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# The role of Bcl-2 modifying factor (Bmf) in airway epithelial cell death

Amelia H.T. Unione

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Amelia Unione

Candidate

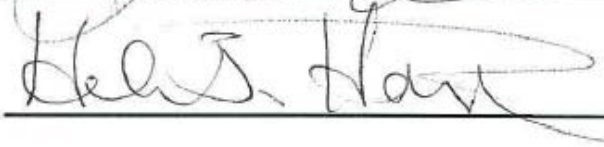
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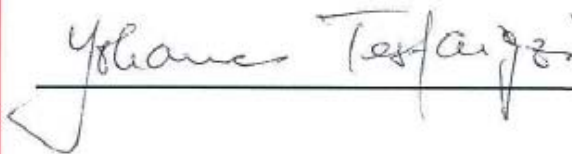
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The Role of Bcl-2 Modifying Factor (BMF) in Airway  
Epithelial Cell Death

by

Amelia H.T. Unione

B.S. Health Science Studies, Boise State University, 2001

DISSERTATION

Submitted in Partial Fulfillment of the  
Requirements for the Degree of

Doctor of Philosophy  
Biomedical Sciences

The University of New Mexico  
Albuquerque, New Mexico

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## **Cell Death**

**By**

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### **ABSTRACT**

One of the components that obstruct the airway in asthma is the sudden and increased secretion of mucus from metaplastic mucous cells in small airways. Because previous studies have shown that the resolution of mucous cell metaplasia (MCM) during prolonged exposure of mice to allergen is mediated by IFN $\gamma$  and the Bcl-2 family of proteins, we investigated the regulation and the role of a pro-apoptotic BH3 domain-only protein, Bmf, in airway epithelial cells (AECs) treated with IFN $\gamma$ . We hypothesize that Bmf is crucial for IFN $\gamma$ -induced cell death. Our findings show that IFN $\gamma$  suppressed Bmf expression in a p53-dependent manner. IFN $\gamma$  treatment caused nuclear accumulation, increased association of p53 with HDAC1, the reduction of acetylated p53, and the interaction of p53 with the Bmf promoter. This reduction was primarily of the higher molecular weight Bmf that was found to increase cell colony formation *in vitro*. Bmf

was important for the resolution of MCM during prolonged exposure to allergen because the mucous cell numbers did not decline in *bmf*<sup>-/-</sup> mice compared to wild-type controls during prolonged exposure to allergen. In addition, *bmf*<sup>-/-</sup> mouse airway epithelial cells (MAECs) were resistant to IFN $\gamma$ -induced cell death. Previous studies in cancer cell lines have shown that Bmf mediates cell death in response to histone deacetylase inhibitors (HDACis). We found that HDACis increased expression of Bmf in AECs through the displacement of HDAC1 and the acetylation of histones 3 and 4 associated with the Bmf promoter. Bmf-deficient AECs were resistant to HDACi-induced cell death, while expressing Bmf in the *bmf*<sup>-/-</sup> MAECs restored this cell death process demonstrating that Bmf is a critical mediator of this cell death process. In conclusion, these findings show that Bmf mediates IFN $\gamma$ - and HDACi-induced cell death in AECs and implies that HDACis may be useful for treatment of cancers and for reducing AEC hyperplasia and MCM, thereby reducing mucous hypersecretions and airway obstruction in chronic diseases.

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**Figure Appendix F Lymph node cell numbers in allergen exposed *bmf*<sup>-/-</sup> and *bmf*<sup>+/+</sup> mice. 102**

## List Of Abbreviations

Ad-Bmf	Adenoviral overexpression system for Bmf
AEC	Airway epithelial cell
Bad	Bcl-2 associated death promotor
Bak	Bcl-2 agonist killer 1
Bax	Bcl-2 associated x protein
Bcl-2	B-cell lymphoma
Bcl-XL	B-cell-2 related gene long isoform
BH	Bcl-2 homology
Bid	BH3-interacting-domain death agonist
Bik	Bcl-2 interacting killer
Bim	Bcl-2 interacting modulator of apoptosis
Bmf	Bcl-2 modifying factor
Caspase	Cysteine aspartyl-specific proteases
CCSP	Clara cell secretory protein
ChIP	Chromatin immunoprecipitation
DLC	Dynein light chain
DMEM	Dulbecco's modified eagle medium
DNA	DeoxyRibonucleic acid
DRB	5,6-dichloro-1- $\beta$ -D-ribobenzimidazole
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
HAEC	Human airway epithelial cell
HAT	Histone acetyltransferase
HDAC	Histone deactylase
HDAC2	Histone deacetylase 2
HDACis	Histone deactylase inhibitors
Hrk	Harakari
IFN $\gamma$	Interferon-gamma
LCM	Laser capture microdissection
MAEC	Mouse airway epithelial cell
Mcl-1	Myeloid cell leukemia sequence-1
MCM	Mucous cell metaplasia
mRNA	Messenger ribonucleic acid
OMM	Outer mitochondrial membrane
PI	Propidium Iodide
PRD	Proline rich domain
Puma	p53-upregulated modulator of apoptosis
RNA	Ribonucleic acid
RIPA	Radioimmunoprecipitation assay buffer
SAHA	Suberoylanilidehydroxamic acid
TNF	Tumor necrosis factor
UV	Ultraviolet

# **Chapter 1**

## **Introduction**

## 1.1 Mechanisms involved in apoptotic cell death

The Nomenclature Committee on Cell Death has described four modalities of cell death: apoptosis, necrosis, autophagy, and cornification <sup>1</sup>. They are all forms of programmed cell death because they are regulated at the genetic and cellular level. Another important modality is senescence, a state in which growth and proliferation are arrested. In vivo studies report that senescence may be important for tumor suppression <sup>2</sup>. Apoptosis is a process by which tissues remove unwanted or damaged cells. This type of cell death is an important part of healthy tissue homeostasis and mammalian development, but when dysregulated can result in disease, such as cancer and autoimmunity <sup>3</sup>. Two well-characterized pathways mediate apoptosis: the extrinsic and the intrinsic. In either case, caspase proteases are ultimately responsible for killing the cell. Apoptosis manifests as cellular blebbing and shrinkage, DNA fragmentation, or changes in the plasma membrane <sup>4</sup>.

Extrinsic cell death otherwise known as the death receptor pathway is initiated when specific ligands bind to cell death receptors located on the cell surface. These receptors belong to the tumor necrosis factor (TNF) family and include Fas receptor and TNF receptor 1 (TNFR-1). This interaction activates initiator caspase-8 and -10, which in turn activate the appropriate effector caspases <sup>5</sup>.

The intrinsic cell death pathway or mitochondrial death pathway is caused by various cellular stressors, such as DNA damage, cytoskeletal damage, loss of adhesion, growth factor withdrawal, macromolecular synthesis inhibition, and others <sup>6</sup>. Complex interactions between the anti-apoptotic and the pro-apoptotic members of the Bcl-2 family of proteins regulate the integrity of the outer mitochondrial membrane (OMM).

The permeabilization of the mitochondrial membrane is the initial step in this process, allowing for the release of several pro-apoptotic factors such as cytochrome c, second mitochondria-derived activator of caspases (Smac/Diablo), apoptosis-inducing factor (AIF), endonuclease G, and HtrA2/omi<sup>7</sup>. Cytochrome c oligomerizes with adaptor protein (Apaf-1), and forms a scaffold called the apoptosome complex. The apoptosome binds and activates pro-caspase-9, activating executioner caspases-3 and -7, and killing the cell<sup>4, 7, 8</sup>.

The endoplasmic reticulum (ER) can induce apoptosis as a way to protect from stresses, such as unfolded protein response (UPR) characterized by the accumulation and aggregation of proteins in the ER lumen or inhibition of protein transport from the ER to the Golgi. An early part of the ER-induced apoptotic response is the release of calcium into the cytosol. Although initiated by the ER, this type of apoptosis is dependent on the mitochondria and members of the Bcl-2 family of proteins. Health conditions such as ischemia, viral infections, and neurodegenerative diseases are associated with a reduction in cell number caused by ER stress-induced apoptosis<sup>9</sup>.

## **1.2 Regulation of apoptosis by the Bcl-2 family**

The Bcl-2 family of proteins consists of both anti-apoptotic and pro-apoptotic members. These proteins share conserved  $\alpha$ -helical regions called Bcl-2 homology (BH) regions<sup>10</sup>. Structurally, these proteins are categorized as folded globular proteins and intrinsically unstructured proteins<sup>4</sup>.

The first subgroup consists of five anti-apoptotic proteins with all four BH regions, which are B-cell lymphoma 2 (Bcl-2), Bcl-2-related gene long isoform (Bcl-x<sub>L</sub>), B-cell-2-like 2 (Bcl-w), myeloid cell leukemia sequence 1 (Mcl-1), and Bcl-2-related

gene A1 (A1). All of these anti-apoptotic Bcl-2 proteins are anchored in the OMM or in the ER membrane, or are found in the cytoplasm. These proteins protect from cell death by inhibiting the function of pro-apoptotic proteins<sup>1,4</sup>.

Bcl-2 antagonist killer 1 (Bak) and Bcl-2-associated x protein (Bax) are pro-apoptotic and referred to as effector proteins. They possess homology domains BH1-BH3, although a BH4 motif has recently been reported<sup>11</sup>. Upon activation of Bax and Bak, they homo-oligomerize and insert into the pores of the OMM causing mitochondrial membrane permeabilization, the essential initiation step for apoptosis. Bax and Bak are found on both the mitochondrial and ER membranes<sup>1,4</sup> and are central in the cell death pathway because deletion of both genes renders cells resistant to death induced by ER stressors and DNA damage<sup>12</sup>.

Members of the third subgroup of the Bcl-2 family are also pro-apoptotic but contain only one homology region and are therefore called the BH3 domain-only proteins. Eleven BH3 domain-only proteins have been identified<sup>7</sup>, some of which include BH3-interacting-domain death agonist (Bid), Bcl-2-associated death promoter (Bad), Noxa or PMAIp1, Bcl-2 interacting killer (Bik), Bcl-2-modifying factor (Bmf), Harakari (Hrk), p53-upregulated modulator of apoptosis (Puma), and Bcl-2 interacting mediator of cell death (Bim)<sup>1,4</sup>. The BH3-only proteins are regarded as sentinels, responding to stress signals and developmental cues<sup>7</sup>. A conserved leucine residue within the BH3 domain, when mutated, renders these proteins unable to interact with the anti-apoptotic Bcl-2 family members<sup>13,14</sup>.

Two models have been proposed to categorize the function of the Bcl-2 family members: the indirect activation model and the direct activation model<sup>7</sup>. Indirect



activation implies that an anti-apoptotic protein sequesters Bax and Bak to block their pro-apoptotic effects. An apoptotic stimulus activates a BH3 domain-only protein, which binds to the hydrophobic cleft of the anti-apoptotic protein, releasing Bax and Bak to kill the cell<sup>1</sup>. Direct activation implies that Bax and Bak are activated directly by binding to BH3 domain-only proteins. Within this model, the BH3-only proteins are subdivided into two groups: direct activators and sensitizers/de-repressors. Bim and Bid are considered direct activators, while Bad, Bik, Bmf, Hrk, Noxa, and Puma are sensitizers/de-repressors<sup>1,4</sup>. These sensitizers bind and inactivate an anti-apoptotic protein and thereby unleash the pro-apoptotic potential of a direct activator. Similarly, according to the derepression theory, the activator remains sequestered or bound by the anti-apoptotic protein and is released only after an apoptotic stimuli causes the sensitizer protein to compete for the binding site that the activator occupies, freeing the activator to bind and activate Bak and Bax, leading to cell death<sup>1,4</sup>.

In addition to the apoptotic signal causing specific BH3 only-domain proteins to be activated, cell type also dictates the function of the BH3 domain-only protein. For instance, the BH3 domain-only Bik is not important for cell death in hematopoietic cells<sup>15</sup>; however, it is critical for IFN $\gamma$ -induced cell death in AECs<sup>16</sup>.

### **1.3 The BH3 domain-only protein Bmf**

Bmf is an important mediator for a diverse group of cell death-causing stimuli including detachment- (anoikis), arsenic trioxide-, TNF $\alpha$ -, TGF $\beta$ -, and HDACi-induced cell death<sup>17-21</sup>. Bmf sensitizes mammary epithelial cells to anoikis, but cannot initiate cell death on its own<sup>20</sup>. The gene for Bmf is located on chromosome 15q14, which is also the site of a tumor suppressor gene thought to be involved in metastasis<sup>22</sup>. Because

anoikis may inhibit metastatic tumor growth<sup>23</sup>, Bmf is believed to be involved in metastatic cancers. Studies demonstrating that Bmf may function as a mammary epithelial tumor suppressor include findings that show the down-regulation of Bmf suppresses anoikis and luminal cell death, promoting anchorage independent growth. Bmf is also said to be a target for a proliferative oncogene, HPV16E7<sup>10</sup>.

Bmf possesses a conserved sequence motif that binds to the dynein light chain (DLC), a part of the myosin V motor complex in the cytoskeleton. In response to particular stresses, Bmf detaches and translocates to the mitochondria (Figure 1-1) where it interacts with Bcl-2<sup>10</sup>. Cytosolic translocation of Bmf is also observed by MEK inhibition that induces cell death in melanoma cells. However, in MEK-inhibition-resistant melanoma cells Bmf remains tethered to the cytoskeleton, and mutation of the region that tethers Bmf to the cytoskeleton increases the killing potential of Bmf<sup>24</sup>. *In vitro*, biochemical assays in HEK293 cells demonstrate that apoptotic activity of Bmf is mediated by JNK phosphorylation at Ser<sup>74</sup> for Bmf<sup>25,26</sup>. Although, *in vivo* experiments in mice with knock-in mutations disrupting this phosphorylation site demonstrate that phosphorylation is not essential for normal Bmf activity<sup>26</sup>.

Within the hematopoietic compartment Bmf is important for B cell homeostasis. Bmf-deficient mice exhibit B-cell hyperplasia as well as an increased sensitivity to  $\gamma$ -radiation-induced B-cell lymphoma<sup>27</sup>. In addition, Bmf prohibits C-Myc- driven B cell lymphomagenesis, and the loss of Bmf in transgenic E $\mu$ -myc/*bmf*<sup>-/-</sup> mice results in tumor formation because of the accumulation of preneoplastic pre-B and immature IgM<sup>+</sup> B cells in the bone marrow of these mice<sup>21</sup>.

We and others have observed that Bmf is regulated at the transcriptional level in various cell types<sup>19, 20, 27</sup>, but expression is also regulated post-transcriptionally through microRNAs and post-translational modifications<sup>10, 28, 29</sup>. *In vitro* experiments in B-chronic lymphocytic leukemia cells demonstrated that the Bmf gene is alternatively spliced and produces three isoforms: Bmf, Bmf II, and Bmf III. Bmf is the only isoform possessing a BH3 domain, while all three have a DLC- binding domain<sup>18</sup>.

In cancer cell lines, transcription of Bmf is regulated by the chromatin remodeling enzymes histone deacetylases (HDACs) and histone acetyltransferases (HATs)<sup>19</sup>. HDACs fall into three classes, I, II, and III, and are important for cellular regulation through the transcriptional control of genes<sup>30</sup>. HDACs catalyze the removal of acetyl groups from the amino-terminal residues of histones causing chromatin condensation, which shuts off transcription; HATs increase transcription through the transfer of acetyl groups to histones and relaxing the chromatin. Therefore, inhibiting HDAC activity increases transcription. Bmf is critical for cell death caused by the HDAC inhibitors (HDACis) FK228 and CBHA in cancer cell lines<sup>19</sup> and by SAHA (suberoylanilidehydroxamic acid) in thymocytes and pre-B cells. However, Bmf does not mediate TSA or CBHA-induced cell death in T or pre-B cells<sup>27</sup>. The present studies elucidate the role of HDACis in regulating Bmf expression and mediating HDACi-induced cell death in AECs.

#### **1.4 Regulation of airway epithelial cell number in asthma**

The prevalence of allergen-induced asthma is dramatically increasing in children and adults worldwide<sup>31</sup>. Cytokines that are important in the allergen-specific inflammatory response in asthma are produced by Th2 helper cells and include IL-4, IL-

5, IL-9, and IL-13. IL-2 and IFN $\gamma$  are produced by Th1 helper cells<sup>32</sup>. IL-13 induces the differentiation of AECs into mucous-secreting cells, termed MCM, thereby increasing the mucus content in the airway epithelium and causing obstruction of the airways<sup>33, 34</sup>. In addition to MCM, patients with asthma have a fragile airway epithelium, in which the epithelial cells are more susceptible to shedding<sup>35</sup>. In AECs obtained by bronchial brushing, we found that Bmf mRNA is significantly induced in patients with asthma compared to non-diseased controls (unpublished data). In the present studies, we used an animal model of asthma to study Th2 cytokine-induced changes in epithelial cell numbers. Mice are sensitized with alum/OVA and exposed to ovalbumin aerosols to induce an allergic Th2 response<sup>31</sup>. This exposure causes airway epithelial cell hyperplasia and MCM in C57BL/6 mice<sup>36, 37</sup>. When mice are repeatedly exposed to allergen for prolonged periods, MCM is reduced, and this reduction involves cell death of epithelial cells. The resolution of these hyperplastic cells appears to involve detachment of the airway cells from the basement membrane.

### **1.5 The role of IFN $\gamma$ in the resolution of MCM**

Studies show that instillation of IFN $\gamma$  causes an accelerated resolution of allergen-induced MCM. When IFN $\gamma^{-/-}$  and Stat1 $^{-/-}$  mice are sensitized and exposed to allergen for prolonged periods, MCM is not resolved. This demonstrates that the cytokine IFN $\gamma$  through Stat 1 activation is a key mediator in the reduction of allergen-induced MCM by inducing apoptosis<sup>16, 37</sup>.

IFN $\gamma$  is the only cytokine belonging to the Type II interferon family and is secreted by natural killer cells and T lymphocytes in response to specific antigens and mitogens<sup>38</sup>. IFN $\gamma$  binds to IFN $\gamma$ -R1, inducing the Jak-Stat-signaling pathway<sup>39</sup>. Cell

death is induced by IFN $\gamma$  in many different cell lines, including colon adenocarcinoma cells, A549 lung epithelial cells, primary human keratinocytes, HeLa cells, breast tumor cells, fibroblasts, and AECs<sup>37</sup>. The IFN $\gamma$ -induced apoptotic pathway varies among cell types; the extrinsic pathway is the pathway for apoptosis in erythrocytes, keratinocytes, and T-lymphocytes<sup>39,40</sup>. However, in AECs IFN $\gamma$  induces cell death<sup>37</sup> by involving certain members of the Bcl2 family, but mitochondrial cytochrome c release was not detected<sup>41</sup>. This type of cell death is accompanied by changes at the ER; IFN $\gamma$  causes translocation of the effector protein Bax to the ER causing ER dilation and reduction of calcium stores<sup>41</sup>. Recent work has shown that pro-apoptotic BH3 domain-only Bik is a critical mediator of IFN $\gamma$ -induced AEC death in a STAT1 dependent manner. *In vivo* studies demonstrating that allergen-induced AEC hyperplasia is not resolved in Bik-deficient mice during prolonged exposure to allergen<sup>16</sup> confirms the importance of Bik in this resolution process. The anti-apoptotic Bcl-2 is also important for resolution of hyperplastic as well as metaplastic mucous cells<sup>36,42,43</sup>. The present studies investigated the role of Bmf in regulating AEC numbers.

## **1.6 p53 and apoptosis**

Mutations in the tumor suppressor protein 53 (p53) have been implicated in half of all human cancers suggesting that this protein plays a major role in cellular homeostasis<sup>44</sup>. In the nucleus, p53 is responsible for regulating hundreds of genes involved in cell cycle progression, apoptosis, preventing apoptosis, senescence, DNA repair, metabolism, and autophagy<sup>44,45</sup>, and is therefore a critical component of the cellular response to stress, including DNA damage and the UPR. In the cytosol, p53 triggers mitochondrial membrane permeabilization by binding and activating Bax or

inhibiting Bcl-2 and Bcl-x<sub>L</sub><sup>6</sup>. p53 is regulated through MDM2-mediated ubiquitination followed by proteosomal degradation. Post-translational modifications including phosphorylation, methylation, and acetylation also regulate the function of p53<sup>44</sup>. Biochemical and cell culture studies have shown that 36 different amino acids can undergo modifications in response to cellular stresses<sup>44</sup>. Because p53 is central in cell death processes<sup>46</sup>, we examined its role in regulating Bmf during the cell death process of AECs.

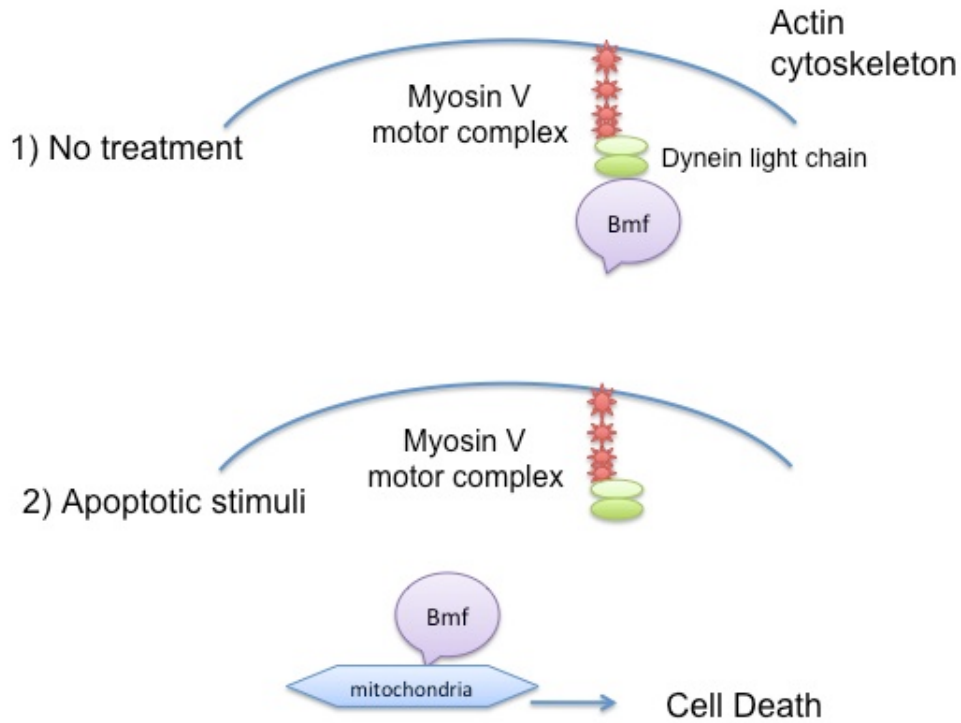
## **1.7 Hypothesis**

We hypothesize that p53 suppresses IFN $\gamma$ -induced Bmf expression, and that Bmf is critical for both IFN $\gamma$ - and HDACi-induced cell death in airway epithelial cells.

Our specific aims are:

- To investigate the regulation of Bmf by IFN $\gamma$  and the suppressive activity of p53.
- To investigate the regulation and role of Bmf in AECs in response to inhibition of HDAC.
- To investigate whether Bmf is necessary for the IFN $\gamma$ -induced resolution of AECs during prolonged exposure of mice to allergen.

## 1.8 Model



**Figure 1-1.** Model demonstrating how Bmf responds to apoptotic stimuli Puthalakath et al. 1) Under normal conditions Bmf resides in the actin cytoskeleton attached to the dynein light chain. 2) In response to apoptotic stimuli such as UV exposure, Bmf translocates from the cytoskeleton to the mitochondria and causes cell death.

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## **Chapter 2**

### **IFN $\gamma$ Deacetylates p53 to Suppress Bmf Expression**

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## 2.1 Abstract

IFN $\gamma$  induces cell death not only in various cancer cells but also in primary keratinocytes, fibroblasts, and airway epithelial cells (AECs). Therefore, it has been used for the treatment of various cancers and our studies show that IFN $\gamma$  reduces airway epithelial cell hyperplasia during prolonged exposure of mice to allergen. However, the effect of IFN $\gamma$  on p53, a major tumor suppressor gene, has not been studied. When screening for Bcl-2 family of proteins involved in the IFN $\gamma$ -induced cell death pathway we found that IFN $\gamma$ , in a p53-dependent manner, downregulated expression of the BH3 domain-only protein, Bmf, in human (HAECs) and mouse (MAECs) primary airway epithelial cells. p53 also suppressed Bmf expression in response to other cell death stimulating agents including UV light and histone deacetylase inhibitors. IFN $\gamma$  did not affect Bmf mRNA half-life but increased nuclear p53 levels and increased the interaction of p53 with the Bmf promoter. IFN $\gamma$  suppressed Bmf expression also in MAECs derived from mice that lack the proline-rich domain of p53, and increasing p53 levels with nutlin 3 failed to modify the suppressive effect of IFN $\gamma$  or UV light. Immunoprecipitation with p53 antibodies showed that an increased interaction of HDAC1 with p53 in IFN $\gamma$ -treated cells was accompanied by a reduction in the acetylated form of p53. Collectively, these studies show that IFN $\gamma$  treatment increases nuclear p53 accumulation, interaction of p53 with HDAC1, and p53 deacetylation to suppress Bmf expression.

## 2.2 Introduction

IFN $\gamma$  induces apoptosis in a variety of cell types, including carcinoma<sup>1,2</sup> and primary keratinocytes<sup>3</sup>. IFN $\gamma$  also induces cell death in AECs<sup>4</sup> to remove hyperplastic epithelial cells following inflammation-induced epithelial cell hyperplasia by activating STAT1<sup>5</sup>. This cell death pathway requires the BH3-only protein Bik<sup>6</sup> and the translocation of Bax to the endoplasmic reticulum (ER)<sup>7,8</sup>. However, the effect of IFN $\gamma$  on p53 has not been reported.

The Bcl-2 family of proteins is characterized by the Bcl-2 homology (BH) domains. The pro-survival proteins Bcl-2, Bcl<sub>xL</sub>, and Mcl-1 have four BH domains (BH1-4). The first group of pro-apoptotic proteins, Bax, Bak, and Bok are characterized by three (BH1-3), while the second group contains only the BH3 domain and, therefore, is also designated the BH3-only group of proteins<sup>9</sup>. One of the BH3-only proteins, Bmf, was first reported to cause cell death upon loss of cell attachment by being released from the DLC and inhibiting the function of the pro-survival Bcl-2<sup>10</sup>. However, this was not confirmed in Bmf-deficient mice<sup>11</sup> that display defects in uterovaginal development<sup>12</sup>. The expression of Bmf induced during formation of the lumen in mammary epithelial acinar structures and the involution of the mouse mammary gland<sup>13</sup>. Bmf was identified as a requirement for necroptosis induced by either zVAD.fmk or TNF $\alpha$  in L929 cells<sup>14</sup> and for HDAC inhibitor-induced apoptosis<sup>13</sup>. Because we have observed that resolution of airway epithelial cells may involve anoikis, and IFN $\gamma$  causes resolution of epithelial cell hyperplasia<sup>7,4</sup> we investigated the effect of IFN $\gamma$  on Bmf expression in airway epithelial cells.

While the function of Bmf has been studied more extensively, understanding is lacking on how its expression is regulated. Bmf expression is increased 500-fold during maturation of oligodendroglial progenitor cells to mature oligodendrocytes<sup>15</sup>. Bmf expression is induced by arsenic trioxide in multiple myeloma cells and mediates cell death<sup>16</sup>. The mechanism involved in Bmf expression in oligodendrocyte maturation, by arsenic trioxide, or mammary cells was not reported. However, histone deacetylase inhibitors (HDACis) preferentially induce Bmf expression in a broad range of cancer cells by hyperacetylating histones H3 and H4 at the Bmf promoter region<sup>17</sup>. In addition, loss of Bmf protein renders resistance to lymphocytes against glucocorticoid- or HDAC inhibitor-induced cell death<sup>11</sup>. While the designation HDACs is based on these proteins being initially known to primarily target histones, it is now clear that many other non-histone proteins are substrates for the various HDACs. p53 was the first non-histone protein known to be regulated by acetylation and deacetylation<sup>18,19</sup>. In the present study, we found that in airway epithelial cells IFN $\gamma$  causes deacetylation and nuclear accumulation of p53 to promote interaction with the Bmf promoter and suppress expression.

## 2.3 Experimental Procedures

### Animals

Pathogen-free *STAT1*<sup>-/-</sup>, *p53*<sup>+/-</sup> mice were purchased from the Jackson Laboratory (Bar Harbor, ME), *bik*<sup>-/-</sup> mice were made available by Dr. Andreas Strasser (Walter and Eliza Hall Institute) and mice with modified proline-rich domain p53<sup>ΔP</sup>, and p53<sup>AXXA</sup> were from Dr. Geoffrey M. Wahl (The Salk Institute for Biological Studies, La Jolla, CA). These mice along with the wild-type littermates were bred at the Lovelace Respiratory Research Institute (LRRI) under specific pathogen-free conditions and genotyped as described previously <sup>11</sup>. All animal experiments were approved by the Institutional Animal Care and Use Committee at Lovelace Respiratory Research Institute, a facility approved by the Association for the Assessment and Accreditation for Laboratory Animal Care International.

### Cell Culture

The preparation of MAECs was carried out as described <sup>20</sup>. The immortalized human airway epithelial cells, AALEB cells <sup>8</sup>, and HAECs (Clontech, Walkersville, MD) were maintained in bronchial epithelial growth medium (BEGM, Lonza, Walkersville, MD)

### RNA Extraction and quantitative RT-PCR

Extraction of RNA from cell pellets was carried out using the RNeasy kit (Qiagen, Valencia, CA) and concentrations were determined using the Thermo Scientific Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The primer/probe sets for Bmf, CDKN1B, and 18s were obtained from Applied Biosystems

(Foster City, CA). Target mRNAs were amplified by quantitative real-time PCR in 20  $\mu$ l reactions on the ABIPRISM 7900HT Real-Time PCR System using the One-Step RT-PCR Master Mix (Applied Biosystems). Relative quantities from duplicate amplifications were calculated by normalizing averaged  $C_T$  values to CDKN1B and/or 18s to obtain  $\Delta C_t$ , and the relative standard curve method was used for determining the fold change as described<sup>21</sup>.

### **Western Blot Analysis**

Preparation of protein lysates and cytosolic and nuclear fractions were prepared<sup>6</sup> and analyzed by Western blotting as described<sup>8</sup>. We used the rat anti-Bmf monoclonal antibody, a gift from Dr. Andreas Strasser (the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) at 2  $\mu$ g/ml, the rabbit anti-p53 polyclonal antibody (FL-393, Santa Cruz Biotechnology Inc., CA) or acetyl-p53 (Lys 382) (Cell Signaling Inc., Boston, MA) at a 1:1000 dilution. Mouse actin polyclonal antibody was used at 1:5000 dilution (Santa Cruz Biotechnology, Inc.) and rabbit anti-lamin polyclonal antibody was used at 1:1000 dilution (#2032 Cell Signaling, Inc. Boston, MA), and rabbit anti-HDAC1 polyclonal antibody was used at 1:1000 dilution (Millipore, Billerica, MA). Goat anti-rat or goat anti-rabbit secondary antibodies were used for visualizing proteins with chemiluminescence (Perkin Elmer, Waltham, Massachusetts) using the FujiFilm Image Reader LAS-4000 (Valhalla, NY).

### **Immunofluorescent Imaging and Analysis**

For immunofluorescent staining AALEB cells were grown on Lab-Tek-II 8-chamber slides (Nalge Nunc International, Rochester, NY) and treated with 50 ng/ml of IFN $\gamma$  or left untreated. Cells were fixed using 3% paraformaldehyde with 3% sucrose in



PBS and permeabilized using 0.2% Triton X-100 with 0.2% Saponin in a blocking solution containing 3% IgG-free BSA, 1% gelatin, and 2% normal donkey serum. The cells were then probed with anti-p53 (#sc126, clone DO1, Santa Cruz Biotech, CA), anti-acetyl-p53 (Lys382) (#2525, Cell Signaling, Inc. Boston, MA) or isotype controls at 1:100 dilution. The immunolabeled cells were detected using F(ab)<sub>2</sub>-fragments of respective secondary antibodies conjugated to Dylight<sup>TM</sup>-649 (Jackson ImmunoResearch, West Grove, PA) at 1:1000 dilution and mounted with Fluormount-G<sup>TM</sup> 22-20, (SouthernBiotech, Birmingham, AL) containing 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining. Fluorescently labeled cells were analyzed as described<sup>8</sup> using the Axioplan 2 imaging system (Carl Zeiss Microimaging, Inc., Thornwood, NY) equipped with a ORCA-ER CCD camera (Hamamatsu Photonics, Japan) coupled with a Lambda DG-4 wavelength switch (Sutter Instrument, Novato, CA) and acquisition software Slidebook<sup>TM</sup> 5 (Intelligent Imaging Innovations, Inc., Denver, CO). p53-positive and acetyl-p53-positive cells were quantified by counting at least 400 cells/well with  $n \geq 4$  for each treatment by a person unaware of the treatment groups.

### **Adenoviral Overexpression**

Adenovirus expression vectors for p53 were developed by cloning p53 cDNA into the shuttle vector. Shuttle CMV\_p53 was packaged into virus-producing cells, and adenovirus particles were harvested as described<sup>23</sup>.

### **Bmf mRNA Half-life**

Cells were treated with the RNA polymerase inhibitor 5,6-dichloro-1-beta-D-riboenzimidazole (DRB) (Sigma-Aldrich, St. Louis, MO) at a final concentration of 50 ng/ml for SAOS-2, Calu-3, A549, and Calu6, and at 100ng/ml for AALEBs to stop RNA

polymerase activity. The relative mRNA abundance was calculated using the  $\Delta\Delta C_t$  method, and the mRNA half life was calculated using Greenberg formula<sup>24</sup>.

### **Chromatin Immunoprecipitation**

After treating with 50 ng/ml hrIFN $\gamma$  for 48 h, cells were fixed with 1% formaldehyde; the reaction was quenched using 1.25 M glycine and scraped into cold PBS-containing protease inhibitors. Chromatin immunoprecipitation was performed using the MAGNA0002 Magna ChIP G kit (Millipore, Billerica, MA) as described by the manufacturer. Sonicated nuclear fractions were incubated with mouse IgG1 monoclonal antibody to p53 (Santa Cruz sc-98 Pab1801), rabbit polyclonal antibody to HDAC1 (06-720 Millipore), rabbit polyclonal antibody to acetyl H4 (06-598 Millipore), rabbit polyclonal antibody to acetyl H3 (06-599B Millipore), mouse IgG1, or rabbit as control (CBL600 Millipore). DNA identification was confirmed with PCR using the following primers specific for the Bmf promoter at region -560 5'ACCTAAGGGCTCCCCTGGA-3', 5'-GCAGGTCGGAAGAAAAGTGCAGC-3', and region -97 5'-TTGGCGCTTCACTCGCCATT-3', 5'-ATCCCGCAAACAGCTGAT-3'.

### **Immunoprecipitation**

Cells were treated with IFN $\gamma$  for 48 h, fixed, rinsed twice with cold PBS, and scraped in cold PBS plus protease inhibitors. Immunoprecipitation was performed using the Pierce Crosslink IP Kit (26147) as described by the manufacturer.

Immunoprecipitated proteins from nuclear extracts were analyzed by Western blotting for HDAC1, anti-p53 (FL-393 Santa Cruz), and acetyl-p53 (Lys382) (#2525 Cell Signaling, Inc., Boston, MA).

## Statistical Analysis

Fold changes or scanned density values were averaged and compared for significance between groups using *Student's t*-test. Data were analyzed using Prism statistical analysis software, and  $p < 0.05$  was considered statistically significant.

## 2.4 Results

Our previous studies demonstrate that physiological relevant levels of IFN $\gamma$  induce cell death in primary normal human airway epithelial cells (HAECs), murine airway epithelial cells (MAECs), and AALEB cells, a cell line derived from HAECs<sup>7,8</sup>. IFN $\gamma$ -induced cell death is mediated by STAT1-dependent Bik expression<sup>6</sup>. While screening for the effect of IFN $\gamma$  on the expression of BH3-only proteins, we found that IFN $\gamma$  reduced Bmf mRNA levels in AALEB cells by 5-fold over 24 h (Figure 2-1A), and this reduction was replicated in both HAECs (Figure 2-1B) and MAECs (Figure 2-1C). Western blot analysis also showed that Bmf protein is reduced at 48 h of IFN $\gamma$  treatment in MAECs; this protein is 30 kDa in size and represents likely the Bmf II<sup>25</sup> (Figure 2-1D). Treatment of *STAT1*<sup>-/-</sup> (Figure 2-1E), *bik*<sup>-/-</sup> (Figure 2-1F), or wild-type MAECs with IFN $\gamma$  also reduced Bmf mRNA levels suggesting that downregulation of Bmf was independent of IFN $\gamma$  activating STAT1 or Bik expression.

To further investigate this unexpected finding, we explored the effect of IFN $\gamma$  on Bmf mRNA levels in epithelial cell lines derived from various cancers as previous studies have described Bmf expression in cancer cell lines<sup>10,13</sup>. This analysis uncovered a striking correlation between basal Bmf mRNA expression levels and p53 status. The p53-deficient cell lines SOAS-2 and Calu-6 cells showed significantly higher Bmf mRNA levels compared to the p53-sufficient cells, A549, and AALEB cells (Figure 2-2A). The role of p53 in affecting Bmf expression was validated by expressing p53 in Calu-6 cells using an adenoviral overexpression system (Figure 2-2B). Interestingly, p53 expression alone was not sufficient; additional treatment with 10 mJ UV light was necessary for reducing Bmf levels, while the same treatment significantly increased Bmf

mRNA levels in cells infected with adenoviral GFP as control (Figure 2-2C). Identical results were obtained when p53 was expressed in Calu-6 cells using a lentiviral expression system after treatment with UV light (data not shown). Consistent with previous reports, exposure of MAECs to 10 mJ of UV light increased p53 levels (Figure 2-2D) and reduced Bmf mRNA levels to 50% of non-treated controls in both MAECs (Figure 2-2E) and AALEB cells (Figure 2-2F) 6 h after treatment.

Previous reports have established that HDAC1 suppresses Bmf expression by inhibiting the promoter activity<sup>17</sup>. Therefore, we wanted to investigate whether HDACis affect Bmf expression in a p53-dependent manner. The HDACis, sodium butyrate, TSA, or MS-275 significantly increased Bmf mRNA levels in AALEB cells (Figure 2-3A), and TSA increased Bmf protein levels in MAECs (Figure 2-3B). While Bmf mRNA levels were increased by TSA in both *p53*<sup>-/-</sup> and *p53*<sup>+/+</sup> MAECs, Bmf mRNA expression was 2-fold higher in *p53*<sup>-/-</sup> compared to *p53*<sup>+/+</sup> MAECs (Figure 2-3C) suggesting that the inhibitory role of p53 may be independent of the regulation by HDACs.

These findings showed that IFN $\gamma$  may affect p53 to cause the reduction of Bmf expression. Therefore, we investigated the effect of IFN $\gamma$  on primary MAECs from *p53*<sup>-/-</sup> and *p53*<sup>+/+</sup> mice and found that Bmf mRNA levels were significantly increased by IFN $\gamma$  in *p53*<sup>-/-</sup> MAECs (Figure 2-4A). Furthermore, Bmf protein was detected in IFN $\gamma$ -treated *p53*<sup>-/-</sup> but not *p53*<sup>+/+</sup> MAECs (Figure 2-4B). Similarly, suppression of p53 levels in AALEB cells using shRNA resulted in IFN $\gamma$  failing to suppress Bmf mRNA levels (data not shown).

Having established that p53 suppresses Bmf expression, we investigated the possibility that p53 may affect Bmf mRNA stability. The Bmf mRNA has an unusually

long 4kb 3'UTR indicating that it may be a crucial factor in regulating expression levels. In general, 3'UTRs have conserved sequences that regulate mRNA stability<sup>26</sup>. For these studies, transcription was blocked with DRB, and cells were harvested at 0, 0.5, 1, 2, and 4 h with or without IFN $\gamma$  treatment. On average, the half-life of Bmf mRNA in non-treated, p53-sufficient A549 and AALEB cells was 1.94 and 2.43, respectively, and in the p53-deficient Calu-6 and SAOS cells, 2.36 and 1.73, respectively. Bmf mRNA half-life in IFN $\gamma$ -treated, p53-sufficient and p53-deficient cells was 1.5 and 1.8 h, respectively. These data suggest that p53 did not affect Bmf mRNA stability.

Because IFN $\gamma$  did not affect Bmf mRNA half-life, we next evaluated whether IFN $\gamma$  affects the interaction of p53 with the Bmf promoter. The -97 region was responsible for acetylation in cancer cell lines in response to HDACi treatment<sup>17</sup>. Therefore, we selected the -97 region to test for p53 interaction. The -560 region was selected as an additional region assuming that there would be less acetylation activity than the -97 region yet strong HDAC1 interaction. ChIP assays showed that p53 interacted with the Bmf promoter only in IFN $\gamma$ -treated AALEB cells but not in non-treated controls (Figure 2-5A). This interaction was observed only for the region close to the transcriptional start site at -97, while no interaction was observed at -560. To further investigate the IFN $\gamma$ -induced modification on p53, we analyzed the distribution of p53 in the cytosolic and nuclear fractions, and found that nuclear p53 levels were greater in IFN $\gamma$ -treated AALEB cells compared to non-treated controls (Figure 2-5B). Similarly, MAECs showed increased p53 levels in the IFN $\gamma$ -treated cells (data not shown). Increased nuclear localization of p53 in IFN $\gamma$ -treated cells was also observed by immunofluorescence (Figure 2-5C), thus confirming the findings by Western blotting.

Together, these studies showed that IFN $\gamma$  treatment modified p53 causing nuclear accumulation of this protein.

In humans, the proline-rich domain of p53 (PRD) is defined by residues 58–98 that contains 15 prolines and five repeats of the amino acid motif PXXP (where P designates proline and X any amino acid). The histone acetyl transferase p300 binds to PXXP-containing peptides derived from the proline repeat domain, and the PXXP motif in p53 is required for p53 acetylation<sup>27</sup>. Therefore, we investigated whether this region plays a role in the IFN $\gamma$ -induced Bmf suppression. In mice, this PRD consists of two PXXP motifs, and we obtained mice with a deletion of the PRD (p53 $\Delta$ P) or lacking the four critical proline residues at loci 79, 82, 84, and 87 that make up the tandem PXXP sites (p53 $^{\Delta$ XXA)<sup>28, 29</sup>. MAECs isolated from p53 $\Delta$ P, p53 $^{\Delta$ XXA, and wild-type littermates were treated with IFN $\gamma$  and analyzed for Bmf levels, which were reduced in these cells as was observed in wild-type MAECs (Figure 2-5D) suggesting that this region is not crucial for the modification in p53 to be enriched in the nucleus.

The stability of p53 is regulated primarily by the E3 ubiquitin-ligase activity of Mdm2<sup>28</sup>, and phosphorylation of p53 disrupts interactions between p53 and Mdm2 leading to p53 stabilization<sup>30</sup>. Nutlin-3, a synthetic cis-imidazoline analog specifically blocks the interaction between p53 and Mdm2 by interacting with a large hydrophobic pocket in the Mdm2 protein which p53 occupies. This results in the stabilization of p53, enhancing p53 activity, such as induction of apoptosis and cell cycle arrest of cancer cells. Numerous studies showed that nutlin-3 is very specific for Mdm2; in addition, experiments using the enantiomer of nutlin 3 as a control determined that the enantiomer is 150 times less active and does not cause stabilization of p53<sup>31</sup>. Bmf mRNA levels were

induced in AALEB cells treated with nutlin-3 for 24 h compared to untreated cells (Figure 2-5E), or remained unchanged in the MAECs (data not shown). In a separate set of studies, AALEB cells were treated with nutlin, nutlin and IFN $\gamma$ , and nutlin and UV light to examine whether nutlin treatment affects the suppression of Bmf mRNA when p53 was modified by these cell death-inducing stimuli. Interestingly, the disruption of p53 from Mdm2 by nutlin3 did not affect suppression of Bmf expression in response to IFN $\gamma$  or UV light treatment (Figure 2-5F). These findings suggested that p53 must have been modified in a different manner from the disruption from Mdm2 interaction.

Several of our findings suggest that modification of p53 by IFN $\gamma$  may involve acetylation. First, the effect of HDACis on Bmf expression is suppressed by p53 and second, Bmf promoter is known to interact with HDAC1<sup>17</sup>. Therefore, we performed immunoprecipitation assays using anti-p53 antibodies and analyzed the pull-down products for HDAC1 and total p53. HDAC1 levels were increased in pull-down products from IFN $\gamma$ -treated cells compared to non-treated cells (Figure 2-6A) suggesting that IFN $\gamma$  enhanced the p53/HDAC1 interaction. Because p53 was found to be complexed with a histone deacetylase we were interested in assessing the acetylation state of p53 after IFN $\gamma$  treatment. Immunoblotting of the pull-down product using antibodies specific to K382p53 revealed that acetylated p53 levels were significantly reduced in IFN $\gamma$ -treated compared to non-treated cells (Figure 2-6A). These results suggest that the IFN $\gamma$ -induced interaction of HDAC1 and p53 may have resulted in the deacetylation of p53. The fact that IFN $\gamma$  treatment leads to deacetylation of p53 was also observed in MAECs as the cytosolic nuclear p53 showed reduced acetylation in IFN $\gamma$ - compared to non-treated



controls (Figure 2-6B). This finding was confirmed by immunostaining of IFN $\gamma$ -treated MAECs and controls using antibodies specific to acetyl p53 (Figure 2-6C).

## 2.5 Discussion

The present studies show that IFN $\gamma$  promotes p53/HDAC1 interaction, deacetylates p53, and enhances p53 to interact with the Bmf promoter and suppress expression. In addition, this suppressive effect of p53 on Bmf expression occurred when cells were exposed to other cell death-stimulating agents including UV light and HDACis.

Suppression of Bmf occurred within 6 h of treatment with UV light but 48 h after IFN $\gamma$  treatment. These findings suggest that the IFN $\gamma$ -induced deacetylation of p53 may be mediated by events triggered at later time points; however, to our knowledge, the fact that IFN $\gamma$  modifies p53 has previously not been reported. Both STAT1 and Bik or merely disruption of the p53-Mdm2 interaction with nutlin 3<sup>32</sup> were not sufficient to modify the reduction of Bmf expression. In addition, AECs with either deleted PRD or mutations of the proline residues within the PRD of p53 to alanines did not abrogate the IFN $\gamma$ -induced downregulation of Bmf demonstrating that the IFN $\gamma$ -induced deacetylation must occur at sites other than the proline-rich domain (PRD). Downregulation of Bmf by IFN $\gamma$  was also observed in NHBEs that were derived from 3 individuals each that had the Arg/Arg or the Pro/Pro p53 genotype at codon 72 within the PRD (data not shown) that corresponds to the mouse PXXP motif, further supporting the idea that the PRD is not involved in the IFN $\gamma$ -induced modification of p53 and suppression of Bmf expression. In addition, merely increasing p53 expression using an adenoviral or lentiviral expression vectors was not sufficient but treatment with UV light of p53-expressing Calu-6 cells was

necessary to suppress Bmf levels. While p53 can be increased in level due to stabilization in response to the p53-Mdm2 feedback loop, our findings suggest that p53 must undergo posttranslational modifications acetylation/deacetylation to be activated<sup>33</sup>. The effects of IFN $\gamma$  on signaling kinase ERK in AECs has been demonstrated<sup>6</sup>. However, blocking the activation of ERK1/2 using UO126 did not affect IFN $\gamma$ -induced deacetylation of p53 (data not shown). Therefore, the pathway by which IFN $\gamma$  causes deacetylation and nuclear accumulation of p53 remains to be elucidated.

At least three independent pathways activate the p53 network. The first pathway is triggered by ionizing irradiation causing double strand breaks that is recognized by the Ataxia telangiectasia mutated (ATM), Chk2 pathway<sup>34</sup>, the p14<sup>ARF</sup> pathway, and the ATR (ataxia telangiectasia related) and casein kinase II that involves kinases<sup>35</sup>. Many other genes have been reported to be suppressed by p53 including DNA topoisomerase II, cyclin B, Cdc2<sup>36</sup>, MMP-1 and -13<sup>37</sup>, presenilin-1<sup>38</sup>, myc, and Map-4<sup>39</sup>. In general, transcriptional activation requires p53 to bind a consensus sequence<sup>40</sup>; however, the repression mechanism by p53 is not well studied. For example, p53 directly binds to the Mad1L1 promoter, but no p53 consensus site was found<sup>41</sup>. Similarly, we found that p53 interacts with the Bmf promoter when cells are treated with IFN $\gamma$ , although a consensus-binding sequence for p53 was not found in the upstream region of the *bmf* gene. While DNA binding is required for p53 to suppress *cdc2*<sup>36</sup> or *cdc20*<sup>42</sup>, p53 can also suppress by interfering with transcriptional factors, which usually transactivate the genes<sup>43</sup>.

We found that HDACis consistently induced Bmf expression in primary human and mouse airway epithelial cells. However, extent of induction was suppressed in the presence of p53. These findings suggested that induction of Bmf is independently

regulated by histone acetylation and by p53 deacetylation. The fact that p53 dampens HDACi-induced Bmf expression further supports our findings that deacetylated p53 interacts with the Bmf promoter to suppress its activity. Similar to our findings, the HDACis induce acetylation of histones H3 and H4 at the Bmf promoter region in various human cancer cell lines and HDAC1 expression reduces Bmf expression<sup>17</sup>. The previous studies also showed that overexpression of histone acetyltransferase p300 mimics the effects of the HDAC inhibitors, suggesting that Bmf expression in cancer cells is primarily regulated by histone hyperacetylation<sup>44</sup>, and this approach was the basis to promote HDACis for cancer therapy. However, our findings that these agents also induce Bmf in primary airway epithelial cells may suggest side effects and that the use of these compounds for cancer therapy should be approached with caution.

Because IFN $\gamma$  treatment or the presence/absence of p53 had no effect on Bmf mRNA half-life and results from ChIP assays showed that IFN $\gamma$  modified the interaction of p53 with the Bmf promoter it is likely that p53 and HDAC1 affect promoter activity by affecting chromatin remodeling. Because HDAC1 associates with the Bmf promoter in an untreated condition<sup>44</sup> and p53 deacetylation correlates with the increased interaction of p53 with HDAC1 we conclude that IFN $\gamma$  treatment causes the deacetylation likely by promoting p53/HDAC1 interaction. While the modification of p53 by HDACis has been studied in lung cancer cells<sup>18</sup>, the state of p53 acetylation in airway epithelial cells has not been studied. Interestingly, acetylated p53 was present in non-treated cells suggesting that acetylated p53 may be crucial for the regular proliferation of HAECs and MAECs.

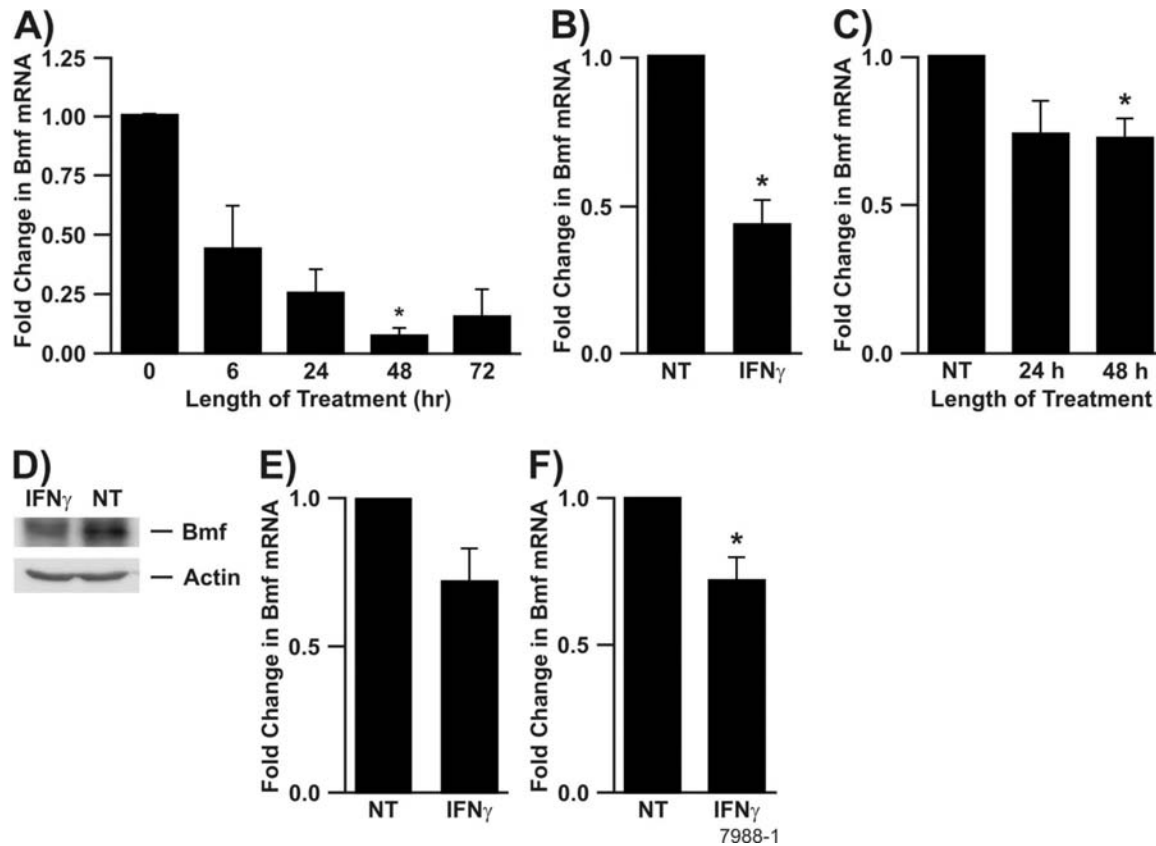
IFN $\gamma$  caused deacetylation and nuclear accumulation of p53 suggesting that acetylation may be involved in the proper transport of p53 out of the nucleus. When p53 is acetylated on the 8 lysine residues in the DNA binding and C-terminal regions the interaction with Mdm2 is prevented leading to stabilization and increase in p53 protein <sup>33</sup>. Marchenko et al. report that the classic importin- $\alpha/\beta$  pathway is responsible for import of only non-ubiquitinated p53 into the nucleus during the early stages of stress response such as DNA damage <sup>45</sup>. Future studies will investigate the lysine residues that are deacetylated by IFN $\gamma$  and whether this modification minimizes the interaction of p53 with nuclear export proteins such as MDM2-mediated ubiquitination <sup>46</sup> to increase nuclear p53 levels.

The fact that p53 mediates the IFN $\gamma$ -induced Bmf suppression in HAECs and MAECs suggests that p53 is not involved in the IFN $\gamma$ -induced cell death pathway. This hypothesis is supported by our previous studies demonstrating that IFN $\gamma$  induces Bik expression in *p53*<sup>+/+</sup> and *p53*<sup>-/-</sup> MAECs and causes cell death in a p53-independent manner <sup>6</sup> and that p53 levels remain unaffected by IFN $\gamma$  <sup>8</sup>. Bmf (Bmf I) has a pro-apoptotic function, while Bmf II and Bmf III have functions contributing to survival and growth <sup>25</sup>. Western blot analysis showed that a 30kDA Bmf protein was reduced by IFN $\gamma$  treatment that corresponds to the Bmf II, a protein that in contrast to the Bmf-I had no effect on apoptosis and instead increased colony formation <sup>25</sup>. Therefore, we conclude that IFN $\gamma$  by suppressing the isoform that enhances colony formation enhances the effect of Bmf I to enhance the cell death pathway. Bmf I is a pro-apoptotic protein <sup>13, 11</sup> and its localization determines its role to induce cell death <sup>10</sup>. Therefore, functional studies on the possible translocation of Bmf I in IFN $\gamma$ -induced cell death are in progress.

## **Acknowledgements**

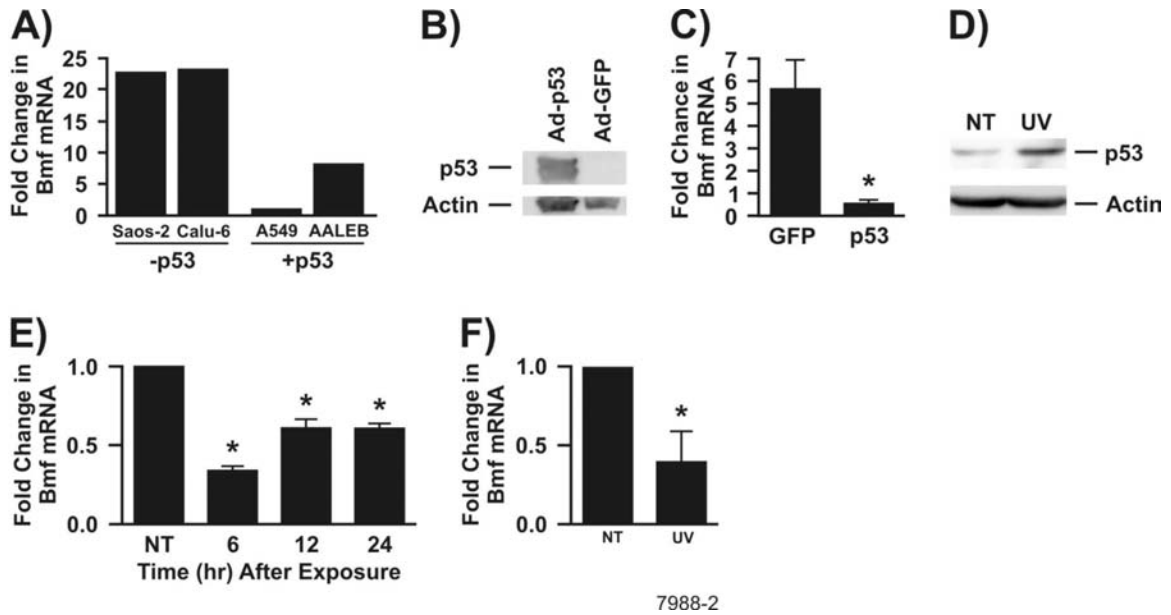
The authors thank Elizabeth Bennett and Kurt Schwalm for technical assistance on selected experiments.

## 2.6 FIGURES AND LEGENDS



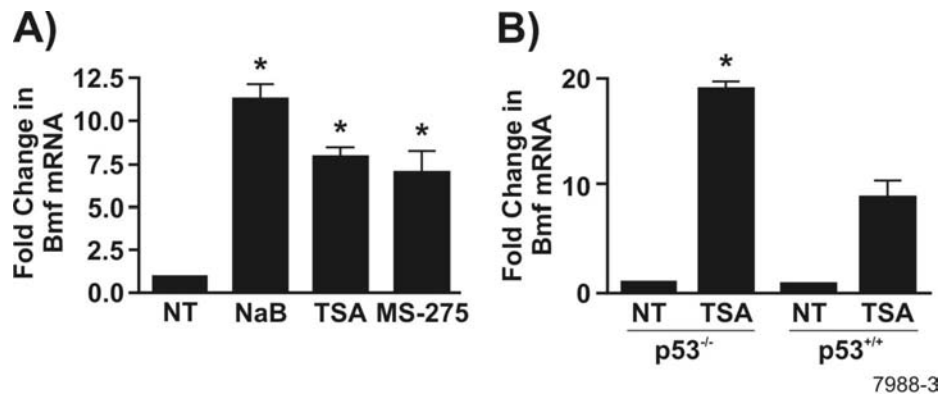
**Figure 2-1. IFN $\gamma$  down regulates Bmf expression.**

Bmf mRNA levels were quantified by qRT-PCR in A) AALEB cells, B) HAECs, C) wild-type MAECs E) *STAT1*<sup>-/-</sup> MAECs, or F) *Bik*<sup>-/-</sup> MAECs after treatment with IFN $\gamma$  (50 ng/ml) and harvested at indicated time points or at 48 h when not stated in the figure. D) Bmf protein levels in lysates of MAECs treated with IFN $\gamma$  for 48 h. The relative standard curve method was used for analysis of unknown samples, and data are presented as fold change after averaging the  $\Delta$ CT values for the untreated samples. Data presented are means  $\pm$  SEM for three independent experiments. \*  $P < 0.05$ ; statistically significant difference from the untreated control.



**Figure 2-2. Bmf expression is suppressed by p53.**

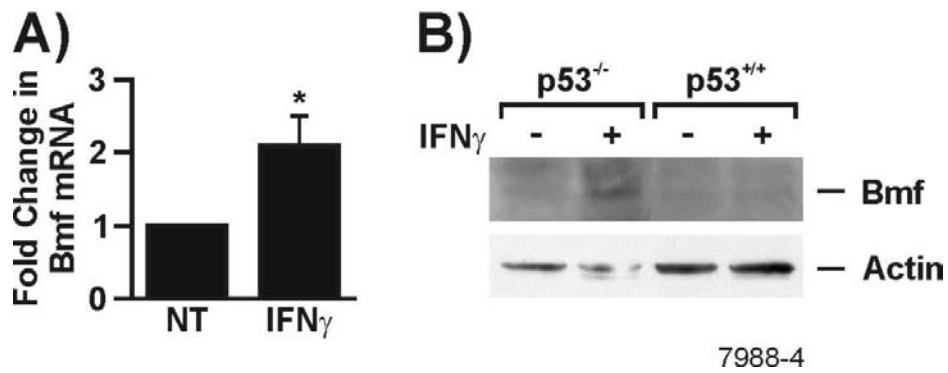
A) Bmf expression levels in p53-sufficient and -deficient cell lines were evaluated using qRT-PCR; Bmf mRNA levels relative to p53-sufficient A549 are shown for AALEB cells, along with p53-deficient SAOS-2 and Calu-6 cells. B) Western blot analysis of p53 in Calu-6 cells infected with an adenoviral expression vector for p53. C) Relative Bmf mRNA levels quantified by qRT-PCR in Calu-6 cells infected with an adenoviral expression vector for GFP or p53 and exposed to 10 mJ of UV light compared to the respective non-treated controls. D) p53 protein levels in MAEC lysates 6 h after exposure to 10 mJ of UV light. E) MAECs exposed to 10 mJ of UV light and analyzed for Bmf mRNA 6 h after exposure to 10 mJ of UV light compared to non-treated controls. F) AALEB cells exposed to 10 mJ of UV light and analyzed for Bmf mRNA 6, 12, and 24 h after exposure compared to non-treated controls. Data presented are means  $\pm$  SEM for three independent experiments. \*  $P < 0.05$ ; statistically significant difference from the control.



**Figure 2-3. p53 suppresses HDACi-induced increase in Bmf mRNA and protein levels.**

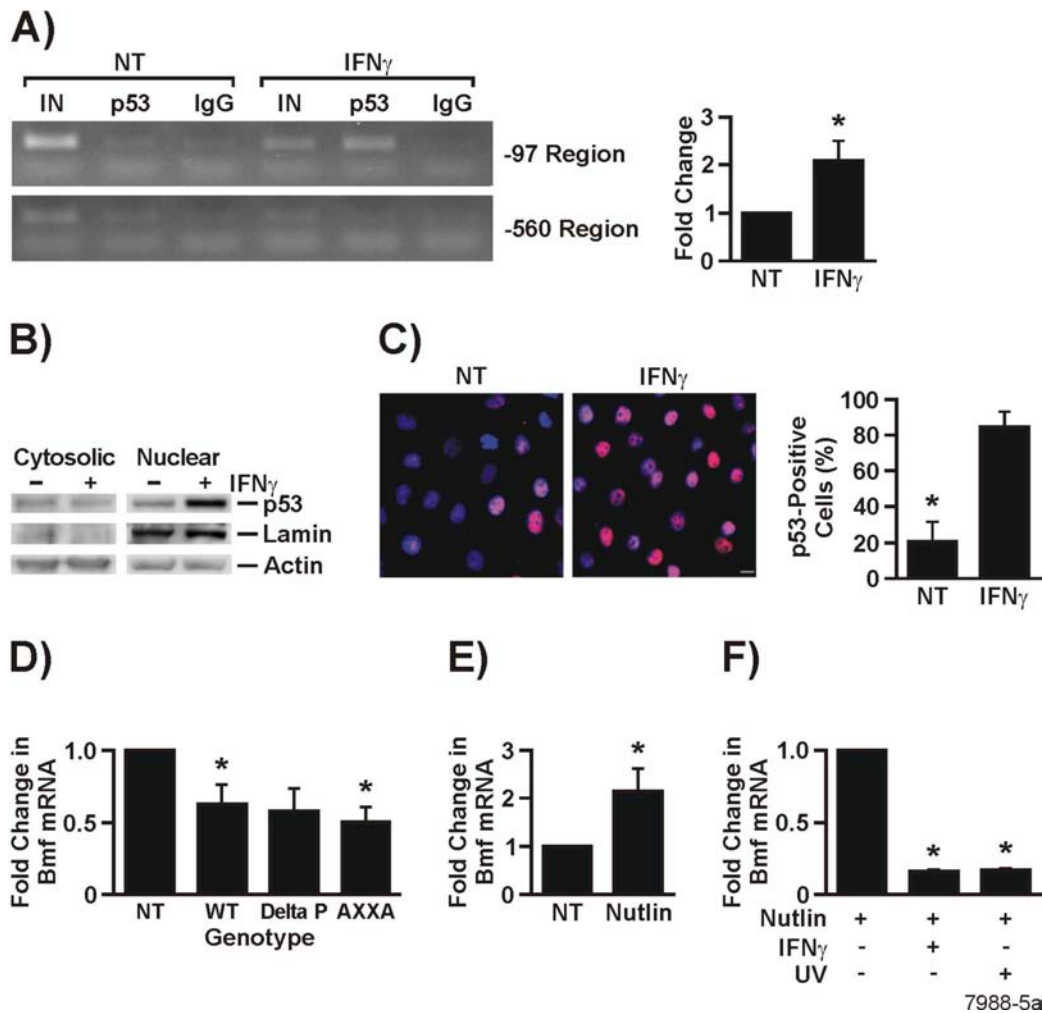
A) Bmf mRNA levels in AALEB cells treated with 5 mM sodium butyrate, 300 nM TSA, and 5  $\mu$ M of MS-275 for 18 h compared to non-treated controls. B) Bmf mRNA expression in *p53*<sup>-/-</sup> and *p53*<sup>+/+</sup> MAECs treated with 300nM TSA for 18 h. Error bar indicates  $\pm$  SEM. \*, P<0.05; statistically significant difference from the control.





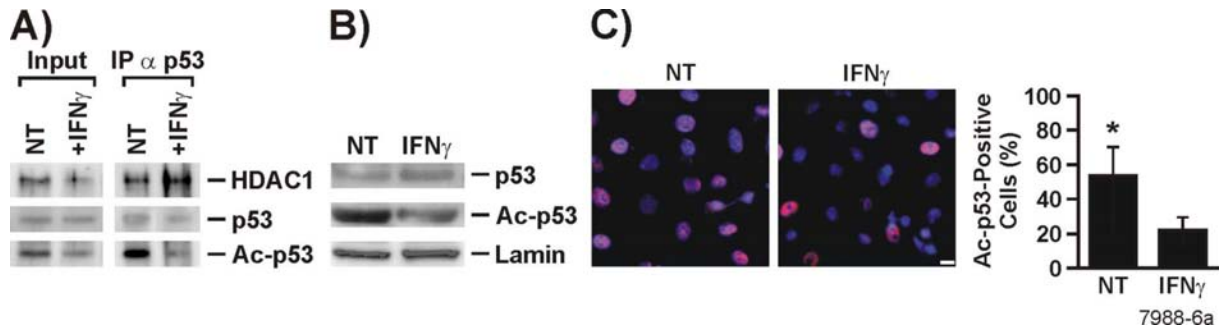
**Figure 2-4. IFN- $\gamma$  induces Bmf mRNA and protein expression in p53-deficient MAECs.**

A) Bmf mRNA in IFN $\gamma$ -treated and non-treated  $p53^{-/-}$  MAECs. Error bar indicates  $\pm$  SEM. \*,  $P < 0.05$ ; statistically significant difference from the control. B) Bmf protein levels in  $p53^{-/-}$  and  $p53^{+/+}$  MAECs treated with nothing or IFN $\gamma$  for 48 h.



**Figure 2-5. p53 is enriched in the nucleus and interacts with the Bmf promoter.**

A) ChIP assays performed on AALEB cells treated with IFN $\gamma$  for 48 h using a monoclonal antibody to p53 or mouse IgG1 as control. DNA was identified using PCR with primers specific for Bmf promoter regions at -97 or -560 regions. Densitometry was used to quantify the amount of DNA in the PCR products from six independent experiments. Quantification of the DNA associated with p53 was normalized to IgG1, and IFN $\gamma$ -treated values were normalized to the untreated values. B) Increased p53 protein levels in the nuclear fractions of AALEB cells 48 h after treatment with IFN $\gamma$  compared to non-treated controls. Nuclear and cytosolic extracts were analyzed for p53, lamin, and actin. Figure is representative of three independent experiments. C) Immunostaining of AALEB cells treated with IFN $\gamma$  for 48 h with anti- p53 antibody and quantification of p53-positive nuclei. D) Bmf mRNA levels in MAECs isolated from p53 $\Delta^P$  and p53 $^{AXXA}$  and wild-type littermate mice. E) Bmf mRNA levels in AALEB cells treated with Nutlin 3 for 24 h. F) Bmf mRNA levels in AALEB cells treated with nutlin 3 and then treated with IFN $\gamma$  for 48 h or exposed to UV for 6 h and compared to their respective untreated control.



**Figure 2-6. IFN $\gamma$  deacetylates p53.**

A) HDAC1 interacts with p53. Nuclear lysates were prepared from AALEB cells after treatment with IFN $\gamma$  for 48 h and were immunoprecipitated using a p53-specific monoclonal antibody. The nuclear lysates (input) and immunoprecipitates were resolved by SDS PAGE and analyzed using Western blotting using antibodies to HDAC1, total p53, and acetyl p53. B) Nuclear extracts prepared from IFN $\gamma$ -treated and non-treated MAECs and probed for total p53, acetyl p53, and lamin. C) AALEB cells treated with IFN $\gamma$  for 48 h immunostained with anti-acetyl p53 and quantified.

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## **Chapter 3**

# **IFN $\gamma$ Inhibits HDAC1 and Induces Expression of Bmf to Reduce Allergen-Induced Mucous Cell Metaplasia**

### 3.1 ABSTRACT

Reports show that histone deacetylase 2 (HDAC2) levels are reduced in macrophages and other inflammatory cells of patients with severe asthma and COPD, and that HDAC2 suppresses expression of inflammatory genes. However, the role of HDACs in airway epithelial cells and how they affect epithelial cell hyperplasia (ECH) is unknown. Our previous studies have shown that the resolution of MCM during prolonged exposure to allergen is mediated by IFN $\gamma$  through STAT1-mediated effect on the Bcl-2 family of proteins. In cancer cells, Bmf, a pro-apoptotic member of the Bcl-2 family, is suppressed by HDAC1 and histone modifications. Therefore, we investigated the role of HDACs and Bmf in regulating airway epithelial cell death and ECH. We found that various HDAC inhibitors including sodium butyrate, trichostatin A<sup>1</sup>, and MS-275 induce Bmf expression not only in cancer cells but also in primary mouse airway epithelial cells (MAECs) and in an immortalized airway epithelial cell line (AALEB cells). Chromatin immunoprecipitation (ChIP) assays demonstrated that TSA enhanced acetylation of histones 3 and 4 but reduced the association of HDAC1 with the Bmf promoter in AALEB cells. MAECs from *bmf*<sup>-/-</sup> but not *bmf*<sup>+/+</sup> mice were protected from TSA-induced cell death and restoring Bmf using an adenoviral overexpression system (Ad-Bmf) in *bmf*<sup>-/-</sup> MAECs restored TSA-induced death. Bmf expression was increased in hyperplastic epithelial cells of mice exposed to allergen for prolonged periods, and MCM was resolved, while resolution of MCM was abrogated in *bmf*<sup>-/-</sup> mice. IFN $\gamma$  blocked HDAC1 interaction with the Bmf promoter in airway epithelial cells. Together, these findings suggest that the resolution of allergen-induced MCM is mediated by IFN $\gamma$  disrupting HDAC1 interaction with the Bmf promoter and inducing expression of



this pro-apoptotic protein. These findings suggest that inhibitors specific for HDAC1 may be useful to reduce MCM in asthma and COPD.

## 3.2 INTRODUCTION

The transfer and removal of acetyl groups from proteins and lysine residues of histones is a critical factor in the regulation of cellular functions and is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs)<sup>2</sup>. HDACs are targets for cancer and chronic disease therapies because of their important role in the transcriptional control of large numbers of genes, that may be aberrant in tumors or chronic disease<sup>3</sup>. Histone deacetylase inhibitors (HDACis) inhibit HDACs causing changes in gene transcription. Trichostatin A<sup>1</sup> and Na-Butyric acid are pan-HDAC inhibitors meaning that they inhibit all three classes of HDACs<sup>4</sup> while more recently MS-275 and FK228, class-specific HDACis, have been developed with the goal of increasing specificity and reducing toxicity<sup>3</sup>.

HDAC2 mRNA levels are reduced in the bronchial biopsies and alveolar macrophages of patients with severe asthma and COPD<sup>5</sup>. HDAC2 is also reported to suppress the expression of inflammatory genes. Furthermore, HAT activity is increased in the bronchial biopsies from the severely asthmatic population. These observations suggest that histone modification play a role in the regulation of inflammatory conditions of the lung<sup>5</sup>.

Aerosol exposure of mice to allergen is an *in vivo* approach to studying a major characteristic of human allergen-induced asthma. Exposure of ovalbumin-immunized mice to ovalbumin aerosols elicits allergic airway inflammation that causes epithelial cell hyperplasia and mucous cell metaplasia (MCM); as a result of prolonged exposure, hyperplastic AECs and mucous cells are removed via IFN $\gamma$  -induced cell death<sup>6</sup>.

The resolution of MCM is regulated by cell death regulator proteins that belong to the Bcl-2 family<sup>7</sup>. Bcl-2 homology (BH) regions characterize proteins from this family, Bcl-2, Bcl-xl, and Mcl-1 have four BH regions and carry out an anti-apoptotic function, while Bax and Bak with three BH regions are pro-apoptotic<sup>8</sup>. BH3 domain-only proteins as their name so implies possess one BH region, and include Bik, Bmf, Noxa, Puma, Bim, and others<sup>9</sup>. Interaction between anti-apoptotic and pro-apoptotic members is critical for apoptosis to occur<sup>8</sup>. A cell type specific function has been reported for many of the BH3-only proteins. For instance, BH3-only Bik was dispensable for cell death in cells of the hematopoietic compartment<sup>10</sup>, but is a critical mediator of IFN $\gamma$ -induced AEC death<sup>11</sup>. Bik is critical for the IFN $\gamma$ -induced resolution of MCM, as this process is disrupted in the *bik*<sup>-/-</sup> mice<sup>11</sup>. Pro-apoptotic Bax is also important for IFN $\gamma$ -induced cell death; it translocates to the ER, resulting in the release of calcium stores. Resolution is delayed in *bax*<sup>-/-</sup> mice<sup>12</sup>, demonstrating that Bax is also an important protein for the resolution of MCM.

In gastric carcinoma cell lines, pro-apoptotic BH3 domain-only Bmf has been shown to be important for cell death in response to the HDACis, FK228, and CBHA. In these cells, Bmf expression is increased as a result of increased acetylation of the histones H3 and H4 at the Bmf promoter<sup>13</sup>. While Bmf-deficient thymocytes and pre-B cells are undergoing apoptosis in response to the HDACi SAHA, they are not resistant to the other HDACis TSA or CBHA<sup>14</sup>. These results are indicative of different types of HDACs playing a role in Bmf-induced cell death.

Our findings suggest that AECs are very sensitive to pan-HDAC inhibitors, such as TSA and Na-Butyric acid, while slightly less sensitive to class I-specific MS-275. In AECs

we find that Bmf is strongly induced by HDACis through the displacement of HDAC1 from its promoter and is directly responsible for cell death in response to TSA.

Studies suggest that Bmf is sequestered to the DLC of the cytoskeleton and released in response to cellular stressors causing detachment-induced cell death in MEFs<sup>15</sup>. Translocation to the mitochondria is said to follow release from the cytoskeleton<sup>15</sup>. Further studies in Bmf-deficient mouse embryo fibroblasts and gastrointestinal epithelial cells did not support a critical role for Bmf during anoikis<sup>14</sup>. However, Bmf as a sensitizer to anoikis was reported in mammary epithelial cells<sup>16</sup>.

Because preliminary microscopic studies suggest that hyperplastic AECs detach from the basement membrane during resolution (unpublished), and previous studies suggested that Bmf is important for detachment-induced cell death, we investigated whether Bmf is involved in the IFN $\gamma$ -induced resolution of MCM. The present report shows that Bmf mediates HDACi-induced cell death in AECs, IFN $\gamma$  displaces HDAC1 from the Bmf promoter, and Bmf mediates IFN $\gamma$ -induced cell death. In addition, we show that Bmf is important for the resolution of MCM in the mouse model of asthma.

### **3.3 EXPERIMENTAL PROCEDURES**

#### **3.3.1. Cell Culture**

The preparation of MAECs was carried out as described<sup>11</sup>. The immortalized human airway epithelial cells, AALEB cells<sup>17</sup> were maintained in bronchial epithelial medium (BEGM, Lonza, Walkersville, MD)

#### **3.3.2 Animals**

Pathogen-free *bmf*<sup>-/-</sup> on the C57BL/6J background were a gift from A. Strasser (Walter and Eliza Hall Institute, Melbourne Australia). The *bmf*<sup>-/-</sup> and wild-type C57BL/6J mice were bred at the Lovelace Respiratory Research Institute (LRRRI) under specific pathogen-free conditions and genotyped as described<sup>18</sup>. All animal experiments were approved by the Institutional Animal Care and Use Committee. LRRRI is a facility approved by the Association for the Assessment and Accreditation for Laboratory Animal Care International. Sensitization and exposure of 8-10 wk old mice to ovalbumin was performed as described<sup>12</sup>. Preparation of lung tissues for histopathological examination and staining with Alcian blue and periodic acid Schiff or hematoxylin and eosin as described<sup>6</sup>. The number of Alcian blue-positive cells per millimeter of basal lamina was quantified using a light microscope (BH-2; Olympus) equipped with the Image analysis system (National Institutes of Health) as described<sup>19</sup>.

#### **3.3.3 Immunohistochemistry**

Lung tissue sections were deparaffinized, rehydrated, washed, and after antigen retrieval incubated with monoclonal rat anti-mouse Bmf (17A9 a gift from A. Strasser, Walter and Eliza Hall Institute, Melbourne, Australia) primary antibody at 1:500 dilution and anti-rat secondary antibody as described<sup>20</sup>. Immunohistochemical stains were

imaged using a microscope (Eclipse E600W; Nikon) with a Plan Fluor 60x NA 0.85 objective and a digital camera (DXM1200F; Nikon) with ACT-1 acquisition software (version 2.621 Nikon)

### **3.3.4 Laser Capture Microdissection**

While the left lungs were sectioned for histological analysis, the right lungs of these animals were used for laser capture microdissection (LCM). Right lungs from exposed animals were inflated by carefully injecting OCT (1:4 dilution in PBS) into the large airway, embedded in OCT, and snap frozen. Tissues were cryosectioned and prepared as is described in the manufacturer's protocol. AECs from five large airways were captured using a laser onto CapSure HS LCM Caps (Arcturus MDS Analytical Technologies, Sunnyvale, CA) using the Arcturus system (Arcturus, Bioscience, Inc. Mountain View, CA). Cellular lysate was extracted from the cap using the PicoPure RNA Isolation Kit as described by the manufacturer (Arcturus, Bioscience, Inc.).

### **3.3.5 mRNA Extraction and Analysis**

The RNeasy kit (Qiagen, Valencia, CA) was used for the extraction of RNA from cell pellets, and concentration was determined using the Thermo Scientific Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The primer/probe sets for Bad, Bak, Bax, Bcl-2, Bcl-xL, Bik, Bmf, CDKN1B, Muc5ac, Noxa, and Puma were obtained from (Applied Biosystems, Foster City, CA). Target mRNAs were amplified by quantitative real-time PCR in 20  $\mu$ l reactions on the ABI PRISM 7900HT Real-Time PCR System using the One-Step RT-PCR Master Mix (Applied Biosystems). Relative quantities from duplicate amplifications were calculated by normalizing

averaged  $C_T$  values to CDKN1B to obtain  $\Delta C_T$ , and the relative standard curve method was used for determining the fold change as described <sup>21</sup>.

### **3.3.6 Western Blot**

Total protein lysates were prepared using RIPA buffer (10 mM Tris, pH 7.4, 15 mM NaCl, 1% Triton X-100, 0.1% SDS, 5 mM EDTA, and protease inhibitors). For histone extraction experiments, lysates were prepared as described <sup>22</sup>. Protein concentration was determined by BCA kit (Pierce, Rockford, IL), and 25  $\mu$ g of protein lysate were analyzed by Western blotting as described <sup>17</sup>. We used the rat anti-Bmf 17A9 monoclonal antibody, a gift from Dr. Andreas Strasser (the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) at 2  $\mu$ g/ml. Mouse anti-actin polyclonal antibody (Santa Cruz Biotechnology, Inc.) was used at 1:5000. Goat anti-rat and rabbit anti-mouse secondary antibodies were used for visualizing proteins with chemiluminescence reagents (Perkin Elmer, Waltham, MA) using FujiFilm Image Reader LAS-4000 (Valhalla, NY).

### **3.3.7 Chromatin Immunoprecipitation**

After treating with 300 nM of TSA or 50 ng/ml hr IFN $\gamma$  for 18 or 48 h, respectively, cells were fixed with 1% formaldehyde, the reaction quenched using 1.25 M glycine, and scraped into cold PBS-containing protease inhibitors. Chromatin immunoprecipitation was performed using the MAGNA0002 Magna ChIP kit (Millipore, Billerica, MA) as described by the manufacturer. Sonicated nuclear fractions were incubated with polyclonal antibodies to acetyl H3 (06-599B Millipore), acetyl H4 (06-598 Millipore), rabbit polyclonal antibody to HDAC1 (06-720 Millipore), and rabbit IgG as control. DNA identification was confirmed with PCR using the following primers

specific for the Bmf promoter at region -560 5'-ACCTAAGGGCTCCCCTGGA-3', 5'-GCAGGTCGGAAGAAAAGTGCAGC-3', and region -97 5'-TTGGCGCTTCACTCGCCATT-3', 5'-ATCCCGCAAACAGCTGAT-3'.

### **3.3.8 Quantitative Analysis of Apoptosis**

Cells treated in 6-well tissue culture plates as described earlier were harvested using cold Trypsin-EDTA, washed twice in cold PBS, and resuspended in staining buffer (10 mM HEPES, 0.14M NaCl, 2.5 mM CaCl<sub>2</sub>, pH 7.4). Double staining with FITC-Annexin V and Propidium Iodide (PI) was carried out according to the manufacturer's recommendations and then analyzed by FACS to quantitate early apoptotic cells (Annexin V+, PI-) and late apoptotic cells (Annexin V+, PI+).

### **3.3.9 Adenoviral Expression**

RNA (1µg) extracted from AECs using The RNeasy kit (Qiagen, Valencia, CA) was reverse transcribed using oligo (dT) primers. The following primers were used for PCR, 5'-AGGTACCCACCATGGAGCCACCTCAGTGTG-3' and 5'-AGTCTAGATCACACAGGGCCCCACCCCTTC-3'. PCR was performed using the following conditions: 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. Bmf and GFP cDNAs were subcloned into the adenovirus expression shuttle vector. Shuttle CMV\_Bmf and Shuttle CMV\_GFP were packaged into virus-producing cells, and adenovirus particles were harvested as described <sup>23</sup>.

### **3.3.10 Statistical Analysis**

Fold changes, scanned density values, or differences in cell numbers between two groups were averaged and compared for significance between groups using *Student's t*-



test. Data were analyzed using Prism statistical analysis software, and  $p < 0.05$  was considered statistically significant.

## **RESULTS 3.4**

### **3.4.1 Histone deacetylase inhibitors (HDACis) induce Bmf expression through increasing histone acetylation and displacement of HDAC1.**

Based on previous reports in gastric carcinoma cell lines that Bmf is induced by the HDACi FK228 acetylating histone tails on the promoter region, we examined whether methylation or acetylation caused induction of Bmf mRNA in lung cancer cell lines. H1568, H2023, and H2228 cell lines treated with TSA showed a strong induction of Bmf mRNA (Figure 3-1A), while the methylation agent 5-aza-2' deoxycytidine (decitabine, DAC) produced no change in mRNA levels (data not shown). To determine whether this induction is specific to cancer cell lines only, we tested whether primary MAECs reacted to TSA in a similar fashion and found that both Bmf mRNA and protein were induced in response to TSA (Figure 3-1B). Experiments that looked at global acetylation levels of histone 4 in histone-containing lysates extracted from TSA-treated AALEBs showed dramatic increases of acetylated histone 4 (Figure 3-1C). ChIP assays confirmed the acetylation of histones H3 and H4 and the association of HDAC1 at the Bmf promoter. Primer sets were designed for DNA identification to span two different 100 base pair regions of the Bmf promoter, 97 base pairs and 560 base pairs upstream from the start site. We found that the acetylation levels of both histone 3 and histone 4 are increased in response to TSA treatment, and that that TSA diminished the interaction of HDAC1 with both of the Bmf promoter regions (Figure 3-1D).

### **3.4.2 Expression of pro-apoptotic Bmf induces apoptosis in AECs.**

Because Bmf has a pro-apoptotic function<sup>24</sup> and HDACi-induced cell death was mediated by Bmf in B cells<sup>14</sup>, we were interested to see if Bmf mediates HDACi-induced

cell death in AECs. AALEB cell numbers were reduced by 30-50% when treated with various HDACis including TSA, sodium butyrate, and MS-275 (Figure 3-2A). Reports that Bmf is a sensitizer to cell death but cannot induce it on its own<sup>16</sup> led us to test whether Bmf is pro-apoptotic in AECs. We developed an adenoviral expression vector (Ad-Bmf) (Figure 3-2B) and infected primary MAECs with Ad-Bmf and Ad-GFP at 50 MOI; this MOI was selected because expressed levels reproduced the level of Bmf expression that results from TSA treatment. Overexpression of Bmf reduced cell numbers by 50% compared to GFP control at 72 h (Figure 3-2B) suggesting that Bmf is pro-apoptotic in AECs. The extent of cell death was similar to that observed in response to TSA. Annexin V staining, which identifies the translocation of phosphatidylserine from the inner leaflet of the cell's membrane to the outer leaflet and is an early event in apoptotic cells<sup>25</sup>, showed that Ad-Bmf caused apoptotic cell death compared to Ad-GFP-infected controls. The apoptotic profile is very similar to that observed in response to TSA treatment (Figure 3-2C). Treatment of *bmf*<sup>+/+</sup> and *bmf*<sup>-/-</sup> MAECs with TSA showed that while 50% of *bmf*<sup>+/+</sup> MAECs undergo apoptosis, the *bmf*<sup>-/-</sup> MAECs were resistant to TSA treatment for 18 h (Figure 3-2D). However, when we reintroduced Bmf into the Bmf-deficient MAECs using Ad-Bmf, the TSA-induced cell death was restored, confirming that Bmf is crucial for HDACi-induced cell death in AECs (Figure 3-2E).

### **3.4.3 *bmf*<sup>-/-</sup> MAECs are resistant to IFN $\gamma$ -induced cell death.**

To investigate the relevance of Bmf expression causing cell death in an *in vivo* system, we chose the mouse model of asthma in which MCM is resolved after prolonged exposure to allergen. Our findings have repeatedly shown that IFN $\gamma$  is crucial for the resolution process<sup>6, 9, 11, 26</sup>. Therefore, *bmf*<sup>+/+</sup> and *bmf*<sup>-/-</sup> MAECs were treated with murine

recombinant IFN $\gamma$ , and viability was assessed after 96 h of treatment. Bmf-deficiency rendered AECs more resistant to IFN $\gamma$  (Figure 3-3A), suggesting that Bmf mediated IFN $\gamma$ -induced cell death. Because Bmf expression was regulated by HDACs interacting with its promoter, we tested the involvement of HDACs by chromatin immunoprecipitation assays using HDAC1 antibodies. AALEB cells treated with IFN $\gamma$  for 48 h showed decreased association of HDAC1 with the Bmf promoter compared to non-treated controls (Figure 3-3B), suggesting that IFN $\gamma$  inactivated HDAC1 to induce Bmf promoter activity.

#### **3.4.4 Bmf expression is important during the resolution of mucous cell metaplasia.**

These results indicated that the resolution of allergen-induced MCM may be mediated by IFN $\gamma$ . Therefore, we postulated that Bmf expression may be increased during resolution when dying cells appeared to detach from the basement membrane (unpublished). Indeed, Bmf protein was detected by immunohistochemistry in the lung tissues of mice exposed to ovalbumin for 15 d (Figure 3-4A), when epithelial cell numbers are reduced<sup>11</sup>. To further address the question as to whether Bmf is important during the resolution of MCM, 15 *bmf*<sup>+/+</sup> and 15 *bmf*<sup>-/-</sup> mice were exposed to allergen. Lung tissues were harvested at 5, 10, and 15 d after exposure, and the number of mucous cells per mm of basal lamina was quantified. The *bmf*<sup>-/-</sup> mice did not show a statistically significant decrease in mucous cell numbers at 15 d post allergen exposure as did the *bmf*<sup>+/+</sup> mice, suggesting that Bmf expression is important for the reduction of mucous cell numbers (Figure 3-4B).

RNA was extracted from AECs captured by LCM and analyzed using qRT-PCR<sup>21</sup>. *Muc5ac*, a mucin gene that constitutes most of the induced mucin following

inflammatory responses<sup>27</sup>, showed strong induction at 5 d post allergen exposure as expected and was decreased at 10 and 15 d in both the *bmf*<sup>+/+</sup> and *bmf*<sup>-/-</sup> mice (Figure 3-4C). This suggests that the increased presence of mucous cells in the *bmf*<sup>-/-</sup> mice at 15 days is not a result of increased *muc5ac* mRNA levels.

Eleven Bcl-2 family members that are important in AEC regulation including Bik, Bax, and others were analyzed. While no significant change was observed for other Bcl-2 family members, Bax and Bik mRNAs were significantly increased in the *bmf*<sup>-/-</sup> airways at 15 d post-exposure compared to *bmf*<sup>+/+</sup> airway cells (Figure 3-4D), while in the *bmf*<sup>+/+</sup> mice *bax* mRNA levels decreased at 15 days and *bik* mRNA levels remained unchanged (data not shown). A delayed increase in Bax and Bik in *bmf*<sup>+/+</sup> mice expression suggests that mucous cell death may be delayed in the knockout animals.

### 3.5 DISCUSSION

In the present study we show that HDAC inhibitors induce Bmf expression that is sufficient to induce apoptosis in AECs. Studies show that the Bcl-2 family of proteins and the intrinsic cell death pathway are important in HDACi-induced cell death in malignant cells<sup>28</sup>, but it is likely that HDACi-induced cell death is cell type specific, because the extrinsic pathway is important in cell death for glucocorticoid-resistant acute lymphatic leukemia cells<sup>1</sup>. Studies suggesting that nonmalignant cells undergo apoptosis, while normal cells are protected lead to questions about how normal primary lung epithelial cells respond to HDACi treatment. Recent reports show that TSA has apoptotic effects in primary human eosinophils, neutrophils, and macrophages<sup>29</sup>. It has also been reported that TSA treatment increases apoptosis in neuronal cells rendering these cells more vulnerable to certain neurotoxins and increasing susceptibility to Parkinson's disease<sup>30</sup>. It is clear that HDACis have varying effects in different cell types and that the exact mechanisms of HDACi-induced cell death are still being elucidated. It is important to understand the effects of HDACis on normal cells, because these agents are in clinical trials for the treatment of human diseases and have implications for the treatment of chronic lung disease.

These studies also demonstrate that TSA inhibits HDAC1 association with the Bmf promoter, increasing acetylation levels of histones 3 and 4. To thoroughly explore the effects of HDACis on Bmf mRNA expression, HDACis from three major classes of inhibitors were tested: TSA of the hydroxamic acid class, sodium butyrate of the carboxylic acid class, and MS-275 of the benzamide class<sup>28</sup>, all of which were very strong inducers of Bmf mRNA (our manuscript in review). Of particular interest was the

induction of Bmf by MS-275, which unlike the other inhibitors specifically targets Class I HDACs, narrowing down the HDAC responsible for repression of Bmf to HDAC1, HDAC2, or HDAC8<sup>28</sup>. This information as well as data suggesting that the overexpression of HDAC1 in gastric carcinoma cells reduces Bmf expression<sup>13</sup> led us to investigate the effects of HDAC1 in Bmf regulation in AECs. HDAC1 is important for transcriptional repression of genes in AECs such as, Cyclooxygenase 2 (Cox 2). HDAC1 degradation occurs in response to diesel particle exposure, thus upregulating Cox 2, causing inflammation, and consequently leading to asthma exacerbations and COPD<sup>31</sup>. HDAC1 is targeted for degradation preferentially over HDAC2 and HDAC3<sup>31</sup>, and may be a very important enzyme for gene regulation in AECs.

Bmf-deficient MAEC numbers are not reduced in response to IFN $\gamma$  treatment. Because *bmf*<sup>-/-</sup> MAECs behave similarly in response to HDACi treatment, the idea that HDAC1 regulates Bmf in response was tested. ChIP demonstrated that HDAC1 is displaced from the Bmf promoter in response to IFN $\gamma$ . Although HDAC1 is displaced from the Bmf promoter in response to IFN $\gamma$  treatment similarly to what is seen in response to HDACi treatment, we do not detect an induction of Bmf mRNA or protein when treating wild-type MAECs with IFN $\gamma$ . Future studies that focus on capturing the dying cells after IFN $\gamma$  treatment by using a caspase inhibitor will be important to assess whether the dying cells may express Bmf. IFN $\gamma$ -induced cell death is a slow process showing a 30% reduction in AEC numbers after 72 h of treatment in nonconfluent cells<sup>17</sup>. In AECs, HDACi treatment is a potent killer; 50% of AECs undergo cell death after 18 h of treatment. Because AECs are naturally exposed to IFN $\gamma$ , it is a physiologically relevant<sup>6, 11, 12, 17</sup> system and perhaps more subtle and regulated in its effects. AECs are

not normally exposed to HDACis, possibly explaining why the effects of Bmf induction are so widespread. Another interesting point is that although we see displacement of HDAC1 in response to IFN $\gamma$  treatment, it may not be accompanied by hyperacetylation of histone tails. Research suggests that HDACs 1, 2, and 3 are necessary for IFN $\gamma$  signaling through STAT1<sup>32</sup>, and HDAC inhibition as a result of HDACi treatment prevents nuclear translocation of STAT1. HDACs may be recruited to another location other than the Bmf promoter within the nucleus in response to IFN $\gamma$  for specific functions relating to STAT1 signaling. IFN $\gamma$  causes many effects on AECs, some of which are not fully understood at this time; therefore, further studies looking at the functional response of Bmf to IFN $\gamma$  are necessary.

The findings from the in vivo mouse asthma model show that Bmf expression is important for the resolution of MCM, because in its absence the clearance of hyperplastic mucous cells is abrogated. Although Bmf protein was detectable at 15 d of allergen exposure, the mRNA level showed no overall change. Bmf protein is expressed at 15 d post exposure, but there is no induction of Bmf mRNA at 10 and 15 d when compared to 5 d. Immunohistochemistry studies demonstrate that Bmf protein is induced in approximately 20% of epithelial cells; induction of mRNA levels in this small population may be too subtle when considering all epithelial cells for mRNA analysis. It also may be that induction of Bmf mRNA is actually seen at times other than 5, 10, or 15 d; it may occur at an earlier or later time point. Further experiments with a more expansive time course will provide insight into this question. A heterogeneous mixture of cells is collected for LCM from the epithelium, including ciliated and nonciliated mucus-secreting cells, which may offer another explanation as to why Bmf mRNA levels do not



appear to be induced in the exposure model. The different cell types of the epithelium may have different expression levels of Bmf. The initial increase in total AEC number seen in the *bmf*<sup>+/+</sup> mice, which reflects increased mucous cell numbers at 5 d, is not observed in the *bmf*<sup>-/-</sup> mice. These perplexing results lead to the hypothesis that the total cell number does not increase because the ciliated cells are turning over and the nonciliated mucous secreting cells are maintained in the Bmf-deficient airways. One type of cell is dying while the other type is simultaneously proliferating, so no overall effect is detected. Evidence suggests that epithelial cells have different expression patterns that affect their identities. For instance, the loss of transcription factors E2f4 or foxj1 results in a loss of ciliated cells in the epithelium<sup>33,34</sup>. Other examples of ciliated cell-specific genes include structural proteins such as  $\beta$ -tubulin IV<sup>27</sup>. Examples of genes specific for mucous-secreting cells include Muc5ac, Clara Cell Secretory Protein (CCSP), and Rab27a<sup>27,35</sup>. These mechanisms are complex; however, they support the idea that cell-type specific regulation is a factor in the development and maintenance of the airway epithelium. Further studies are important to illuminate the validity of this hypothesis. Immunofluorescence experiments are underway to locate Bmf protein in sections of allergen-exposed airways, while co-staining for markers specific for ciliated cells and nonciliated mucus-secreting cells. The development of AEC cultures that consist predominantly of mucus-secreting cells or ciliated cells will help to elucidate whether Bmf regulates cell death in these cells.

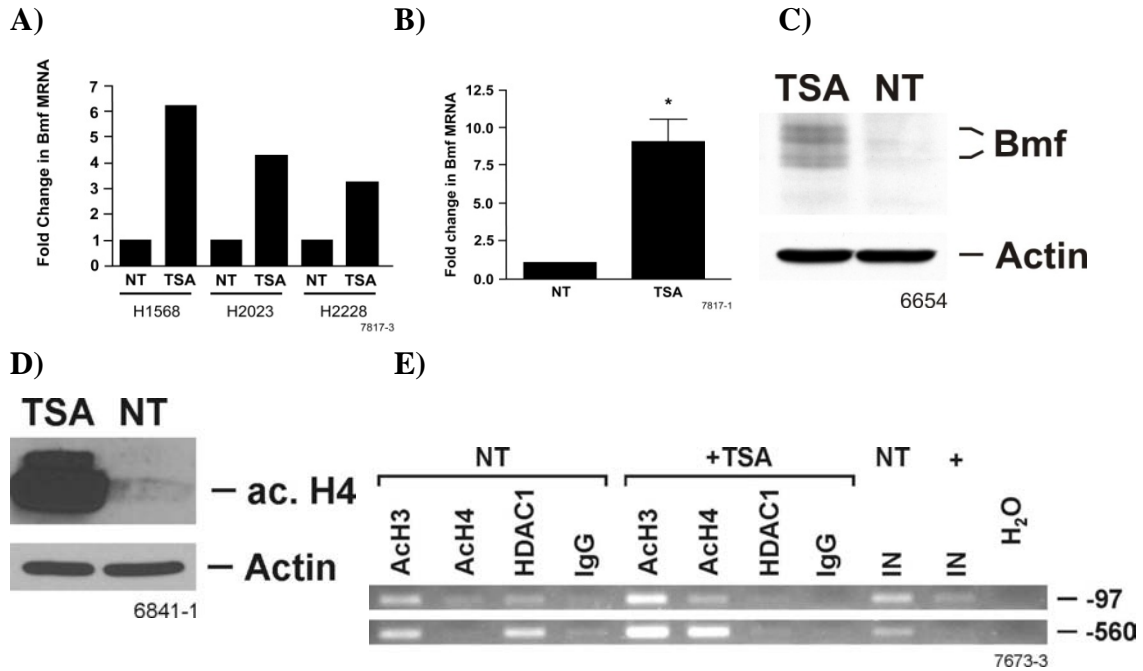
Mucous cell metaplasia in response to allergen exposure is characterized by the induction of Muc5ac mRNA levels, which is the predominant mucin gene induced in AECs<sup>27,36</sup>. Although mucous cells are sustained at 15 d post exposure in the Bmf-

deficient animals, Muc5ac mRNA levels reflect those observed in the wild-type animals; levels are high at 5 d and reduced at 10 and 15 d. This indicates that resolution is important for the reduction of cell numbers; mucin protein can still exist within the mucous cells while Muc5ac mRNA levels have returned to pre-allergen exposure levels.

Further investigation into the finding that there are differences in expression of Bcl-2 family members, which are major regulators of IFN $\gamma$ -induced cell death, and the resolution of MCM may provide information as to why MCM is not resolved in the *bmf*<sup>-/-</sup> mice. LCM and real-time PCR results in the Bmf-deficient animals show an induction of Bik and Bax at 15 d, an induction not seen in the wild-type animals. Suggesting that regulation differs in the Bmf-deficient animals, this finding leads to the question as to whether Bax is translocating to the ER in the *bmf*<sup>-/-</sup> animals, as is seen in wild-type animals or whether the function of Bik is abrogated in the absence of Bmf, delaying cell death. Other data suggest there may be an important relationship between Bik and Bmf; when overexpressing Bmf in *bik*<sup>-/-</sup> MAECs cell death is delayed by 24 h (unpublished data) in comparison to wild-type MAECs. Cell death is also delayed when Bik is overexpressed in *bmf*<sup>-/-</sup> MAECs compared to wild-type cells. These two proteins may function together to carry out cell death in AECs. This is an important area that requires further investigation and the development of a *bmf*<sup>-/-</sup>*bik*<sup>-/-</sup> mouse to test this hypothesis. In conclusion, these results show that HDACs induce Bmf expression through the displacement of HDAC1 from the Bmf promoter, causing cell death in AECs through the induction of Bmf. Furthermore, resolution of allergen-induced MCM is mediated by IFN $\gamma$  blocking HDAC1 interaction with the Bmf promoter, causing an induction in Bmf

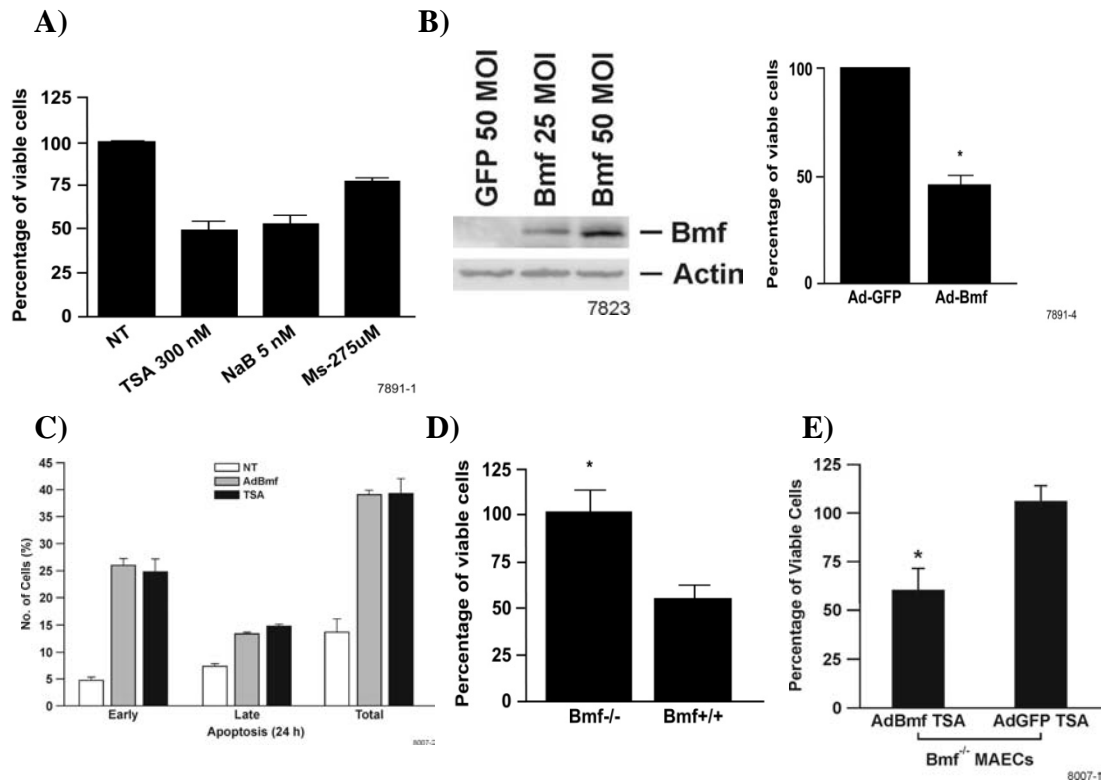
expression. These findings support further investigations to evaluate whether HDAC1-specific inhibitors may be effective for reducing MCM in asthma and COPD.

### 3.6 FIGURES AND LEGENDS



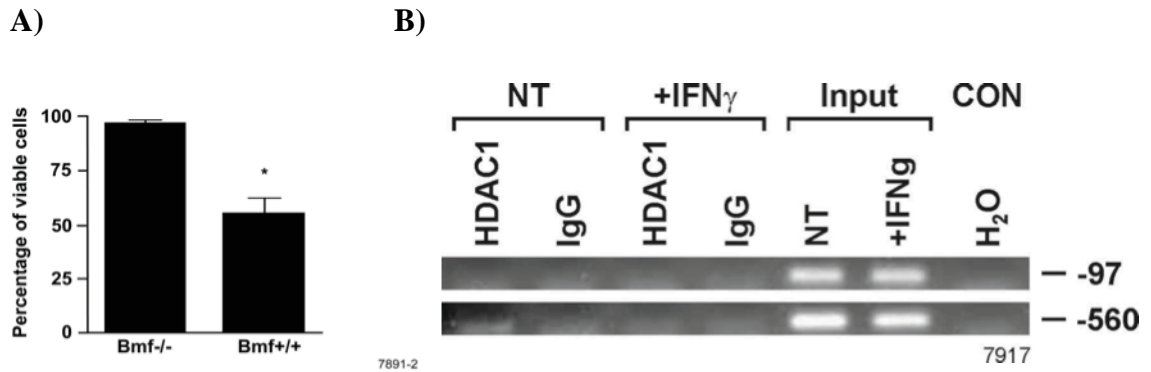
**Figure 3-1. Histone deacetylase inhibitors (HDACis) induce Bmf expression through increasing histone acetylation and displacement of HDAC1.**

(A) qRT-PCR results show that Bmf mRNA is induced in response to HDACi, TSA in three different lung cancer cell lines: H1568, H2023, and H2228. (B) TSA induces Bmf mRNA and (C) protein levels in MAECs. (D) Lysates containing extracted histones from TSA-treated AALEBs show global acetylation of histone 4. (E) Chromatin immunoprecipitation of nuclear lysates prepared from AALEB cells treated with TSA, using anti-acetylhistone 3, anti-acetylhistone 4, and anti-HDAC1 reveal that HDACis enhance acetylation of histone 3 and histone 4, and diminish the association of HDAC1 with the Bmf promoter.



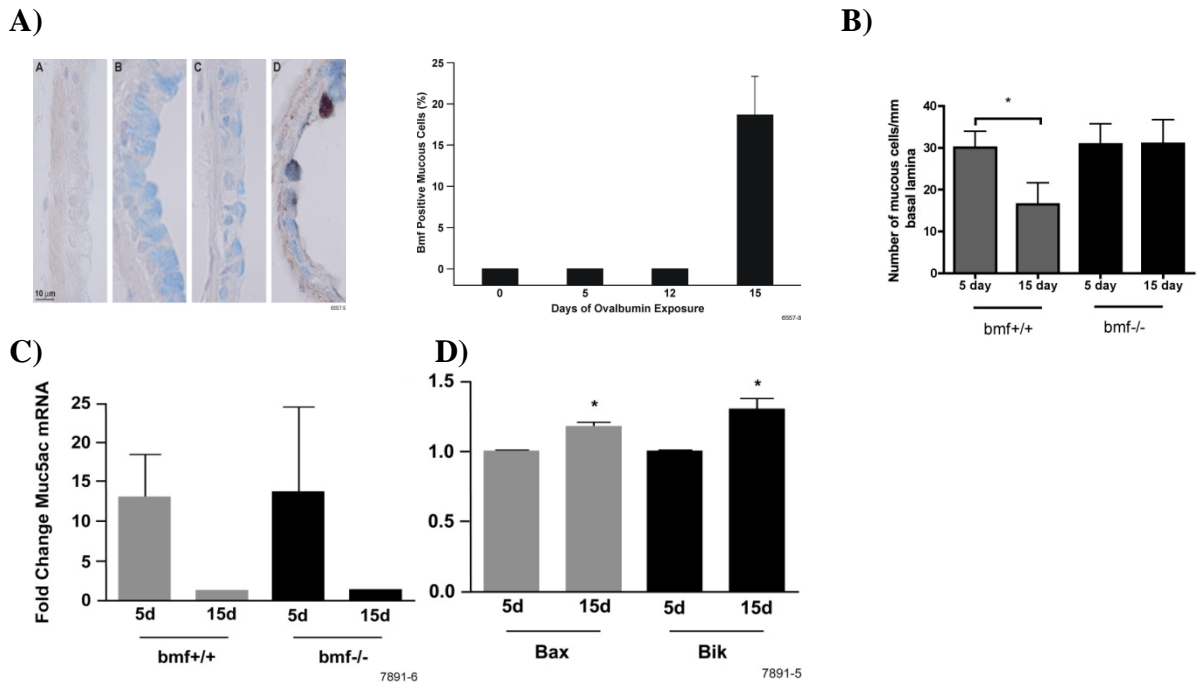
**Figure 3-2. Bmf causes apoptosis.**

(A) AALEB cells were treated with nothing, 300 nM TSA, 5 mM NaB or 5  $\mu$ M of MS-275 for 18 hr. Cells were harvested and quantified using trypan blue exclusion. Cell numbers were reduced significantly in HDACi-treated groups. (B) MAECs overexpressing Bmf protein as a result of infection with Ad-Bmf at 50 MOI show a reduction in cell number compared to MAECs infected with Ad-GFP at 50 MOI. (C) Annexin V staining of TSA-treated MAECs compared to non-treated and Ad-Bmf-infected MAECs compared to Ad-GFP infected MAECs. (D) HDACis induce cell death in *bmf*<sup>+/+</sup> MAECs; however, *bmf*<sup>-/-</sup> MAECs are resistant to HDACi-induced cell death. *bmf*<sup>+/+</sup> and *bmf*<sup>-/-</sup> MAECs were treated with 300 nM of TSA for 18 hr. Cells were quantified by trypan blue exclusion assay. (E) *bmf*<sup>-/-</sup> MAECs, were infected with 50 MOI Ad-Bmf or Ad-GFP and treated with TSA for 18 hr. Cells were harvested and quantified. Ad-Bmf restores cell death compared to Ad-GFP-infected cells treated with HDACi.



**Figure 3-3. IFN $\gamma$ -induced cell death is reduced in Bmf-deficient MAECs.**

A) The percentage of viable cells is not decreased after 96 hr of IFN $\gamma$  treatment in *bmf*<sup>-/-</sup> MAECs compared to *bmf*<sup>+/+</sup>. Cells were harvested and quantified using trypan blue exclusion. B) Chromatin immunoprecipitation of nuclear lysates prepared from AALEB cells treated with IFN $\gamma$  show that HDAC1 is displaced from the Bmf promoter after 48 h treatment.



**Figure 3-4. Bmf is important during the resolution of MCM.** A) Bmf protein is induced in vivo during resolution of MCM. Immunohistochemistry was used to stain for Bmf in 0 d, 5 d, 10 d, and 15 d sectioned tissues of ovalbumin-exposed mice. A statistically greater number of cells are positive for Bmf in the 15 d tissues. B) Resolution of MCM as a result of prolonged exposure to allergen is inhibited in *bmf*<sup>-/-</sup> MAECs. ABH&E was used to stain sectioned tissues from 5 d, and 15 d *bmf*<sup>-/-</sup> and *bmf*<sup>+/+</sup> mice. Tissues from five mice from each group were blinded, and mucous cells/mm basal lamina were quantified. There is a statistically significant decrease in mucous cell numbers at 15 d compared to 5 d in the wild-type animals. This difference is not observed in the *bmf*<sup>-/-</sup> mice. C) qRT-PCR showing Muc5ac mRNA levels from LCM captured AECs from the right lungs of 5 and 15 d ovalbumin-exposed *bmf*<sup>+/+</sup> and *bmf*<sup>-/-</sup> mice. D) LCM captured AECs from ovalbumin-exposed mice show altered mRNA expression levels of other Bcl-2 family members in the Bmf-deficient mice. qRT-PCR results show that Bax and Bik mRNA levels are significantly induced at 15 d in the AECs of ovalbumin-exposed *bmf*<sup>-/-</sup> mice compared to 5 d.

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## **Chapter 4**

### **Discussion**

In the present studies, we investigated the regulation and function of Bmf in AECs with a focus on two apoptotic stimuli, IFN $\gamma$  and HDACis. IFN $\gamma$  is important for the regulation of cell numbers in the airway epithelium and causes the resolution of MCM. Because MCM is a crucial component of chronic lung disease, including asthma, these studies contribute to clarifying the role of Bmf in asthma. Our studies on the regulation of Bmf by HDACis was based on previous studies in cancer cells and led us to identify Bmf as a mediator of HDACi-induced cell death. Therefore, HDACis may be useful for treatment of chronic lung disease to reduce MCM.

**Specific questions generated from the major findings of these studies.**

- 1) Is the suppressive effect of p53 on Bmf important for IFN $\gamma$ -induced cell death?
- 2) Although Bmf is downregulated by IFN $\gamma$  it appears to be important in IFN $\gamma$ -induced cell death; therefore, we are interested to investigate what role Bmf plays in IFN $\gamma$ -induced cell death.
- 3) Why do Bmf expression levels vary greatly between tissue and cell types, and what is the role of the different Bmf isoforms in AECs?
- 4) The fact that Bmf is important during the resolution of MCM suggests that Bmf functions in conjunction with other pro-apoptotic Bcl-2 family members that are also crucial for this resolution.
- 5) Do mouse airway epithelial cells (MAECs) from Bmf-deficient mice attach to cell culture plates better than Bmf-sufficient MAECs because their survival is longer during the time they are detached from the basement membrane?

6) Can HDACis be used to induce Bmf specifically in mucous cells to initiate cell death in metaplastic mucous cells?

#### **4.1 The role of p53 in the regulation of Bmf expression by IFN $\gamma$ and HDACis**

Experiments presented here show that IFN $\gamma$  treatment causes nuclear accumulation of p53 and increased association with HDAC1. This association resulted in the reduction of acetylated p53 and the interaction of p53 with the Bmf promoter (Figure 4-1). p53 is activated through posttranslational events, such as acetylation and phosphorylation<sup>1</sup>. The acetylation of p53 by histone acetyltransferases (HATs) induces p53-dependent growth arrest and apoptosis, while deacetylated p53 promotes cell survival in cancer cell lines<sup>1</sup>. In support of these previous findings, IFN $\gamma$  deacetylated p53 and suppressed Bmf expression, suggesting that p53 has an inhibitory role in IFN $\gamma$ -induced cell death. This cell death inhibitory role of p53 requires further investigation, because of the overwhelming evidence for the pro-apoptotic role of p53 in response to many stimuli<sup>2</sup>. However, there is support that p53 does play an anti-apoptotic role as well. Physiological-relevant levels of p53 protect fibroblasts from undergoing serum withdrawal-induced apoptosis<sup>3</sup>. In addition, anti-apoptotic roles for p53 in cellular processes such as DNA repair and the cell cycle have been established<sup>4</sup>. Experiments designed to identify the region of p53 that interacts with HDAC1 will allow for the development of a p53 mutant that is unable to interact with HDAC1. IFN $\gamma$  would be unable to deacetylate this mutant p53 and sustaining acetylated p53 would enhance cell death, if this pathway is suppressing cell death.

IFN $\gamma$  affects the airway epithelium in several ways; it mediates the resolution of airway epithelial hyperplasia by inducing apoptosis<sup>5,6</sup>, it has inhibitory effects on the development of mucous cells<sup>7</sup>, and at low concentrations it enhances the development of mucous cell metaplasia (MCM)<sup>8</sup>. Our studies demonstrate that two Bmf isoforms are expressed in AECs, so it is possible that IFN $\gamma$  affects the regulation of Bmf isoforms differently. Studies in lymphoma cells show that three Bmf transcripts are translated into isoforms of Bmf. Bmf (Bmf I) is pro-apoptotic, and Bmf II and Bmf III promote survival and growth<sup>9</sup>. Through the development of Bmf transcript-specific primers, it will be possible to delineate which forms of Bmf are affected in AECs in response to IFN $\gamma$ . However, because Bmf II, a pro-survival protein, was suppressed by IFN $\gamma$ , expressing the p53 mutant that is deficient in interacting with HDAC1 will likely suppress cell death. These studies will clarify whether the suppressed Bmf will enhance or suppress cell death.

Although Bax is crucial for IFN $\gamma$ -induced cell death<sup>10</sup>, Bax mRNA is also downregulated in response to IFN $\gamma$ . Future studies will investigate whether p53 regulates Bax and Bmf mRNAs by similar mechanisms. Western blot analysis of lysates from *p53*<sup>-/-</sup> and *p53*<sup>+/+</sup> MAECs treated with IFN $\gamma$  will show whether p53 suppresses Bmf or Bax, supporting the idea that p53 may have an inhibitory role in the IFN $\gamma$ -induced cell death pathway.

Previous studies show no difference in IFN $\gamma$ -induced cell death in *p53*<sup>+/+</sup> and *p53*<sup>-/-</sup> MAECs at 96 h<sup>11</sup>. However, it is important to analyze the cell death of *p53*<sup>-/-</sup> MAECs at 24, 48, and 72 h because cell death may be delayed in the p53-deficient cells. It is possible that at earlier time points the *p53*<sup>-/-</sup> MAECs are more resistant to cell death and

that shortly before 96 h a p53-independent mechanism drives the cell death process so that a large number of cells undergo apoptosis in a delayed manner. This may account for the similar cell numbers in the *p53<sup>+/+</sup>* and *p53<sup>-/-</sup>* MAECs after 96 h of IFN $\gamma$  treatment.

IFN $\gamma$  dramatically induces Noxa mRNA and protein levels within 1 h (data unpublished). Therefore we investigated whether Noxa mediates IFN $\gamma$ -induced nuclear accumulation of p53. Western blot analysis of nuclear lysates from *noxa<sup>+/+</sup>* and *noxa<sup>-/-</sup>* MAECs treated with IFN $\gamma$  (data not shown) showed no difference, suggesting that Noxa is not involved in the accumulation of p53 in the nucleus. Future studies will test whether other signaling proteins that are activated by IFN $\gamma$  treatment including JNK or ERK1/2<sup>11</sup> are involved in IFN $\gamma$ -induced effects on p53.

Our studies show that p53 suppressed the Bmf promoter activity not only in response to IFN $\gamma$  but also following UV irradiation (Figure 2-2) or cytochalasin D treatment, (Appendix C). Bmf promoter activity is also suppressed in response to HDAC1 in cancer cell lines<sup>12</sup>. The fact that HDACi treatment acetylated histones 3 and 4 and displaced HDAC1 suggests that chromatin remodeling plays a crucial role for Bmf regulation in AECs as well. Acetylation of histone tails causes DNA to relax, allowing transcription factors to access the Bmf gene<sup>13</sup>. The Bmf gene promoter does not contain any p53-binding motifs; therefore, it is likely that p53 is part of a protein complex that suppresses transcriptional activity. It is important to identify these p53-interacting proteins in order to elucidate the role of p53 in the suppression of Bmf. Identification of these proteins will be the first step in clarifying the molecular events that occur when p53 is deacetylated by HDAC1 in response to IFN $\gamma$ . In addition, these studies will help

clarify the role of p53 in chromatin remodeling that appears to be independent from HDAC-mediated suppression of Bmf transcription.

#### **4.2 The role of Bmf in IFN $\gamma$ -induced cell death**

Our studies show that *bmf*<sup>-/-</sup> MAECs are resistant to IFN $\gamma$ -induced cell death at 96 h (Figure 3-3). Bmf mRNA and protein expression were downregulated at 48 h, however Bmf levels have not been examined at 96 h of IFN $\gamma$  treatment in non-differentiated cells. IFN $\gamma$  induces cell death in 30 % of AECs after 72 h, but in 50% after 96 h. Bmf may be important for the accelerated levels of apoptosis observed between 72 and 96 h. Because Bmf may be induced in expression at later time points when cells undergo cell death, future studies will examine cell death in *bmf*<sup>-/-</sup> MAECs over 24, 48, and 72 h of IFN $\gamma$  treatment. If cell death is delayed over 96 h, we will conclude that Bmf is important for cell death at the later time points in response to IFN $\gamma$ .

The findings that Bmf expression is suppressed by IFN $\gamma$  while Bmf mediates IFN $\gamma$ -induced cell death suggest that the low levels of Bmf protein present in AECs are sufficient to cause cellular death. It is possible that controlling Bmf at low levels may be crucial to ensure the selective induction of cell death in hyperplastic AECs rather than eliminating the majority of AECs that are in the G0 phase if high levels of Bmf were to be expressed. Immunofluorescent staining to observe the translocation of Bmf protein in response to IFN $\gamma$  in MAECs will help identify the cell compartments and the proteins involved.

As discussed in Chapter 3, another explanation for this paradox may be that Bmf is only necessary to facilitate cell death in mucus-secreting cells of the epithelium (Figure 4-2) while being downregulated in the other cell types. Because our *in vitro* studies used



non-differentiated epithelial cells, the regulation may not represent the complexity of diverse cell types found in the airway epithelium.

In an effort to explore the cell type-specific affect of Bmf in mucus-secreting and ciliated cells of the epithelium *bmf*<sup>-/-</sup> and *bmf*<sup>+/+</sup>, MAECs were grown on Transwell membranes until confluent and driven to differentiation in air-liquid interface conditions. These cultures were treated with IFN $\gamma$  for 48 h to simulate the conditions of prolonged allergen exposure in airway epithelia *in vivo*. The purpose of this study was to test whether IFN $\gamma$  has a specific effect on the Bmf-deficient cultures, and to use immunostaining to evaluate whether there is heterogeneous expression of Bmf within the cell types in wild-type cultures. This study is in progress. ABH&E staining will allow for the quantification of cell numbers/mm basal lamina. Immunofluorescent staining will visualize ciliated and mucous cells with antibodies to  $\beta$ -tubulin or Muc5ac, respectively. Co-immunostaining for Bmf in the *bmf*<sup>+/+</sup> MAECs will show whether expression is specific to the different cell types. Our hypothesis is that there will be more mucous cells in the IFN $\gamma$  treated *bmf*<sup>-/-</sup> cultures and fewer ciliated cells, and that Bmf expression will co-localize with Muc5ac expression. Alternatively, future experiments will investigate IFN $\gamma$ -induced Bmf expression in the various cell types of the airway epithelium using cultures that predominantly consist of either mucus-secreting or ciliated cells.

To explore the cell type-specific function of Bmf *in vivo*, transgenic approaches can be used either to overexpress Bmf using cell type-specific promoters or by using the Cre/loxP system to target gene expression in mucus-secreting or ciliated cells. Expression of a gene can be targeted to Clara cells by driving the expression of Cre-recombinase with the CCSP promoter<sup>14</sup>. To selectively delete Bmf in these cells, CCSP

can be flanked by two lox P sites. A mouse expressing Cre-recombinase under the CCSP or the FOXP1 promoters<sup>15</sup> will then be bred into a mouse expressing floxed Bmf. When Cre-recombinase is expressed, the segment of DNA in between the two lox P sites will be excised, knocking out Bmf either in Clara or ciliated cells. Clara cells differentiate into mucus-secreting cells of the small airway and will provide information about the function of Bmf in all mucus-secreting cells. Generating such mice will allow for the investigation of Bmf function in mucus-secreting or ciliated cells *in vivo* because prolonged exposure of these transgenic mice to allergen will allow the delineation of Bmf function in mucous cells. Our hypothesis is that Bmf-deficient mucus-secreting cells will be unable to undergo apoptosis, while apoptotic pathways will be unaffected in ciliated cells.

### **4.3 Bmf expression in the lung**

Protein lysates extracted from various murine organs showed that significant Bmf protein levels are found in the lung liver and spleen tissues but not in the heart (Appendix A and data not shown). Interestingly, while high levels of Bmf protein were detected in lung tissue, they are not detected in MAECs. Previous studies have analyzed various cell types of the hematopoietic compartment for Bmf protein expression; very high expression levels are found in the immature CD4+8+ thymocytes, while much lower levels are found in the mature resting T cells or mitogen-stimulated T cell lymphoblasts. Bmf expression is found throughout the B cell development with the exception of pro-B cells, in which no expression is detected<sup>16</sup>. Because, in cultured AECs, Bmf protein expression is detected at much lower levels than that detected in the lung tissue, we assume that Bmf is expressed in cell types other than epithelial cells. Immunofluorescent

studies are needed to identify the cell types that express Bmf protein within the lung. Should Bmf be expressed in AECs of intact lungs, we will conclude that epithelial cells when cultured suppress Bmf expression. It is also possible that Bmf is expressed in types I and II cells, or in macrophages, both of which are abundant in normal lungs. However, because the lung structure and function of *bmf*<sup>-/-</sup> mice appear normal, Bmf does not seem to have a role in the development of the lung.

Similar to findings of Verna et al. in hematopoietic cells, we find that two isoforms of Bmf are expressed in lung tissue as well as AECs<sup>1</sup>. Because different isoforms of Bmf with potentially different functions are expressed in the lung, we have generated an adenoviral expression system that expresses a single pro-apoptotic isoform of Bmf. However, when AECs are infected with this virus two Bmf-specific protein bands were detected on Western blots (Appendix E). The smaller band corresponds to the molecular weight of Bmf, suggesting that the larger Bmf isoform may actually represent Bmf with posttranslational modifications. Future studies will also generate adenoviral expression vectors to specifically express the larger Bmf isoforms and allow the investigation of these isoforms in AECs.

#### **4.4 The functional role of Bmf during resolution of MCM**

Results from the allergen exposure study suggest that mucous cell numbers remain higher in the *bmf*<sup>-/-</sup> compared to *bmf*<sup>+/+</sup> mice after 15 d of allergen exposure. However, Bik and Bax were induced in epithelia of *bmf*<sup>-/-</sup> mice exposed to allergen for 15 d. These findings suggest that resolution of AEC hyperplasia was delayed in *bmf*<sup>-/-</sup> mice and may occur if mice are exposed for longer periods. Therefore, in the future, 30 mice will be exposed to allergen and groups of 5 sacrificed at 0, 2, 5, 10, 20, and 30 d. Earlier

time points will be included to determine whether Bmf mRNA may be increased in *bmf*<sup>-/-</sup> mice at 2 or 5 d. Analysis of the airway epithelium of *bmf*<sup>-/-</sup> mice at 20 and 30 d will allow us to evaluate whether the delayed induction of Bik and Bax will drive the resolution of MCM.

IFN $\gamma$ -induced cell death is a complex process involving different members of the Bcl-2 family of proteins such as, Bax, Bak, Noxa, and Bik. The finding that IFN $\gamma$ -induced cell death is inhibited in *bmf*<sup>-/-</sup> MAECs suggests that Bmf mediates this cell death process. In addition, overexpression of Bik protein, a central mediator of IFN $\gamma$ -induced cell death in AECs<sup>11</sup>, induced Bmf mRNA (data not shown). In *bik*<sup>-/-</sup> MAECs, Bmf expression causes cell death 24 h later than it does in *bik*<sup>+/+</sup> MAECs (unpublished). Cell death is also delayed when Bik is expressed in *bmf*<sup>-/-</sup> compared to *bmf*<sup>+/+</sup> MAECs. These results support the idea that Bik and Bmf may function in tandem. Because Bik is induced in *bmf*<sup>-/-</sup> MAECs (Figure 3-4), we hypothesize that Bik is upstream of Bmf. Bik is induced at 24 h<sup>11</sup> and may cause Bmf to translocate to specific organelles to cause cell death. Immunofluorescent staining in time course experiments after IFN $\gamma$  treatment will assess the time of Bmf translocation. In addition, infection of cells with adenoviral vectors expressing Bik will assess whether Bik mediates Bmf translocation. Control studies will use *bik*<sup>-/-</sup> MAECs to elucidate whether Bik mediates IFN $\gamma$ -induced Bmf translocation.

To further evaluate the functional relationship between Bmf and Bik, it is important to generate cells in which both genes are deleted. Breeding of *bmf*<sup>-/-</sup>*bik*<sup>-/-</sup> mice would allow for the investigation as to whether these proteins are dependent on one another to carry out cell death. We assume that the mutual deletion of these two proteins would

render AECs unable to undergo apoptosis in response to IFN $\gamma$ . Reintroducing expression of each of these proteins individually will allow us to examine the role and function of each protein and determine whether both proteins are required to restore cell death in response to IFN $\gamma$ .

Preliminary data from the first allergen exposure study suggest that inflammatory cell numbers are higher in the lung lymph nodes from Bmf-deficient mice compared to wild-type mice (Appendix F). When stimulated these *bmf*<sup>-/-</sup> lymphocytes trend toward producing more IL-13 and IFN $\gamma$  than the wild-type controls after 10 d of allergen exposure. In addition, it is important to evaluate the inflammatory response during the time course of allergen exposure in *bmf*<sup>+/+</sup> and *bmf*<sup>-/-</sup> mice. This can be determined with differential cell counts and analysis of Th2 cytokines in the bronchoalveolar lavage collected at the time of lung harvest. This is important because previous studies show that Bmf is involved in cell death of hematopoietic cells<sup>16</sup>, and mucous cells may be sustained in *bmf*<sup>-/-</sup> mice due to prolonged survival of inflammatory cells that produce Th2 cytokines.

Results from the allergen exposure study presented in Chapter 3 (Figure 3-4B) suggest that mucous cells in the *bmf*<sup>-/-</sup> mice have a survival advantage over the mucous cells in the *bmf*<sup>+/+</sup> mice. Total AEC numbers at 5 d in the *bmf*<sup>-/-</sup> allergen-exposed mice were not elevated as they were in wild-type animals; however, mucous cell numbers were increased. There are several possible explanations for this apparent discrepancy. First, the fact that the total cell numbers were not increased suggests that Bmf may have an important role in promoting survival of ciliated cells. In the absence of Bmf, these cells may turnover at an accelerated rate. Second, studies suggest that cytokines such as, IL-13

can alter the differentiation of ciliated cell<sup>17</sup> and increase the proportion of secretory cells<sup>18</sup>, so it is possible that Bmf-deficiency alters the rate of ciliated cell differentiation into mucous cells.

#### **4.5 The role of Bmf in attachment of AECs to the basement membrane**

Deficiency in Bmf greatly improved attachment and growth. This was observed when *bmf*<sup>-/-</sup> and *bmf*<sup>+/+</sup> MAECs were grown in serum-free medium and without collagen coating of culture plates, conditions that improve attachment of MAECs, (Appendix D). This finding may be explained by the fact that Bmf may have an important role in the death of cells during the attachment process. Studies show that Bmf sensitizes mammary epithelial cells to detachment-induced cell death<sup>19</sup>. Therefore *bmf*<sup>-/-</sup> MAECs may have a longer life span and a better chance to attach to the culture plate and grow. Alternatively, Bmf may be a crucial protein in sustaining isolated stem cells that can proliferate and establish MAEC cultures<sup>20</sup>.

Disruption of tight junction proteins, such as occludin and claudin-1, cause cell death and epithelial cell extrusion, a process in which contraction of actin and myosin in neighboring cells extrudes dying cells from the epithelial layer<sup>21, 22</sup>. Experiments in differentiated HAECs show that the disruption of cell-to-cell junctions causes apical extrusion, which is shown to be anoikis, or detachment-induced cell death<sup>22</sup>. Because we have observed epithelial cells that appear to be extruded during the reduction of hyperplastic epithelial cells, we assume that anoikis and apical extrusion may be involved in the removal of airway epithelial cells. In the future, immunofluorescent staining and 3-D confocal images will be used to visualize whether Bmf-expressing cells show apical extrusion in differentiated MAECs and in murine airways *in vivo*. In addition, expressing

Bmf using Ad-Bmf in differentiated cultures combined with immunofluorescent staining for occludin and claudin-1, as well as a marker for apoptosis such as caspase-3, may establish that Bmf expression causes AEC extrusion. These studies will clarify whether Bmf expression can be used as a marker for cells that are extruding from the epithelium. Because these cells that have the potential for metastasis<sup>23</sup>, the studies may have significance in identification of metastatic cells in cancer.

#### **4.6 The potential utility of HDACi therapy in treating chronic lung disease**

Asthma, cystic fibrosis, and COPD are chronic airway diseases characterized by mucous hypersecretion, which obstructs airflow and increases wheezing, coughing, or in the case of sudden secretion can completely obstruct the airway and lead to patient death. In addition, the presence of a large amount of mucus in the airway traps toxins and pathogens in the airway, enhancing damage to the lung<sup>24</sup>. Because our findings suggest that Bmf is important for mucous cell apoptosis, and that HDACis induce apoptosis through the induction of Bmf, HDACis may be useful in the treatment of chronic lung disease. Treatment of differentiated MAECs with IL-13 to induce MCM, followed by treatment with HDACis will show whether this approach reduces MCM. Similarly, we will examine whether HDACis are efficient in reducing allergen-induced MCM in mice.

In summary, our results suggest that Bmf is an important pro-apoptotic protein for regulation and maintenance of AECs. Bmf is important for AEC death in response to IFN $\gamma$  and HDACi; these apoptotic stimuli upregulate the transcription of Bmf by modifying the interaction of HDAC1 with the promoter (Figure 4-3). Although we cannot eliminate the idea that apoptotic stimuli regulate Bmf posttranslationally by causing it to translocate from the cytoskeleton to the mitochondria as proposed by

Puthalakath et al. in Figure 1-1, the regulation of Bmf in AECs may be unique because increased transcription and protein synthesis may provide sufficient Bmf to directly affect mitochondria and cause cell death. In addition, efficiency of Bmf-induced cell death appears to vary depending on cell types of the airway epithelium. Exploring the functional roles of Bmf may provide new means of treating chronic lung diseases.



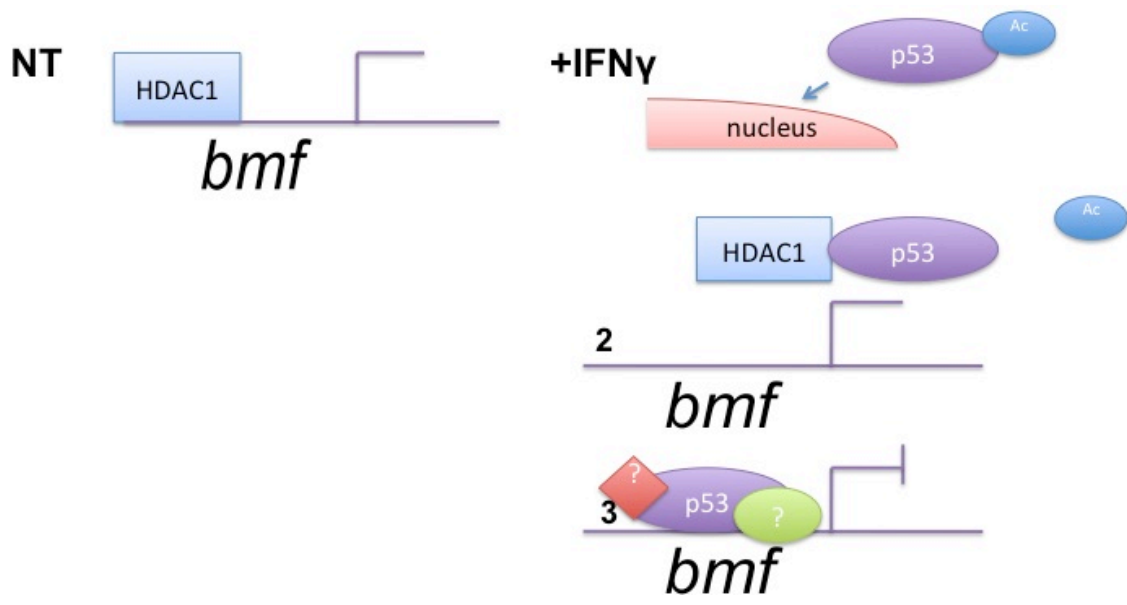


Figure 4-1. Model for regulation of Bmf mRNA by p53 in response to IFN $\gamma$ .

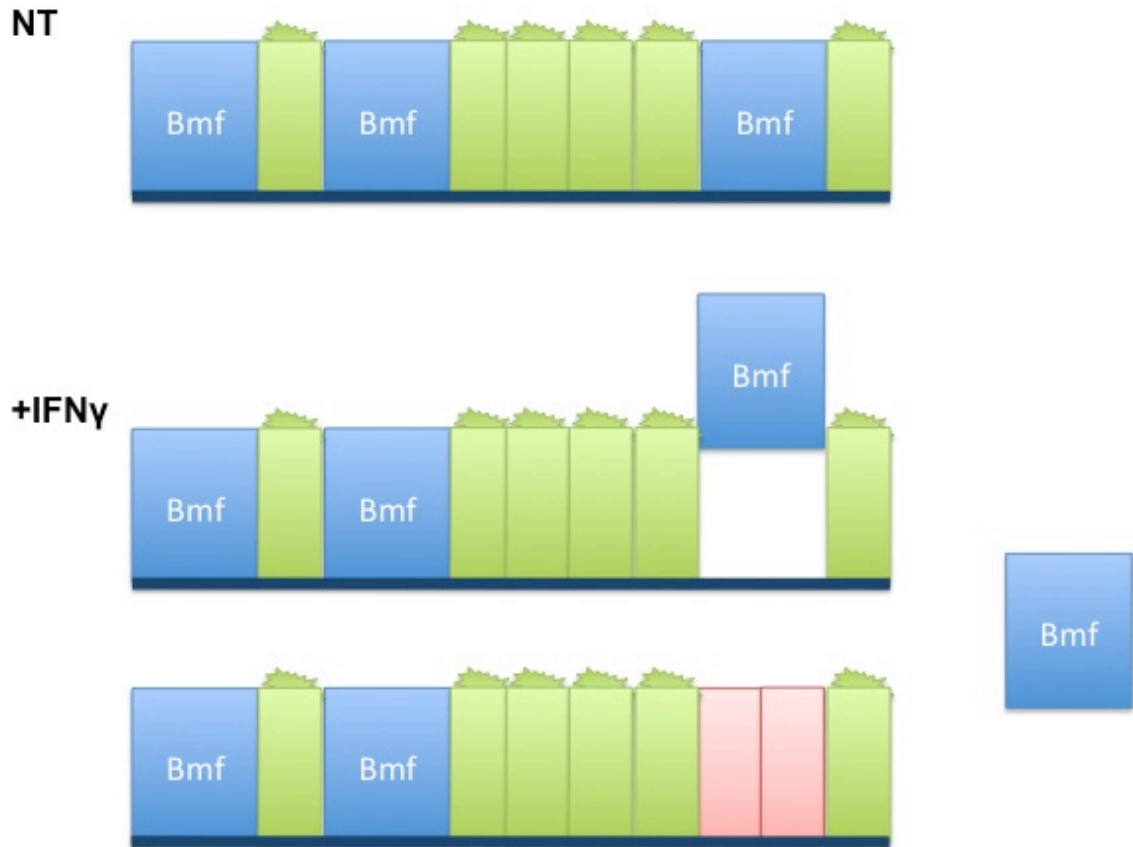


Figure 4-2. Model for the cell-type specific effect Bmf potentially has in mucin-secreting cells of the epithelium. Cells removed from the epithelium are replaced by neighboring cells.

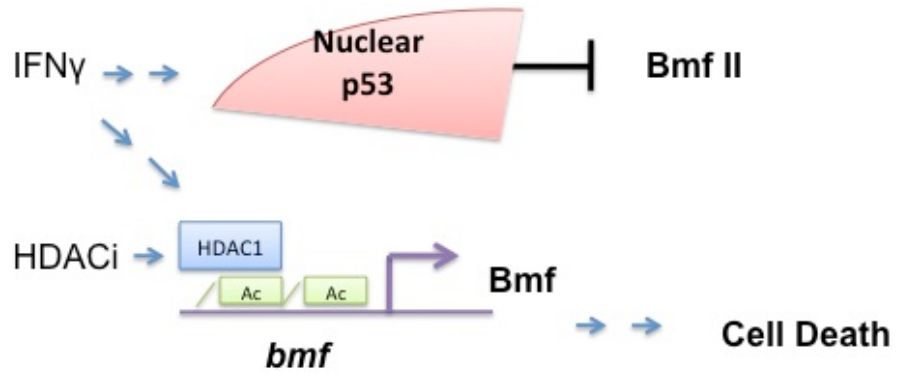


Figure 4-3. Model for the regulation of Bmf by IFN $\gamma$  and HDACis in AECs.

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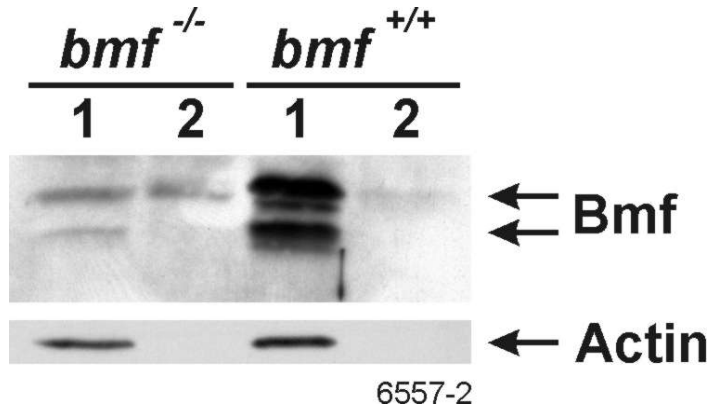
## **Appendices**

## **APPENDIX A:**

### **Expression of Bmf protein in lung tissue**

Studies from Verna et al.<sup>1</sup> demonstrate that Bmf protein expression is variable in different cell types. To characterize the *bmf*<sup>-/-</sup> mice and the anti-murine Bmf 17A9 monoclonal antibody, both of which were gifts from A. Strasser (Walter and Eliza Hall Institute, Melbourne, Australia), a Western blot was run comparing lung and heart tissue from *bmf*<sup>-/-</sup> and *bmf*<sup>+/+</sup> mice. The antibody described in Verna et al.<sup>1</sup> shows two distinct bands, which represent the 25 kD and 30 kD isoforms of Bmf. Two light background bands also appear in the lung extracts from *bmf*<sup>-/-</sup> and *bmf*<sup>+/+</sup> mice. These bands are cross-reacting products and presumably unrelated to Bmf. Bmf protein expression was detected in the lung tissue, while none was detected in the heart tissue.

**Figure and Figure Legend (Appendix A)**



**Appendix A. Bmf protein expression in heart and lung tissue.** Lung (lane 1) and heart (lane 2) tissues from *bmf*<sup>-/-</sup> and *bmf*<sup>+/+</sup> mice were homogenized on ice in 100 µl of saline with protease inhibitors using a tissue homogenizer. After addition of 50 µl of 3x RIPA buffer, samples were incubated on ice for 15 min while the samples were vortexed every 5 min and centrifuged at 14, 000 RPM for 10 min. The soluble fraction was isolated and protein concentration determined using a BCA assay. 50 µg of protein was used for the Western blot as described earlier (3.3.6). Immublot was probed for Bmf (17A9) and the loading control β-Actin.

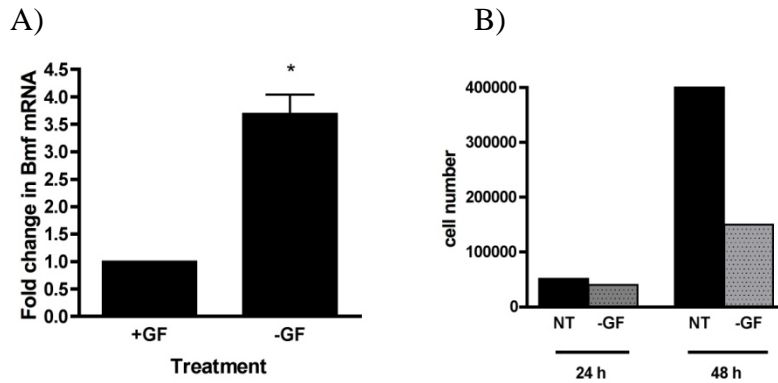


## **APPENDIX B**

### **Growth factor withdrawal induces Bmf mRNA**

The medium used to grow AECs in culture must contain nutrients and growth factors, which include retinoids, epidermal growth factor (EGF), hydrocortisone, transferrin, triiodothyronine, epinephrine and insulin. Studies report that growth factors are an important component in medium for growing and maintaining healthy epithelial cells in culture<sup>2</sup>. Epithelial cells grown in medium without the growth factors, insulin, and EGF, have difficulty attaching to the cell culture plates. Because withdrawal of EGF induced Bmf expression in MCF-10A mammary epithelial cells<sup>3</sup>, we carried out this experiment in AECs. Minimal Bmf induction was observed when either EGF or insulin was individually removed from the medium; however, a significant induction of Bmf mRNA was seen when insulin and EGF were removed from the medium.

## Figure and Figure Legend (Appendix B)



### Appendix B. Growth factor withdrawal reduces AEC numbers and induces Bmf mRNA.

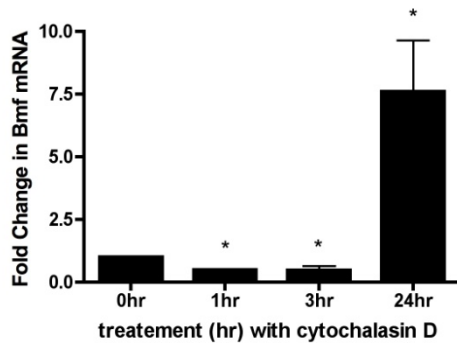
Bmf mRNA is induced in response to growth factor withdrawal. AALEB cells were plated to be 30% confluent and 24 h later maintained with or without insulin and EGF for 48 h. Cells were harvested, RNA extracted using the Qiagen RNA extraction kit, and qRT-PCR was used to analyze Bmf mRNA expression (A). Trypan blue exclusion was used to quantify AECs after the absence of EGF and insulin for 24 and 48 h. Growth factor withdrawal reduces AEC numbers.

## **APPENDIX C:**

### **Cytochalasin D treatment changes Bmf mRNA expression**

The disruption of actin filament integrity with either cytochalasin D or jasplakinolide induces apoptosis in AECs, suggesting that actin may be important in the apoptotic pathway<sup>4</sup>. Latrunculin A treatment, another agent that disrupts actin polymerization, induces Bmf mRNA in MCF-10A cells<sup>3</sup>. Our studies show that the treatment of AECs with cytochalasin D cause the AECs to round up and lose normal morphology, followed by AEC death (unpublished). Whether this is related to the induction of Bmf seen at 24 h is unknown.

### Figure and Figure Legend (Appendix C)



#### **Appendix C: Treatment with cytochalasin D initially reduced but then induced Bmf mRNA levels at 24 hr.**

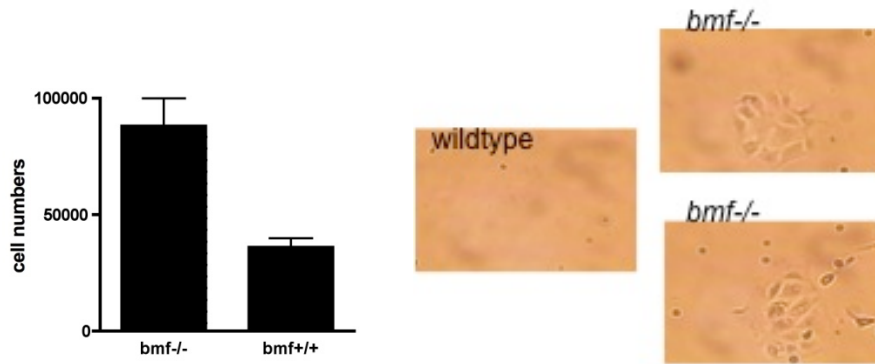
HCCBE cells, an AEC line derived from NHBEs, were plated in 6-well dishes, grown to 35% confluency, and 24 h later were treated with cytochalasin D at a concentration of 1 $\mu$ g/ml. Cells were harvested using trypsin at 0, 1, 3, and 24 h, RNA was extracted using the Qiagen RNA extraction kit, and qRT-PCR was used to analyze Bmf mRNA levels.

## **APPENDIX D:**

### ***bmf*<sup>-/-</sup> and *bmf*<sup>+/+</sup> MAECs grown in attachment-poor conditions**

Collagen-coated culture plates and medium containing fetal bovine serum are important for primary AEC growth and attachment. *bmf*<sup>-/-</sup> and *bmf*<sup>+/+</sup> MAECs were plated on culture plates without collagen and in serum-free medium. These experiments revealed that the *bmf*<sup>-/-</sup> MAECs have an advantage over the *bmf*<sup>+/+</sup> MAECs grown in these conditions.

**Figure and Figure Legend (Appendix D)**



**Appendix: D *bmf*<sup>-/-</sup> MAECs are more resistant to serum deprivation when compared to *bmf*<sup>+/+</sup> MAECs.**

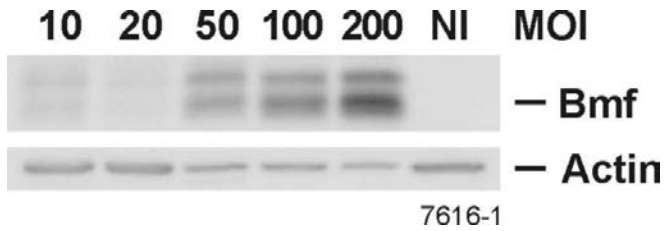
*bmf*<sup>-/-</sup> and *bmf*<sup>+/+</sup> MAECs (75,000) were plated in 6-well culture dishes without collagen coating and in serum-free medium. Photomicrographs were taken at 72 h, and cell numbers were determined after harvest at 96 h.

## **Appendix E:**

### **Expression of Bmf in AALEB cells**

Infecting AALEBs with adenoviral Bmf (as described in 2.2.6) produces two Bmf specific bands when compared to non-infected cells. The adenovirus was designed to produce the 25kD isoform of Bmf; however, another band that is ~30kD was expressed as well. This band is not present in the non-infected cells, nor is it expressed in cells infected with Ad-GFP (data not shown). This second band may be the result of a posttranslational modification of the smaller form of Bmf.

**Figure and figure legend (Appendix E)**



**Appendix E. Infection of AALEB cells with Ad-Bmf results in the expression of two Bmf-specific bands.** AALEB cells were infected with Ad-Bmf, and 24 h later cells were harvested and analyzed by Western blotting using the 17A9 anti-Bmf antibody (described in Appendix A);  $\beta$ -Actin was used as a loading control.

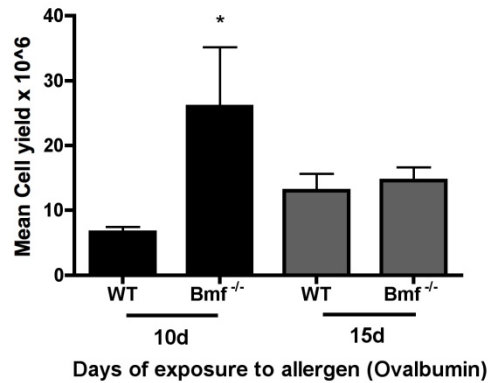


## **APPENDIX F:**

### **Lymph node cell numbers in allergen exposed *bmf*<sup>-/-</sup> and *bmf*<sup>+/+</sup> mice**

Lung lymph nodes from allergen exposed *bmf*<sup>-/-</sup> and *bmf*<sup>+/+</sup> mice were removed at the same time that the lungs were harvested at 5, 10, and 15 d of allergen exposure. We collected these organs to investigate whether allergen exposure produced changes in the immune response in the *bmf*<sup>-/-</sup> mice compared to *bmf*<sup>+/+</sup>.

**Figure and figure legend (Appendix F)**



**Appendix F. Cell numbers are greater in the lung lymph nodes of allergen-exposed *bmf*<sup>-/-</sup> mice.** A single cell suspension was prepared from the lymph nodes using a gentle grinding technique. Red blood cells were lysed, and lymph node cells were resuspended in 0.5 ml of culture medium. Cells were filtered through a sterile cell strainer and then counted. Cells were plated in medium containing either Concanavalin A or ovalbumin to stimulate cytokine production in 48 well dishes in triplicate. Cells were harvested 48 h later, and cytokine levels were assessed with an enzyme-linked immunosorbent assay.

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