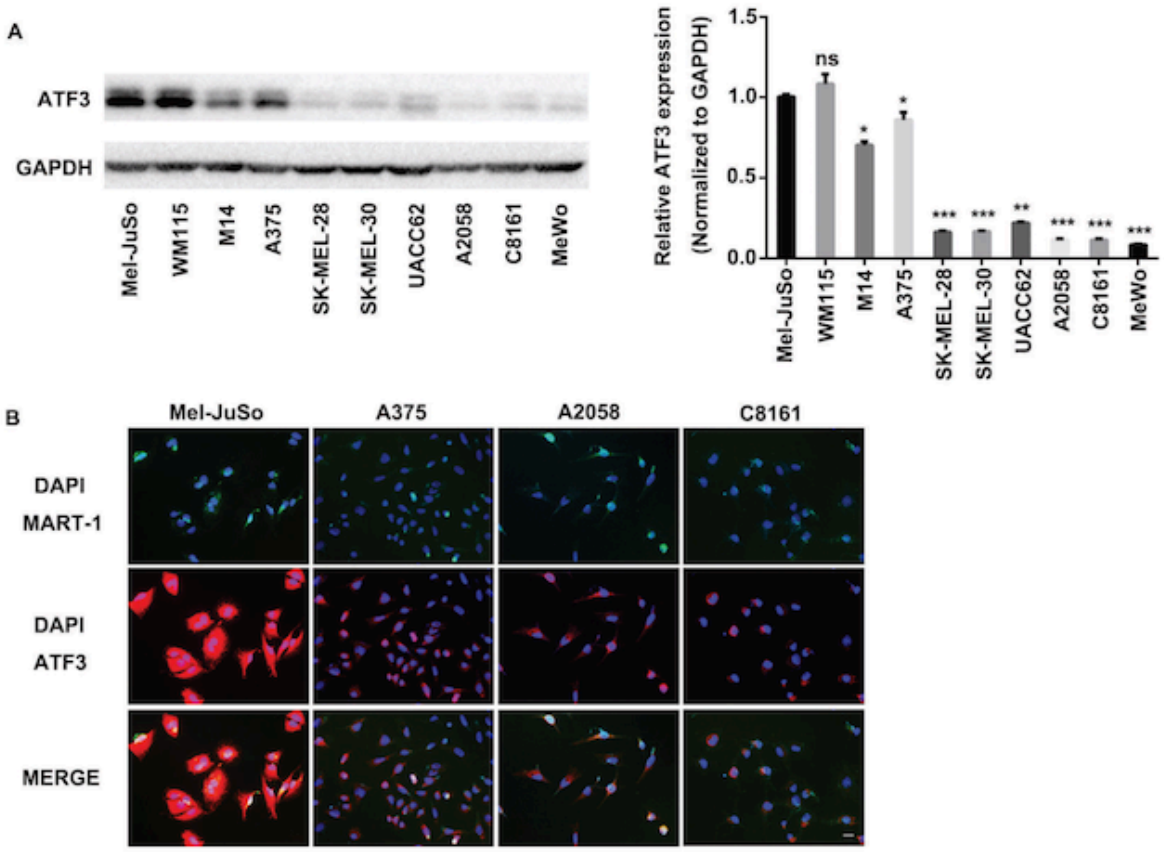
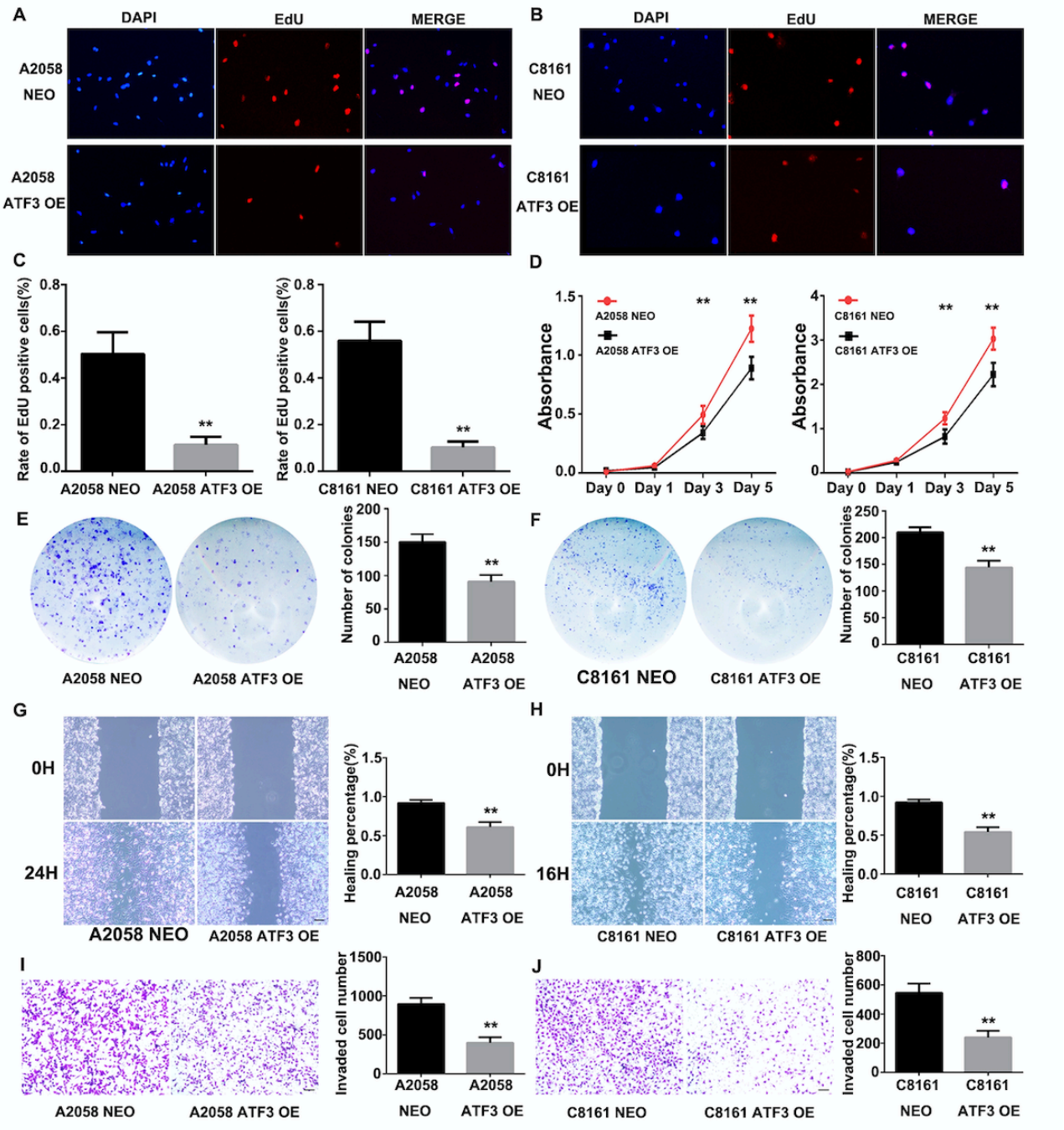


Figure 9. ATF-3 expression decreased in melanoma cell lines



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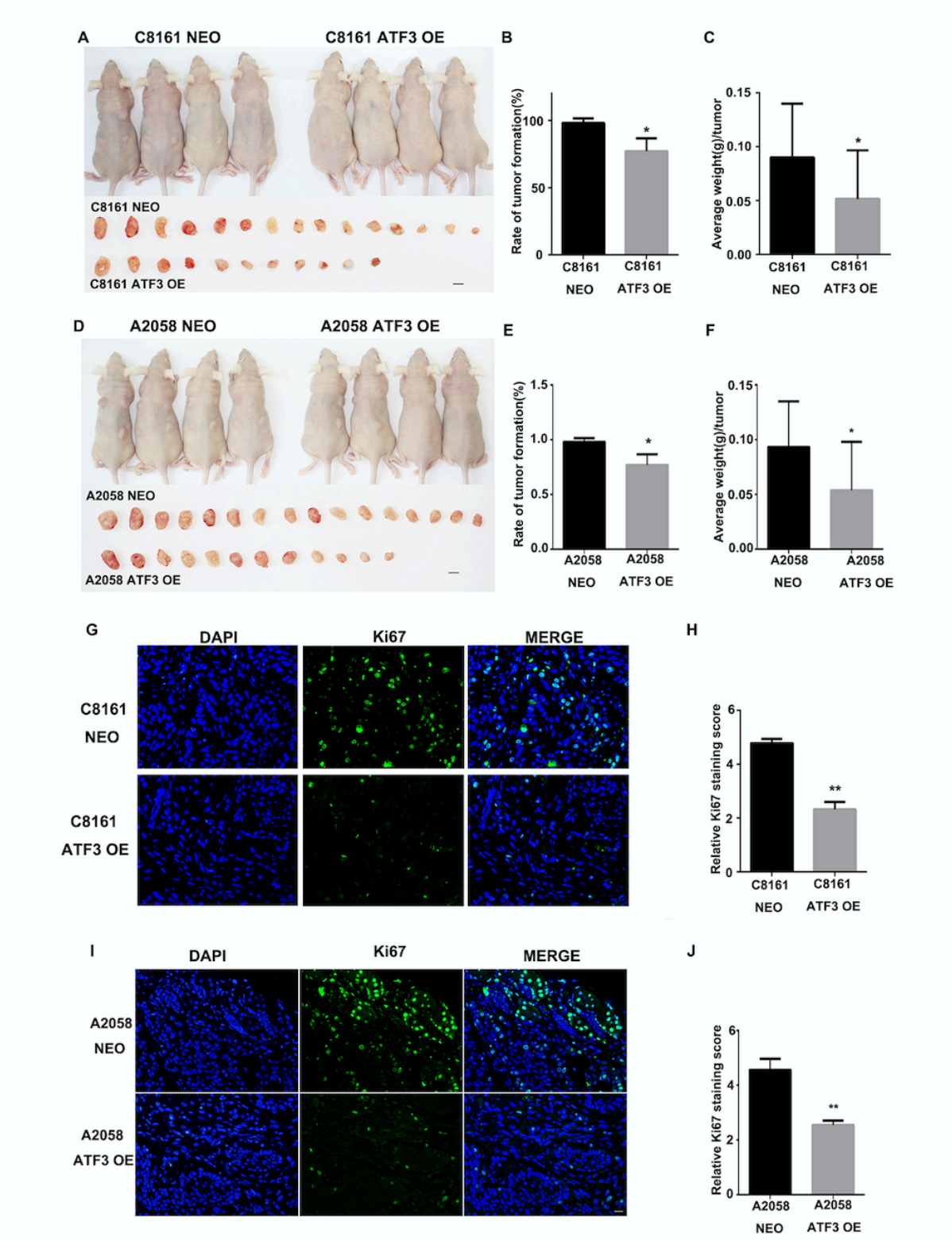

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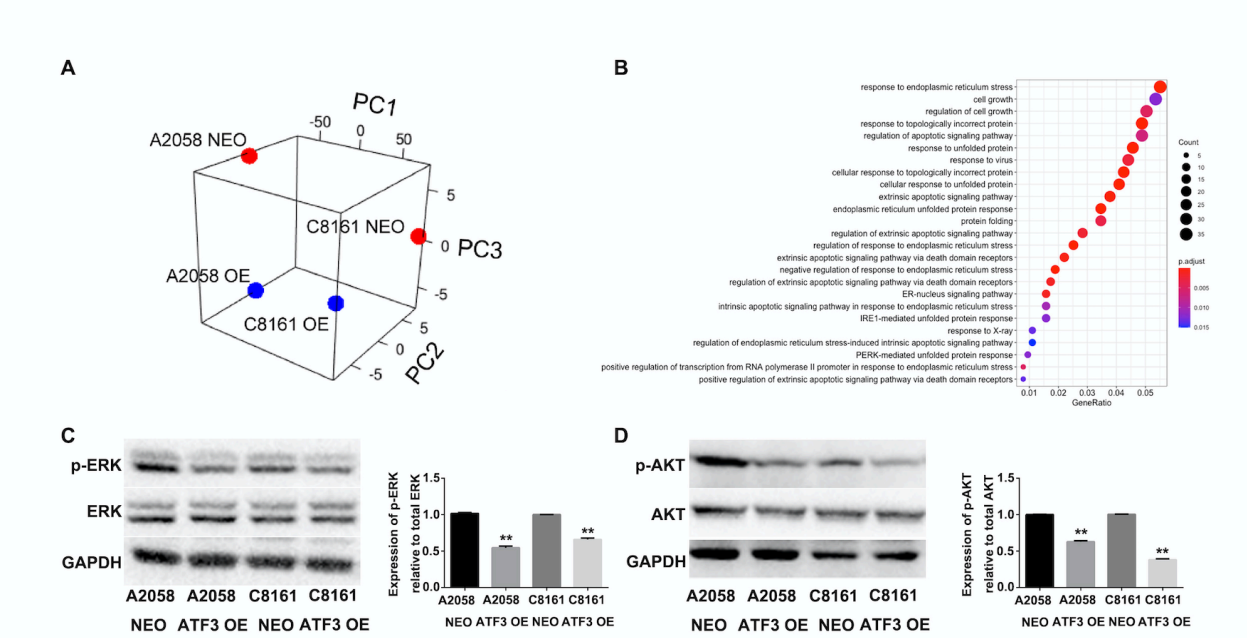

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