UNIVERSITY OF CALIFORNIA
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Epigenetic Regulation of Head and Neck Squamous Cell Carcinoma Chemoresistance, Invasion, and Metastasis

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Oral Biology

by

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Development of chemoresistance, invasive growth, and metastasis remain key challenges in head and neck squamous cell carcinoma (HNSCC) therapy. Recent studies revealed that an activated hepatocyte growth factor receptor (MET) is frequently overexpressed and highly associated with HNSCC invasion and metastasis. Also, autophagy, a highly conservative intracellular recycling system, has shown to play a primary role in cancer cells to attenuate cytotoxicity of chemoreagents in many hematopoietic and solid cancers. However, little is known about the epigenetic regulation of the MET signaling pathway or autophagy induction and whether it plays a role in promoting HNSCC invasion and metastasis or development of resistance to therapy. In our study, we found that histone deacetylase 6 (HDAC6) is a key epigenetic regulator of autophagy that promotes chemoresistance in HNSCC against the proteasome inhibitor, Bortezomib. The depletion of HDAC6 inhibited autophagy activation and enhanced Bortezomib-induced apoptosis in HNSCC cells. Mechanistically, we revealed that HDAC6 mediated activation of autophagy by modulating...
activation of protein kinases such as unc-51 like autophagy activating kinase 1 (ULK1) to promote clearing of large quantities of cytotoxic, unfolded protein aggregates induced by the Bortezomib. In addition, we found histone demethylases KDM6B plays an important role in acquiring cisplatin resistance in HNSCC and in unraveling the mechanism associated with the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. Interestingly, we also identified KDM6B as an essential epigenetic regulator of MET-driven HNSCC metastasis. KDM6B was highly expressed in both the growth factor-induced HNSCC cells and in cells overexpressing the oncogenic translocated promoter region MET (TPR-MET). KDM6B knockdown significantly decreased the HNSCC invasion and metastasis by regulating the expression of ETS proto-oncogene 1 (ETS1) and the high mobility group AT-Hook 2 (HMGA2) genes, known as the drivers of metastasis. Mechanistically, KDM6B facilitated the binding of the transcription factor ELK1, a downstream target of c-MET signaling pathway, to the promoters of ETS1 and HMGA2. In conclusion, our study provides insight into the epigenetic regulation of HNSCC chemoresistance, invasion, and metastasis and suggests that HDAC6 and KDM6B could be an important therapeutic target to improve chemotherapeutic efficacy and to decrease the tumor burden of HNSCC patients.
The dissertation of Insoon Chang is approved.

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### EDUCATION/TRAINING

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### PUBLICATIONS

INTRODUCTION

1.1 Head and Neck Cancer

1.1.1 Head and Neck Squamous Cell Carcinoma (HNSCC)

Cancers of the head and neck are collectively referred to as a group of heterogeneous cancers affecting the lip, oral cavity, pharynx, larynx, paranasal sinuses, and nasal cavity. More than 90% of head and neck cancers are comprised of squamous cell carcinoma (SCC) that originate from squamous cells that line the mucosa of the oral cavity, nasopharynx, oropharynx, and larynx. Although HNSCC arises from the common origin in the squamous mucosa of the upper aero-digestive tract, HNSCC is known to be heterogeneous. The major risk factors for developing HNSCC are exposure to carcinogens through tobacco smoking, alcohol consumption, infection with high-risk types of human papillomavirus (HPV), and genetic predisposition. The diagnosis is mainly confirmed by a tissue biopsy collected from the patient along with medical imaging and blood tests. The clinical presentation of premalignant lesions of the mucosal surfaces of the upper aerodigestive tract includes leukoplakia (white lesion), erythroplakia (red lesion), or speckled leukoplakia (mixed white/red lesion). Among these clinical changes, erythroplakic and speckled lesions are commonly associated with ominous histopathologic alterations that may lead to the development of invasive carcinoma. Histologically, carcinoma in situ exhibits full-thickness mucosal epithelial dysplastic change without the destruction of the basement membrane. The early, superficial, or microscopic invasive SCC is diagnosed as squamous carcinoma when these are interruption of the basement membrane with invasion of the carcinoma into the underlying submucosal compartment. The prognosis for HNSCC patients is closely related to the stage at presentation. The tumor stage is typically determined by the size and extent of the primary tumor, as well as the presence of lymph-node and/or distant metastases. Prognosis also varies according
to the primary tumor site\textsuperscript{5,6}. Studies have shown that newly diagnosed HPV-positive HNSCC in the oropharynx is more chemotherapy-responsive than non-oropharyngeal HNSCC\textsuperscript{3,7}.

1.1.2 Incidence and survival trends

HNSCC is amongst the 10 most common and lethal malignancy worldwide with approximately 560,000 new cases diagnosed and 300,000 deaths each year\textsuperscript{8}. HNSCC exhibits high morbidity and mortality associated with the high recurrence rate and the malignant phenotype, characterized by the extensive local invasion and metastasis to the regional cervical lymph nodes and/or distant organs\textsuperscript{1,2,9,10}. In the U.S., HNSCC represents \textasciitilde 3\% of malignancies and accounts for the ninth most common cancer diagnosed in men\textsuperscript{5}. According to 2017 SEER and the Centers for Diseases Control and Prevention’s National Center for Health Statistics reported by National Cancer Institute, an estimated 346,902 people are currently living with oral cavity and pharynx cancer\textsuperscript{11}. Also, based on 2010-2014 cases and deaths, an estimated 49,670 new cases and 9,700 deaths are predicted to occur in 2017, costing approximately $3.1 billion on treatment\textsuperscript{11,12}. HNSCC is more prevalent in men than women (14.97 per 100,000 in men and 6.24 in women)\textsuperscript{12}. Oral cavity and pharynx cancer is predominantly diagnosed and causes death among people aged between 55 to 64 (30.7\% of new cases and 26.6\% of HNSCC death)\textsuperscript{6,11}. However, an increasing trend in young adults with HPV-positive HNSCC have also been observed in the U. S. and Europe lately\textsuperscript{5,13}. Interestingly, a significant improvement on the 5-year survival rate of HNSCC was observed going from 54.7\% in 1992-1996 to 65.9\% in 2002-2006 in developed countries\textsuperscript{12,14}. However, even with this improvement, HNSCC is still considered to have one of the lowest 5-year survival rates in any major cancers with about a 30-47\% recurrent rate despite the rigorous development and utilization of several therapeutic modalities in the last 30 years\textsuperscript{14-16}.

1.1.3 Current treatments and challenges
Current standard treatment option for HNSCC patients is surgical resection with concurrent chemoradiotherapy (CRT)\textsuperscript{17}. However, despite improved outcomes with CRT, disease recurrence and treatment toxicity continue to be challenges in treating HNSCC patients\textsuperscript{1,13}. Many studies have shown that the 2- and 5-year survival rates are significantly lower in the recurrence patients than in non-recurrent HNSCC patients\textsuperscript{15}. Advanced or recurrent HNSCC patients represent a devastating type of malignancy with a high incidence of local invasion, cervical lymph node and/or distant organ metastasis, and drug resistance\textsuperscript{14,15,17}. Surgical resection and reirradiation of recurrent or advanced cancer patient in the head and neck area is possible, but past clinical trials showed excessive toxicities exerted by reirradiation and surgical re-treatment leading to a high incidence of patient disfigurement and death\textsuperscript{18}. Thus, majority of patients with recurrent or metastatic HNSCC are currently treated with palliative chemotherapy, typically with platinum-based chemotherapy regimens such as cisplatin\textsuperscript{6,17,19}. However, despite the recent quality and performance advancements in therapeutic modality, the detrimental effects of HNSCC are attributable, in part, to the limited understanding of molecular signaling pathways that promote cancer pathogenesis and progression. Also, because of the failure of conventional chemotherapy regimens to improve survival rates in recurrent and metastatic cancer treatment, much attention has been shifted to studying chemo resistance and metastasis-associated molecules in diverse cancers including HNSCC\textsuperscript{17,20,21}. Recently, new insights on molecular pathogenesis of cancer implicated that various cancers including HNSCC are comprised of the accumulation of genetic and epigenetic changes and alterations\textsuperscript{1,22}. These accumulated changes may occur in cancer-related signaling pathways to obtain phenotypes such as immortality, ability to evade apoptosis, invasion and metastasis, etc. during the tumor initiation, progression, and maintenance\textsuperscript{2,23}. Thus, understanding the complex networks of molecules and intracellular and intercellular signal
transduction pathways associated with therapy resistance, invasive growth, and metastasis is of the utmost importance in order to develop effective novel chemotherapeutic regimen.

1.1.4 Epigenetics and cancer

The term “epigenetics” was first introduced by Dr. Conrad Waddington in 1956 to define epigenetics as studies on heritable changes in gene expression and cellular phenotype that occur independently of alterations in the DNA sequence\textsuperscript{22,24,25}. Over time, this definition of epigenetics was continually refined, and now the term epigenetics is most commonly used to describe chromatin-based events that regulate the DNA-templated process\textsuperscript{22,24}. In the cell, the primary functions of chromatin are to compact DNA, reinforce mitosis, repair and prevent DNA damage, and control gene transcription and DNA replication\textsuperscript{26}. Histones are major protein components of chromatin that allow DNA to wrap around and assemble to form nucleosome\textsuperscript{25,26}. Chromatin controls gene expression by altering DNA accessibility through structural changes of the nucleosome, which is mediated by histones\textsuperscript{25}. Genes are actively transcribed when DNA is loosely packaged around histones (referred to as euchromatin) to allow access of transcription-related molecules such as transcription factors and RNA polymerases\textsuperscript{25}. In contrast, gene transcription is inactivated when DNA is more tightly condensed and associated with histones (heterochromatin)\textsuperscript{25}. Previously, many studies have shown that these chromatin structure changes are highly regulated by chromatin-modifying enzymes, which alter noncovalent interactions within and between nucleosomes\textsuperscript{24-26}. There are now at least four different DNA modifiers and 16 classes of histone modifiers that have been found in the eukaryotic cells that can change chromatin structure through methylation, acetylation, phosphorylation, and ubiquitylation\textsuperscript{25,26}. In addition, chromatin modifying enzymes have been found to also serve as docking sites for additional chromatin modifiers and remodeling enzymes\textsuperscript{26}. 

4
Since the information conveyed by epigenetic modifications play a critical role in regulating cell phenotype, aberrant epigenetic changes and accumulation will result in dysregulation of gene expression profiles leading to the development and progression of diseases such as cancer\textsuperscript{24,25,27,28}. The earliest indication of epigenetic changes linking to tumorigenicity were acquired from studies associated with gene expression and DNA methylation\textsuperscript{25}. Consequently, recent whole genome sequencing in vast array of cancers, including HNSCC, revealed that the cancer initiation, maintenance, and progression are accompanied by profound changes in DNA methylation and histone modification patterns as well as altered expression profiles of chromatin-modifying enzymes\textsuperscript{24,25}. However, although substantial progress has been made in understanding the molecular control of cancers such as HNSCC, the underlying mechanisms associated with epigenetic changes that regulate these molecular pathways and signal transductions are not well-investigated. Furthermore, unlike genetic mutations, epigenetic changes are known to be potentially reversible, offering a promising therapeutic candidate for cancer patient treatment\textsuperscript{28}.

1.2 Resistance to chemotherapy

1.2.1 Definition of Chemoresistance

Resistance to the chemotherapy is one of the major reasons for cancer treatment failure and occurs when cancer becomes tolerant to administered therapy regimen\textsuperscript{20,29}. Several research studies have revealed that tumors acquire an ability to attenuate apoptosis to succeed in progressing to state of high-grade malignancy and resistance to therapy\textsuperscript{20}. Although this molecular progression of cancer leading to the development of chemoresistance is not well known, growing evidence suggests that cancer cells may develop direct or indirect resistance to the therapeutic drug through DNA mutations, epigenetic alterations, and metabolic changes\textsuperscript{29}. In tumors, including HNSCC,
resistance to chemotherapy may be either pre-existent, before therapy (intrinsic resistance), or induced by drugs over time (acquired resistance)\textsuperscript{29}. Many molecular mechanisms leading to intrinsic or acquired resistance in cancer have been found, including drug efflux, DNA damage repair, drug target alteration, drug inactivation, epithelial-mesenchymal transition, autophagy activation, etc\textsuperscript{20,29,30}. The manifestation of these mechanisms is cancer-specific and varies amongst cancer type and their stages of progression\textsuperscript{29}. Also, these mechanisms may act independently or in combination with regulation of various signal transduction pathways activated in the cancer cells\textsuperscript{29}. In addition, cancer stem cells (CSCs) have been identified as a rare cell population that can undergo self-renewal/differentiation and enhance tumorigenicity in multiple malignancies including HNSCC and various solid cancers\textsuperscript{20,29,31-33}. In HNSCC, CSCs have shown to highly express CD44 antigen on the cell surface and exhibit high aldehyde dehydrogenase (ALDH) and drug efflux activities\textsuperscript{31,33}. Recently, our lab identified moloney murine leukemia virus insertion site 1 (Bmi1) expressing CSCs (Bmi1\textsuperscript{+} CSCs) in HNSCC and showed that Bmi1\textsuperscript{+} CSCs promote chemoresistance and mediate tumor initiation, invasive growth, and lymph node metastasis in HNSCC using a transgenic mice model\textsuperscript{31}. Thus, further studies are needed on signaling pathways associated with HNSCC chemoresistance and CSCs to identify biomarkers and unravel their detailed mechanism for development of effective therapeutic modalities.

1.2.2 Therapy resistance and Autophagy

Lately, autophagy has received greater attention due to its implications in pathological processes including tumorigenesis, chemoresistance of malignancies, and neurodegeneration\textsuperscript{34-37}. Autophagy is a highly conserved cellular degradation process that eliminates aggregated or unfolded/misfolded proteins and damaged organelles in response to stress or nutrient deprivation\textsuperscript{38}. Autophagy exists in 3 types: Macroautophagy, microautophagy, and chaperone-mediated
autophagy. Macroautophagy (hereafter autophagy) is the most prevalent form of autophagy\textsuperscript{36,38}. Autophagy mediates the orderly degradation and recycling of targeted cellular components by 1) forming a double-membraned structure called autophagosomes to sequester target components and 2) degrading components by acidic lysosomal hydrolases during lysosome-autophagosome fusion\textsuperscript{38}. Autophagy in cancer exhibits context-dependent role in suppressing and promoting cancer\textsuperscript{36,37}. During early stages of cancer, autophagy can be tumor suppressive by eliminating oncogenic protein substrates, damaged organelles, and cytotoxic unfolded protein aggregates (CPAs) to control cellular integrity and alleviate cellular stress\textsuperscript{36}. However, once cancer is established, autophagy promotes the progression of cancer by allowing adaptation to the microenvironmental stress during invasion and metastasis and acquisition of resistant to chemotherapy\textsuperscript{36,37}. It was shown that autophagy provided protection for breast cancer cells (MCF-7) against epirubicin, an anthracycline drug used for chemotherapy, but the inhibition of autophagy re-sensitized the MCF-7 cells to epirubicin\textsuperscript{39}. Moreover, accumulative studies imply that a coordinated pro-survival cellular response known as unfolded protein response (UPR) may involve in induction of autophagy under cellular stress\textsuperscript{40,41}. The UPR is an integral adaptive signaling pathway that has dual roles in cancer\textsuperscript{42,43}. UPR can play a cytoprotective role by restoring the endoplasmic reticulum (ER) homeostasis through chaperone activity, resulting in diminished ER stress induced by the presence of CPAs\textsuperscript{44}. In addition, UPR can play a catabolic role by inducing apoptosis when persistent ER stress exists\textsuperscript{44,45}. However, the detailed mechanisms and interaction between apoptosis, autophagy, and UPR remains largely unknown.

1.2.3 Proteasome Inhibitor and Histone deacetylases

Bortezomib (also known as PS-341 or Velcade) is the first therapeutic proteasome inhibitor approved by U.S. Food and Drug Administration (FDA) to treat hematological malignancies
including multiple myeloma\textsuperscript{46}. Bortezomib (BTZ) targets rapidly dividing cells and has shown to exhibit anti-tumor activity in both solid and hematological malignancies by specifically binding to the catalytic site of the 26S proteasome with high affinity to promote cancer-specific apoptosis\textsuperscript{47,48}. Previously, our lab found that BTZ can induce apoptosis in both primary and cisplatin-resistant HNSCC cells by promoting persistent ER stress through accumulation of CPAs in the cytoplasm\textsuperscript{47}. BTZ-induced ER stress resulted in an increased expression of the pro-apoptotic molecule Noxa, while inhibiting the pro-survival nuclear factor-kappa B (NF-κB) expression\textsuperscript{47}. In addition, our subsequent studies revealed that BTZ treatment in HNSCC cells elicited both ER stress induced apoptosis and UPR response, and inhibition of UPR enhanced BTZ-cytotoxicity\textsuperscript{47}. Our findings proposed potential use of BTZ as an alternative therapeutic regimen to overcome cisplatin-resistance in human HNSCC. However, clinical phase II studies on BTZ-based regimens indicated that BTZ alone exerted minimal cytotoxic effects in HNSCC patients\textsuperscript{19}. The mechanism behind this intrinsic BTZ chemoresistance in HNSCC remains largely unknown and further study is necessary.

Human histone deacetylases (HDACs) are family of enzymes comprising of 18 proteins, which are grouped into 4 classes based on their sequence homology with yeast proteins\textsuperscript{49,50}. HDACs are involved in histone modification. Acetylation of histones is commonly associated with transcriptional activation by epigenetically regulating accessibility of transcription factors to the gene promoter\textsuperscript{50}. However, the effect of histone acetylation highly depends on the nature and site of modifications occurred and may also results in transcription inactivation\textsuperscript{50}. In contrast, the deacetylation performed by HDAC molecules exerts opposite effect of histone acetylation and typically results in transcriptional repression\textsuperscript{50}. In cancer, HDAC has been found to act as an oncogene itself while inhibiting expression of genes such as tumor suppressors, cell-cycle
inhibitors and differentiation factors, or apoptosis inducers. HDACs are also found overexpressed in various solid tumors including HNSCC. Thus, many HDAC inhibitors have been developed for cancer treatment and have displayed significant therapeutic effects specific to cancer cells in vitro and in vivo.

In recent years, our lab identified that the combination treatment of BTZ and the HDAC inhibitor, Trichostatin A (TSA), overcomes BTZ-resistance and synergistically increases BTZ-induced apoptosis in numerous HNSCC cells in vitro and in vivo. However, how TSA re-sensitizes the HNSCC cells and how the crosstalk between UPR, autophagy, and apoptosis leads to the development of chemoresistance remain as important issues which need to be further addressed. Several studies have highlighted the role of HDAC6, an HDAC class IIB cytoplasmic tubulin deacetylase, in the clearance of CPAs through the formation of a single juxtanuclear inclusion body called the aggresome. The subsequent autophagic degradation of the aggresome leads to diminished population of CPAs in the cytoplasm that results in alleviation of ER stress induced by proteasome inhibition. HDAC6 has also been shown to deacetylate heat shock protein 90 (HSP90) and modulate its chaperone activity to restore ER homeostasis. Growing evidence suggests that autophagy is activated as a cytoprotective response after Bortezomib treatment in many solid tumors including prostate cancers and histone deacetylase 6 (HDAC6) is required for the autophagy induction. However, the mechanistic interaction between HDAC6 and autophagy remains largely undiscovered. Moreover, the aberrant expression of HDAC6 has been reported in HNSCC patient tissues. Based on these findings, we hypothesized that HDAC6 might be a critical regulator of the cell protective response, mediating the molecular network between ER stress, autophagy, and apoptosis to develop resistance to chemotherapy in HNSCC.
1.2.4 Cisplatin resistance

Platinum-based chemoreagents such as cisplatin (cis-diamminedichloroplatinum-II or CDDP) or carboplatin are one of the main chemotherapy medications used to treat HNSCC\textsuperscript{20,21}. Cisplatin is a DNA intercalator, which targets rapidly dividing cells\textsuperscript{17,20,21}. Cisplatin mostly binds with the guanine residues of the DNA, leading to multiple inter and intra-strand crosslinks that inhibit DNA replication, break double-strands of DNA, and result in apoptosis\textsuperscript{65}. Cisplatin is effective as a single agent for the non-invasive primary HNSCC or in combination with other drugs such as Cetuximab (epidermal growth factor receptor inhibitor) or Docetaxel (disrupts function of microtubules) in the treatment of more advanced or recurrent HNSCC\textsuperscript{17,21}. However, HNSCC often demonstrates significant resistant to cisplatin, which is either acquired during repeated treatment or presented as an intrinsic characteristic of the cancer\textsuperscript{20,66}. Therefore, cisplatin resistance has become one of the main factors in disease recurrence. Cisplatin resistance also has implications for ongoing treatment because it requires significant dose increase for elimination of resistant cancer cells, which results in increased toxicity to the patients. Hence, cisplatin resistance became a major obstacle to effective HNSCC therapy.

Many studies have been conducted to unravel the molecular mechanism of cisplatin resistance in cancer cells including HNSCC. Recent gene expression comparison between parental and cisplatin resistant HNSCC cell lines have identified 199 genes that were differentially expressed in each group\textsuperscript{9}. Also, the NF-κB signaling pathway has been found to mediate cisplatin resistance through histone acetylation in HNSCC\textsuperscript{67}. The mammalian NF-κB family of proteins is comprised of RelA/p65, NF-κB 1, NF-κB 2, c-Rel, and RelB subunits that form dimers to control downstream gene expression\textsuperscript{68,69}. NF-κB is negatively regulated by the IκB family of proteins and positively regulated by the IκB kinase (IKK) complex\textsuperscript{70}. In normal cells, NF-κB is bound to IκB
proteins and resides in the cytoplasm in its inactive form. However, when cells are stimulated by cytokines, bacterial products, viral expression, growth factors, and/or stress stimuli, IKK complex phosphorylates IκB proteins and induces their degradation, resulting in activation of NF-kB and nucleo-translocation to control gene expression. In cancer cells, expression and activation of NF-κB regulators and effectors are often altered, and NF-κB has been shown to play an important role in various cancer initiation, maintenance, and progression. Recently, NF-κB was identified to act as a modulator of chromatin modifications following cisplatin treatment in HNSCC. The study illustrated that cisplatin treatment activated NF-κB signaling pathway to prevent histone 3 acetylation, leading to condensed structure of tumor chromatin and reduced Breast cancer 1 protein (BRCA1) expression. BRCA1 is a tumor suppressor gene that has been known to promote DNA repairing. Moreover, Nor et al. recently unveiled that cisplatin can induce Bmi1 expression and enhance the CSC population in human primary HNSCC cells. The rare CSC population was isolated from human HNSCC tumors by their ALDH activity and CD44 expression within 12 hours after surgery. Studies on these collected HNSCC CSC showed that they rely on interleukin 6 (IL6) signaling for their survival and self-renewal, and cisplatin treatment further enhanced their self-renewal and survival in vitro. As aforementioned, CSCs are capable of initiating tumors, disseminate from the primary tumor to promote metastasis, and promote resistant to therapy. Taken together, further studies on the molecular mechanisms of cisplatin resistance and the involvement of epigenetic alterations need to be elucidated to strengthen our understanding, identify the biomarkers involved in this process, and develop an effective therapeutic modality.

1.3 Invasion and Metastasis

1.3.1 HGF and MET signaling pathway
Hepatocyte growth factor receptor (HGFR), also known as c-MET or MET, and its physiological ligand hepatocyte growth factor/scatter factor (HGF/SF), represent a signaling pathway that has a critical role in cell proliferation, survival angiogenesis, invasive growth, and metastasis in a variety of human tumors, including breast, prostate, lung, pancreatic, bladder, and head and neck cancer\textsuperscript{72-74}. Activation of MET-dependent signaling pathways controls numerous cellular responses such as cell growth, proliferation, transformation, survival, and migration\textsuperscript{73-75}. Few of the major downstream pathways triggered by MET activation are the mitogen-activated protein kinase (MAPK) signaling pathway, phosphoinositide 3 kinase (PI3K)-Akt pathway, and signal transducer and activator of transcription 3 (STAT3) pathway\textsuperscript{74,75}. MET is frequently overexpressed in nearly all types of HNSCC, correlating positively with more advanced stages of tumor and shortened patient survival\textsuperscript{76}. Also, MET is often found to be constitutively activated in HNSCC due to somatic mutations that occur during cancer development and progression\textsuperscript{77}. Further, the serum HGF/SF levels are retained higher in advanced tumor stages and patients with recurrent HNSCC\textsuperscript{78}. Likewise, several studies indicate that the overexpression of MET and/or its ligand HGF/SF correlates highly with lymph node metastasis, pathologic stages, disease recurrence, and the survival of HNSCC patients\textsuperscript{79,80}. Taken together, this compelling evidence indicates the importance of the HGF/SF-MET axis in the metastasis of HNSCC. Numerous preclinical studies have provided strong experimental foundations for assessing the therapeutic value of HGF-MET inhibition in advanced or metastatic tumors, including breast, prostate, liver, renal, and HNSCC\textsuperscript{74-76,80}. Additional research and ongoing clinical trials testing the efficacy and safety of HGF or Met inhibition in HNSCC and other types of solid tumors will be pertinent in determining whether the HGF-MET signaling axis will prove to be a beneficial therapeutic target in the treatment of HNSCC.
1.3.2 Lysine-specific histone demethylases

Histone methylation occurs on arginine and lysine residues found on histone tails to tighten the coiling of DNA around histones to condense the chromatin. Demethylation of these methylated residues are tightly regulated by specific enzymes, respectively. Arginine residue demethylation occurs via peptidyl arginine deiminase 4 (PADI4); however, this Arginine residue demethylation is not considered a “true” demethylation because the loss of a methyl group takes place by converting arginine to citrulline residue without leaving a free arginine. The “true” demethylation occurs when the methyl group is removed from the lysine residue by two families of histone demethylases proteins: amino oxidase homolog lysine demethylase 1 (KDM1) and Jumonji C (JmJC) domain containing histone demethylases. The KDM1 family is composed of two members, KDM1A and KDM2B. These proteins contain a SWIRM domain and a highly conserved flavin-containing amino oxidase homolog that specifically removes the mono- and dimethylated lysine on histone 3 (H3) and 4 (H4). KDM1 family demethylases are unable to demethylate trimethylated lysine residues due to the limitation on the oxidation reaction. JmjC domain containing proteins (JMJD) form the largest group of the histone demethylases. Nearly 20 of JMJD demethylases have been identified as lysine-specific demethylases. JMJD demethylases belongs to the 2-oxoglutarate-dependent dioxygenases and undergo hydroxylation with Fe$^{2+}$ and oxygen to remove the methyl group from the lysine residues. Unlike KDM1 family demethylases, JMJD demethylases can remove trimethylations on specific lysine residues.

Due to their functions in transcriptional activity, alteration and aberrant expression of histone demethylases are often associated with cancer development and progression in various cancers including HNSCC. Recently, our lab reported that lysine-specific demethylases 4A (KDM4A, also known as JMJD2A) is a key epigenetic regulator of activator protein 1 (AP1)
activation in HNSCC cells to promote invasive growth and metastasis. AP1 is a transcription factor that consists of FOS and JUN (also known as c-Jun) dimers and known as one of the major effectors activated by the signaling mediated by HGF/MET and other oncogenic factors to induce invasive tumor growth and metastasis in various cancers. In HNSCC, we observed that AP1 can be activated by phosphorylation of FOS (especially FOSL1) and JUN family proteins, and JUN and FOSL1 positively regulate their own expression through AP1 binding on their promoters to consequently amplify AP1 activation. Mechanistically, we revealed that KDM4A aids in HGF/MET-stimulated transcriptional upregulation of JUN and FOSL1 by removing trimethyl lysine 9 of histone H3 (H3K9me3) from their promoter regions, which allows recruitment of AP1 to the promoters to initiate the positive feedback loop to enhance expression of both JUN and FOSL1 that eventually dimerizes to form AP1. This KDM4A-dependent induction of AP1 allowed the transcriptional regulation and expression of genes that promote invasive growth and metastasis in HNSCC cells in vitro and in vivo. Moreover, our preliminary data shows that the HGF/MET axis induces KDM6B expressions in HNSCC cells, and the inhibition of KDM6B attenuates HGF/MET-mediated invasive growth and metastasis in vitro and in vivo. However, our understanding on the epigenetic regulation on HNSCC invasion and metastasis still remains limited. Thus, further studies are required to obtain a comprehensive understanding on the molecular network and mechanisms involving epigenetic modifications that control invasion and metastasis of HNSCC for development of an effective therapy treatment.
1.4 Specific Aims

Chemoresistance, invasive growth, and metastasis remain key challenges in HNSCC therapy. Hence, in this thesis dissertation, I will focus primarily on addressing involvement of epigenetic regulations in HNSCC progression and relapses.

First, autophagy, a highly conservative intracellular recycling system, has recently shown to play a primary role in cancer cells to attenuate cytotoxicity of chemoreagents. Emerging evidence suggests that histone deacetylase 6 (HDAC6) could promote autophagy. However, little is known about how HDAC6 mediates autophagy induction when cells are under cytotoxic stress. My study aims to investigate the simultaneous utilization of proteasome inhibitor Bortezomib, a previously proposed chemotherapeutic agent for HNSCC, and HDAC inhibitor Trichostatin A (TSA), as a novel therapeutic regiment to treat HNSCC while uncovering the molecular mechanisms of intrinsic and acquired chemoresistance mediated by autophagy. Our findings provide the first insight that HDAC6 is a key epigenetic regulator of autophagy that promotes chemoresistance in HNSCC. Targeting HDAC6 in combination with traditional therapeutic agents may provide a novel strategy to improve chemotherapeutic efficacy and decrease tumor burden of HNSCC patients through its synergistic cytotoxic effects.

Secondly, the epigenetic regulation on molecular signaling pathways leading to cisplatin resistance in HNSCC is currently poorly understood. Hence, my study aims to unravel the detailed mechanism of cisplatin resistance mediated by NF-κB signaling pathway and CSCs, involving epigenetic regulation via histone demethylases. Investigating the epigenetic regulation of cisplatin resistance may shed light on potential therapeutic targets to improve current HNSCC therapy, and could provide implications for development of a novel therapeutic modality.
Lastly, activated MET is frequently overexpressed and highly associated with HNSCC invasion and metastasis. However, little is known about the epigenetic regulation of the MET signaling pathway and whether it plays a role in promoting HNSCC metastasis. In this study, we aim to investigate the role of lysine-specific demethylase KDM6B and its underlying mechanism in MET-driven HNSCC metastasis. Our study provides insight into the epigenetic regulation of HNSCC invasion and metastasis and suggests that KDM6B could be an important therapeutic target for inhibiting invasive tumor growth and metastasis.
2 MATERIALS AND METHODS

2.1 Cell culture, siRNA transfection, and viral transduction

HNSCC cell lines UM-SCC1, UM-SCC9, and UM-SCC23 were obtained from Dr. Thomas Carey at the University of Michigan, and both parental and cisplatin resistant UM-SCC22B cell lines were obtained from Dr. Jacques Nor at the University of Michigan, School of Dentistry. These cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FBS with 1% penicillin-streptomycin (Invitrogen) at 37°C with 5% carbon dioxide. BTZ (L.L.C. Laboratories) was dissolved in DMSO as a 10mM stock solution and stored at -80°C. Cisplatin (Sigma-Aldrich) was also dissolved in DMSO as a 0.1M stock solution and stored at -80°C. TSA and Bafilomycin A1 were both purchased from Sigma Aldrich, Inc. and were dissolved in DMSO and stored at -20°C.

For siRNA transfections, SCC cells were seeded into 6-well or 6cm plates for 12h and then transfected using Lipofectamine RNAiMAX reagents according to the manufacturer’s protocol (Invitrogen). Two days (48h) after the transfection, the cells were harvested for various assays. KDM3A, KDM6B, ETS1, HMGA2, JUN, FOS, ELK1, HDAC6, and scramble siRNAs were procured from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Each siRNA consists of pools with three to five target-specific 19-25 nt siRNAs designed to knockdown target gene expression.

shRNAs targeting KDM6B and KDM4A were prepared using the lentiviral expression vector pLKO.1 (Addgene). The sequence with no known homology (scramble) is 5’-CCTAAGGTAACTCGCCTCG-3’; the human HDAC6 targeted sequence is 5’-GGATGGATCTGAACCTTGAGA-3’; the human ATG5 targeted sequence is 5’-CTTTGATAATGAACAGTGAGA-3’; the human KDM6B targeted sequence are 5’-GCGGCTCGTGTATGTACAT-3 (KDM6Bsh1) and 5’-CTGTTCGTGACAAGTGAGA-3 (KDM6Bsh2); and the human KDM4A targeted sequence are 5’-CGAGTTATCAACTCAAGAT-3’ (KDM4Ash1) and 5’-GCCACGAGCATCCTATGAGA-3’ (KDM4Ash2). pLKO human ULK1 shRNA 8 (#27633) were purchased from Addgene, Inc. Lentivirus production was performed according to the protocol provided by
Addgene using 293T viral packaging cells. 48 hours after the transfection, the media containing viruses were collected and used for infection. Infected cells were incubated for 48 hours and then selected with antibiotics for at least 1 week, at which point knockdown was confirmed by real-time reverse transcription polymerase chain reaction (RT-PCR) or Western blot analysis.

pBabe-TPR-MET was provided by Dr. Vande Woude, and pCMV-HA (hemagglutinin)-KDM4A was purchased from Addgene and subcloned into the retroviral vector pQCXIII. The human pcDNA-HDAC6-FLAG plasmid was purchased from Addgene and subcloned into the retroviral vector pMSCV. Retroviral vector KDM3A and KDM6B were provided by Dr. Paul Khavari. For viral transduction, retrovirus was generated by GP2-293T retroviral packaging cells. 48 hours after the transfection, the media containing viruses were collected and used for infection. Infected cells were incubated for 48 hours and then selected with antibiotics for at least 1 week, at which point knockdown was confirmed by real-time reverse transcription polymerase chain reaction (RT-PCR) or Western blot analysis.

2.2 Cell viability, proliferation, and apoptosis assays

Control and ATG5, HDAC6, ULK1, KDM3A, KDM6B, or ULK1 knockdown cells were seeded on 6-well or 12-well plates at 2x10^5 cells per well. Cells were then treated with the chemotherapeutic agents. 24, 48, or 72 hours after the treatment, viability of the cells was determined using trypan blue staining or Annexin V apoptosis assay. For trypan blue staining, viable and dead cell numbers were recorded by Countess II FL automated cell counters (Invitrogen). PE Annexin V Apoptosis Detection Kit I was purchased from BD Pharmigen, and the experiments were done according to the manufacturer’s instruction. Data were obtained using flow cytometry analysis using FACS SCAN flow cytometer (Becton-Dickinson, San Jose, CA) at the flow cytometry center at University of California, Los Angeles. For each sample, 10,000 events were counted and recorded. All samples were performed in triplicates. The cell proliferation assay
was done using WST-1 based colorimetric assay (Roche) following the manufacturer’s instruction. Cells were seeded in 96-well plates for 24, 48, or 72 hours. For IC50 determination, cells were treated with various doses of cisplatin (0.1, 0.4, 2, 10, 50, 250 μM) for 24, 48, or 27 hours, respectively. After the treatment, cells were incubated with cell proliferation reagent WST-1 for 2 hours at 37°C with 5% CO2. Absorbance at 420-480 nm, with the reference wavelength more than 600 nm, was recorded with a Bio-Rad Smartspec Plus spectrophotometer. Calculation of the IC50 value was obtained using BiDatafit 1.02 software, a nonlinear curve fitting program.

2.3 Immunoprecipitation and Western blot analysis

Cells cultured in 10-cm dishes were washed with PBS and were then collected using the scraper. The cells were lysed using whole cell lysate buffer (Sigma Aldrich) or radioimmunoprecipitation (RIPA) assay buffer (10mM tris-HCL, 1 mM EDTA, 1% SDS, 1% NP-40, 50 mM b-glycerophosphate, 50 mM sodium fluoride) with 1/100 volume of protease inhibitor cocktail for 1hr on ice. After centrifugation at 4 °C, the supernatants were collected and stored at -80 °C. For immunoprecipitation, the supernatants were incubated with Dynabeads Protein A or G (ThermoFisher Scientific) for at least 2 hours at 4°C. Immunoprecipitates were washed three times with lysis buffer. Proteins bound to the beads were eluted with SDS loading buffer at 98°C for 5 min and then subjected to SDS-PAGE. The protein concentration of each sample was measured colorimetrically using Bio-Rad reagents, and 40 μg of total proteins were resolved and separated on 5-15% SDS-PAGE. The gel was then transferred onto a polyvinylidene difluoride (PVDF) membrane using the Bio-Rad semidry transfer system. The membranes were incubated with blocking solution containing 5% dry-milk in TBS/Tween 20 buffer at room temperature for 1 hour and subsequently probed with primary antibodies at 4°C overnight. Appropriate secondary antibodies were used and detected using ECL reagents (Pierce, Rockford, IL, USA) on the Kodak
BioMax MS film or Bio-Rad ChemiDoc MP system using Image Lab 5.2.1 version. Primary antibodies against the indicated proteins were purchased from the following sources at the indicated dilutions: Cleaved caspase 3 (1:1000; Cell Signaling Technology), Cleaved caspase 9 (1:1000; Cell Signaling Technology), LC3 (1:1000; Cell Signaling Technology), Acetyl tubulin Lys 40 (1:1000; Cell Signaling Technology), phosphor-ULK1 Ser317 (1:1000; Cell Signaling Technology), ULK1 (1:2000; Cell Signaling Technology), phosphor-mTOR Ser2448 (1:1000; Cell Signaling Technology), mTOR (1:1000; Cell Signaling Technology), GAPDH (1:2000; Cell Signaling Technology), phosphor-Beclin1 Ser93/96 (1:1000; Cell Signaling Technology), Beclin1 (1:1000; Cell Signaling Technology), Ubiquitin (1:2000; Santa Cruz Biotechnology), KDM6B (1:1000; Cell Signaling Technology), ETS1 (1:1000; Cell Signaling Technology), HMGA2 (1:1000; Cell Signaling Technology), phosphor-ELK1 Ser383 (1:1000; Cell Signaling Technology), ELK1 (1:1000; Cell Signaling Technology), KDM4A (1:1000; Cell Signaling Technology), KDM3A (1:2000; Bethyl Laboratories), phosphor-p65 (1:2000; Cell Signaling Technology), p65 (1:2000; Cell Signaling Technology), Bcl2A1 (1:1000; Biovision), cIAP2 (1:1000; Cell Signaling Technology), Mcl1 (1:1000; Cell Signaling Technology), Bax (1:1000; Cell Signaling Technology), cleaved PARP (1:1000; Cell Signaling Technology), α-tubulin (1:100,000; Sigma-Aldrich), and TBP (1:2000, Abcam).

2.4 Immunofluorescence staining and GFP-LC3 puncta formation assay

pBABEpuro LC3-GFP plasmid was provided by Dr. Reuben Kim. HNSCC cells were infected with retroviruses expressing LC3-GFP plasmid for 48 hours and selected with antibiotics for at least 1 week. Cells were then collected, plated onto chamber slides, and treated with BTZ and/or TSA for 24 hrs. Consequently, cells were stained with an anti-ubiquitin antibody overnight and then incubated with appropriate secondary antibodies conjugated with fluorophore (Jackson
Laboratories) for 1 hr to visualize ubiquitin localization. To detect nuclei, 4,6-diamidino-2-phenylindole (DAPI; Life Technologies) staining was done for 5 minutes. Slides were examined for ubiquitin positive inclusion bodies using a Nikon Eclipse TE 2000E inverted microscope and captured images with a Leica Sp2 MP-FLIM confocal microscopy at the CNSI Advanced Microscopy core at University of California, Los Angeles. The presence of ubiquitin-positive inclusion bodies was considered indicative of intracellular aggregates formation, and the aggregates were quantified in five different fields. Analysis of LC3 staining was also done with flow cytometry by following the protocols described by Shvets et al.84

2.5 Real-time reverse transcriptase polymer chain reaction (RT-PCR)

Total RNA was isolated from HNSCC cells using Trizol reagents (Invitrogen). 2-µg aliquots of RNAs were synthesized using random hexamers (Sigma) and M-MuLV reverse transcriptase (NEB). The real-time PCR reactions were performed using the iQ SYBR green supermix (Bio-Rad) on the Icycler iQ Multi-color Real-time PCR Detection System (Bio-Rad). The human gene primer sequences used in real-time qPCR for experiments are provided in Table 1. The primers for histone demethylase profiling are provided in Table 2.

<table>
<thead>
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<th>GENES</th>
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Table 2. Primer sequences used in profiling of histone demethylases.

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<th>GENES</th>
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### 2.6 Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using a ChIP assay kit (Upstate, USA) according to the manufacturer’s protocol. Cells were incubated with a dimethyl 3,3’ dithiobispropionimidate-HCl (DTBP) (Pierce) solution (5 mmol) for 10 min at room temperature, followed by 1% formaldehyde treatment for 15 min in a 37°C water-bath. For each ChIP reaction, 2x 10^6 cells were used. All resulting precipitated DNA samples were quantified with Real-time PCR. Data are expressed as the percentage of input DNA. Antibodies for ChIP assays were purchased from the following commercial sources: anti-KDM6B (ChIP grade; Abcam), anti-KDM3A (ChIP grade; Abcam), anti-H3K27me3 (ChIP grade; Abcam), anti-H3K9me3 (ChIP grade; Abcam), anti-p65 (Santa Cruz Biotechnology), anti-ELK1 (ChIP grade; Abcam), and anti-IgG (Cell Signaling Technology). The primer sequences used in ChIP assays are provided in Table 1-3.

#### Table 3. Primer sequences used for ChIP assays.

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<td>MCL1 (7kb up)</td>
<td>TTGAGTCCATCATCTTGACC</td>
<td>CTGCTTTTCAAGCCATCAT</td>
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<td>TGACTTTATGGGATCGTACCC</td>
<td>AGGTGATTGCATCATCATGAG</td>
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<tr>
<td>cIAP2 (7kb up)</td>
<td>GAAATGCAAGCTCTCCTCCA</td>
<td>CACTTGGGCTTGGTAGTAG</td>
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### 2.7 Invasion Assay

To screen cell invasion, 1x10^5 cells (siRNA-transfected or shRNA transduced) were plated onto 24-well BD BioCoat Matrigel Invasion chambers (BD Biosciences) with DMEM media containing no FBS while the bottom of the chamber is filled with DMEM media with 1% FBS. Cells were then treated with human recombinant HGF, BTZ and/or TSA, or Cisplatin, respectively.
After 24 hours, chambers were stained with the HEMA 3 kit (Fisher). The images of invaded cells were recorded and counted in 5 to 10 random fields and averaged. Each group was performed in triplicates.

2.8 CSC isolation and in vitro tumorsphere formation assay

To isolate putative CSCs from cultured HNSCC cell lines, tumor cells were first stained with the ALDHEFLUOR assay kit (STEMCELL Technologies) for 30 min at 37°C following the manufacturer’s guidelines to label the ALDH\textsuperscript{high} populations. Tumor cells were then isolated and incubated with anti-CD44-APC (1:100; BD PharMingen) for 30 min and propidium iodide (PI) for 5 min on the ice. ALDH\textsuperscript{high} CD44\textsuperscript{+} CSCs were sorted by a FACSVantage SE (Beckton Dickson). Sorting results were analyzed with FlowJo software (https://www.flowjo.com).

For tumorsphere formation assays, FACS-sorted cells were cultured in ultra-low attachment plates at a density of 100-500 cells/well. Cells were cultured in serum-free DMEM/F12 (Thermo Fisher Scientific) supplemented with 1% B27 supplement (Thermo Fisher Scientific), 1% N2 supplement (Thermo Fisher Scientific), penicillin-streptomycin (100 mg/ml; Invitrogen), human recombinant epidermal growth factor (EGF; 20 ng/ml; R&D Systems), and human recombinant basic fibroblast growth factor (bFGF; 10 ng/ml; R&D Systems), in a humidified 5% CO2 incubator at 37°C. Fresh aliquots of EGF and bFGF were added every 2 days, and the serum-free media were changed every other day until the spheres formed. For passaging, tumorspheres were collected by centrifugation and dissociated with trypsin-EDTA (Invitrogen) to obtain single cells. Cells were then re-suspended in sphere culture media after removing serum. Spheres with a diameter over 40 mm were counted 1-2 weeks after plating.

2.9 Immunohistochemistry staining
To use of human HNSCC samples for this study was approved by the UCLA Institutional Review Board. Paraffin-embedded sections provided by the UCLA Translational Pathological core were deparaffinized with 100% xylene twice for 5 min and then rehydrated with distilled water through an ethanol series step by step. The slides were stained with polyclonal antibodies against KDM6B (1:100; Cell Signaling Technology) or control IgG (Santa Cruz Biotechnology). Slides were then incubated with HRP-labelled polymer for 1hr, detected the immunocomplexes with AEC+ chromogen (Dako EnVision System), and counterstained with hematoxylin QS (Fisher). The intensity of immunostaining was scored as follows: 0, no staining; +, weak staining; ++, moderate staining; and ++++, strong staining. The Wilcoxon rank sum test was applied to test the significant differences in immunohistochemical staining intensity between different groups. The Pearson correlation coefficient of linear regression was used to determine the correlation between different proteins. All statistical analyses were performed with SPSS 23.0 software.

2.10 Orthotopic mouse model of HNSCC

All animal experiments were performed in accordance with a protocol approved by the UCLA Committee on Animal Care. Both SCC23/MET/Scramble and SCC23/MET/KDM6Bsh1 cells (3x10^5 cells in 0.1ml of PBS) were submucosally inoculated into the floor of the mouth near the base of the tongue in 8 to 10-weeks old nude mice (8 mice per group). Tumor growth was measured using bioluminescence imaging every 3 days after injection. Briefly, mice were anesthetized with ketamine and xylazine, subsequently injected with D-luciferin (300 mg/kg in PBS), and imaged in an IVIS Spectrum Xenogen machine (Caliper Life Sciences). Bioluminescence analysis was performed with live imaging software according to the manufacturer’s instruction (version 4.0; Caliper Life Sciences). To detect cervical lymph node metastasis, cervical lymph nodes from individual mice were harvested, incubated with D-luciferin
solutions in a petri dish. These collected lymph nodes were imaged in an IVIS Spectrum Xenogen machine at the end of experiments. Both the control group mice (n=8) and the KDM6B knockdown group mice (n=8) were sacrificed for isolation of cervical lymph nodes at 20 days after inoculation. The harvested cervical lymph nodes were fixed with 4% paraformaldehyde in PBS overnight. Samples were then rinsed with cold PBS, equilibrated in 30% sucrose in PBS overnight and embedded in paraffin for immunohistochemistry staining. The lymph nodes were stained with pan-cytokeratin antibody (Santa Cruz Biotechnology) or control IgG (Santa Cruz Biotechnology) then counterstained with hematoxylin QS.

2.11 Microarray procedure and data analysis

For microarray, total RNA was extracted with miRNeasy kit (Qiagen) following the manufacturer’s instruction. 1μg of RNA from each sample was hybridized to Affymetrix Human Genome U133 plus 2.0 array at the UCLA DNA Microarray facility. The arrays were scanned with a GeneArray scanner (Affymetrix). The robust multichip average (RMA) method was used to normalize the raw data. Differential gene expression analysis was performed based on the generated metadata txt file. >1.6fold change (37% inhibition) in expression compared to Scrsb was considered significant down-regulation. To investigate enriched biological functions associated with KDM4B/KDM6B-dependent genes, GO analysis was performed using the online Database for Annotation, Visualization and Integration Discovery (DAVID) Bioinformatics Resources v6.7. P values were calculated based on hypergeometric distribution. Only significantly overexpressed (p<0.05) GO terms were included. Heatmap was generated via PermutMatrix v1.9.3 (Caraux and Pinloche, 2005) to reflect fold change relative to median expression of each gene in Log2 ratio.

2.12 Statistical Analysis
Experiments presented in the figures are representative of at least three independent repetitions. Numerical data and histograms were expressed as the mean ± standard deviation. In comparisons between two groups, two tailed Student’s t-test was performed and a difference was considered statistically significant with $P < 0.05$. In multiple group comparisons, one-way ANOVA was employed with Tukey’s methods as post-hoc adjustment for Type I errors. All statistical analyses were performed with SPSS 23.0 software.
3 ROLE OF HDAC6 IN AUTOPHAGY MEDIATED HNSCC CHEMORESISTANCE

3.1 BTZ induces both autophagy and apoptosis in HNSCC

In our previous work, we showed that BTZ induced apoptosis in several HNSCC cells lines including UM-SCC1 (SCC1) and UM-SCC23 (SCC23), which could be synergistically enhanced in combination treatment with TSA\textsuperscript{53}. In this study, we explored whether BTZ induced autophagy in these cell lines. During autophagy activation, microtubule-associated protein 1A/1B-light chain 3 (LC3)-I is conjugated to LC3-II (also known as LC3B) by lipitation\textsuperscript{84}. Thus, LC3 has been widely used as an indicator of autophagy activation\textsuperscript{84,85}. Western blot analysis revealed that both LC3-I and LC3-II expression increased in a time dependent manner in SCC1 cells following BTZ treatment, indicating activation of autophagy (Fig. 1-1A). To determine whether autophagy attenuated BTZ-induced apoptosis, we stably knocked down autophagy protein 5 (ATG5) in SCC1 cells using shRNA (Fig. 1-1B). ATG5 is known to be an essential protein required in the formation of the autophagosome, a cytosolic double-membrane vacuole that fuses with lysosomes\textsuperscript{86}. Treatment with BTZ showed a significant increase in apoptosis in ATG5-knowckdown (ATG5 KD) SCC1 cells compared to the control cells (Fig. 1-1C and 1-1D). Western showed that ATG5 knockdown downregulated autophagy activation but enhanced caspase-9 activation induced by BTZ (Fig 1-1E). Additionally, knockdown of ATG5 using smartpool ATG5 siRNA in SCC1 cells further confirmed that ATG5 knockdown enhanced BTZ-induced apoptosis and caspase-9 activation (Data not shown). Moreover, we found that ATG5 knockdown also enhanced apoptosis in SCC23 cells induced by BTZ (Data not shown). Collectively, these data indicate that autophagy might play an important role in decreased chemosensitivity in HNSCC cells.
FIGURE 1-1. Induction of autophagy attenuates BTZ cytotoxicity in HNSCC cells. A, Western blotting showed that LC3 was induced by BTZ in a time-dependent manner. α-Tubulin (Tubulin) was used as a loading control. B, Real-time RT-PCR showing the mRNA level of ATG5. Scramble, SCC cells infected with viruses expressing scramble shRNA; ATG5KD, SCC1 cells infected with viruses expressing ATG5 shRNA. Values are means ± S.D.; ** p < 0.01. C, The knockdown of ATG5 enhanced BTZ-induced cell death in SCC1 cells. The cell viability assay results are representative of three independent experiments. Values are means ± S.D.; * p< 0.05; ** p < 0.01. D, Apoptosis detection with FITC-Annexin V and propidium iodied (PI) staining using flow cytometry for ATG5KD SCC1 cells treated with BTZ for 24 hrs. E, Western blotting of LC3, cleaved caspase 9, Noxa, and Tubulin in ATG5KD SCC1 and control cells.
3.2 Aggresome formation and autophagy

Accumulation of unstable unfolded or misfolded proteins in the cytoplasm are prone to forming CPAs, which requires efficient disposal to reduce ER stress level and promote cell survival\(^{57,87}\). An increasing number of studies show that autophagy removes these protein aggregates in the form of the aggresome to promote tumor cell survival. We found that BTZ treatment induced the accumulation of ubiquitylated unfolded or misfolded proteins in SCC1 cells (Fig. 1-2A). Immunofluorescence staining with anti-ubiquitin and anti-vimentin antibodies revealed aggresome formation in SCC1 cells as visualized by confocal microscopy. TSA is a broad type potent HDAC inhibitor, which has been known to inhibit zinc-dependent deacetylases including Class I, II, and IV HDACs\(^{88}\). In contrast, the combined treatment of BTZ and TSA failed to form the aggresome (Fig. 1-2B). Instead, ubiquitylated protein aggregates were deposited in the cytoplasm diffusely. The number of the aggresomes formed in TSA- and BTZ-treated SCC1 cells was also significantly reduced compared to BTZ-treated SCC1 cells (Fig. 1-2C). Similarly, TSA also significantly inhibited BTZ-induced aggresome formation in SCC23 cells (Data not shown).

Autophagy activation is associated with aggresome formation. We performed GFP-LC3 puncta formation assays to monitor BTZ-induced autophagy in SCC1 cells using mammalian expression reports containing human \(LC3\) gene fused with the green fluorescent protein (GFP). Whereas BTZ treatment alone induced GFP-LC3 punctate formation, TSA addition significantly inhibited BTZ-induced autophagy activation using immunofluorescent assay (Fig. 1-3A and 3B). The flow cytometry analysis further confirmed the significant reduction of LC3 expression in SCC1 cells treated with BTZ and TSA together compared to the BTZ alone (Fig. 1-3C). Western blot analysis also confirmed that TSA inhibited the expression of LC3-II induced by BTZ, and the blockage of LC3 degradation and autophagosome turnover at the lysosome using Baf revealed that
LC3 was inhibited at the prior stage to the lysosomal degradation (Fig. 1-3D). Consistently, we found that TSA also inhibited autophagy activation induced by BTZ in SCC23 cells as determined by GFP puncta formation assays, flow cytometry, and western blot (Data not shown).

**FIGURE 1-2. TSA inhibits BTZ-induced aggresome formation in HNSCC cells.** A, Western blotting of Ubiquitin in SCC1 cells following BTZ treatment. B, Microscopic images of aggresome using anti-ubiquitin and anti-vimentin antibodies and DAPI staining in SCC1 cells treated with DMSO, TSA, and/or BTZ for 24 hrs. Scale bar, 15 μm. C, The average number of aggresome per 100 SCC1 cells treated with DMSO, TSA, and/or BTZ for 24 hrs. Values are means ± S.D.; ** p < 0.01. Data were collected from three independent experiments, and at least 10 images per slide were analyzed.
FIGURE 1-3. TSA inhibits BTZ-induced autophagy activation in HNSCC cells. A, Microscopic images of GFP-LC3 puncta in SCC1 cells treated with DMSO, TSA, and/or BTZ for 24 hrs. Scale bar, 15 μm. B, Bar graph represents average number of GFP-LC3 puncta/cell in SCC1 cells treated with DMSO, TSA, and/or BTZ for 24 hrs. Values are means ± S.D.; ** p < 0.01. C, Flow cytometry analysis of SCC1 cells infected with lentiviral expressing GFP-LC3. D, Western blotting of LC3 proteins in SCC1 cells treated with DMSO, Baf, TSA, and/or BTZ for 16 hrs.
3.3 HDAC6 regulation on aggresome formation and autophagy induction

Recently, it has been shown that HDAC6 was involved in gathering scattered polyubiquitylated CPAs and transporting them to the microtubule organizing center to promote aggresome formation\textsuperscript{56,59,63}. To determine whether HDAC6 played a role in BTZ-induced aggresome formation and autophagy induction in SCC cells, we stably knocked down HDAC6 using shRNA (HDAC6 KD) in SCC1 cells (Fig. 1-4A). In accordance with other studies, HDAC6 KD SCC1 cells treated with BTZ showed disruption in aggresome formation compared to the control SCC1 cells treated with BTZ (Fig. 1-4B), and the number of the aggresome in HDAC6 KD SCC1 cells was also significantly reduced (Fig. 1-4C). We also used smartpool HDAC6 siRNAs (HDAC6si) in SCC1 cells to provide a control for off-target knockdown effect and obtained the similar results (Data not shown).

Moreover, GFP-LC3 punctate formation assays revealed that HDAC6 KD in SCC1 cells significantly inhibited autophagy induction (Fig. 1-5A and 1-5B) in addition to disruption of BTZ-promoted aggresome formation. Western blot analysis and flow cytometry analysis also confirmed that knockdown of HDAC6 inhibited BTZ-induced autophagy activation (Fig. 1-5C and 1-5D). Additionally, we showed consistent results in HDAC6si SCC1 cells treated with BTZ compared to the control (Data not shown). Further, knockdown of HDAC6 in SCC23 cells also inhibited autophagy activation (Data not shown).

Previously, we found that TSA enhanced BTZ-induced apoptosis in SCC cells\textsuperscript{53}. Interestingly, we found that TSA also attenuated HDAC6 activity that resulted in increased expression of acetyl-tubulin in the presence of BTZ, indicating TSA might inhibit autophagy through HDAC6 activity in SCC1 cells (Fig. 1-6A). Consistently, we found that HDAC6 knockdown in SCC1 cells potently enhanced DNA fragmentation induced by BTZ (Fig. 1-6B). In
addition, the knockdown of HDAC6 also promoted the activation of caspase-9 and -3 induced by BTZ (Fig. 1-6C) and apoptosis (Fig. 1-6D). Apoptosis also increased in HDAC6si SCC1 cells treated with BTZ (Data not shown). Taken together, our results implicate that HDAC6 mediates chemoresistance in HNSCC cells by promoting an aggresome formation and autophagy induction under ER-stress.

**FIGURE 1-4. HDAC6 is required for BTZ-induced aggresome formation in HNSCC cells.**

A, Real-time RT-PCR showing the mRNA level of HDAC6 in SCC1 cells infected with lentiviruses expressing HDAC6 shRNA vector or scramble. **p < 0.01. B, Microscopic images of aggresome using anti-ubiquitin and anti-vimentin antibodies and DAPI staining in HDAC6 knockdown SCC1 cells and control cells treated with BTZ for 24 hrs. Scramble, SCC1 cells infected with lentiviruses expressing scramble shRNA; HDAC6KD, SCC1 cells infected with lentiviruses expressing HDAC6 shRNA. Scale bar, 15 μm. C, Bar graph represents the average number of aggresome per 100 cells in HDAC6KD SCC1 cells and control cells treated with BTZ for 24 hrs. Values are means ± S.D; ** p < 0.01, Data were collected from three independent experiments.
FIGURE 1-5. HDAC6 is required for BTZ-induced autophagy activation in HNSCC cells. **A**, Microscopic images of GFP-LC3 puncta in HDAC6KD SCC1 cells and control cells treated with BTZ for 24 hrs. Scale bar, 15 μm. **B**, Bar graph represents the average number of GFP-LC3 puncta per cell in HDAC6KD SCC1 cells and control cells treated with BTZ for 24 hrs. Values are means ± S.D; **p < 0.01. C**, Western blotting of LC3 proteins in HDAC6KD SCC1 cells and control cells treated with BTZ for 24 hrs. **D**, Flow cytometry analysis of scramble and HDAC6KD SCC1 cells infected with lentiviral expressing GFP-LC3.
FIGURE 1-6. The knockdown of HDAC6 significantly increases BTZ-induced apoptosis in HNSCC cells. A, Western blotting of acetyl α-tubulin and GAPDH in SCC1 cells treated with BTZ or TSA for 24 hrs. B, The HDAC6KD enhanced DNA fragmentation in SCC1 cells as determined by DNA ladder assays. M, 1kb DNA ladder. C, Western blotting of cleaved caspase 3 and cleaved caspase 9 in control and HDAC6KD SCC1 cells following BTZ treatment. D, Apoptosis detection with FITC-Annexin V and propidium iodide (PI) using flow cytometry for HDAC6KD SCC1 cells treated with BTZ for 24 hrs.
3.4 HDAC6 and autophagy inhibitor mTOR

To further assess HDAC6 involvement in the regulation of autophagy initiation under BTZ treatment, we first examined the phosphorylation status of a series of kinases involved in autophagy induction regulation using HDAC6 KD and control SCC1 cells. ULK1 is a kinase regulating autophagy initiation and progression by phosphorylating Beclin 1 and recruiting a pro-autophagic VPS34 lipid kinase and ATG14L to initiate autophagosome formation. Recent studies revealed that ULK1 activation was directly regulated by the different activation statuses of a pro-autophagic 5′ AMP-activated protein kinase α (AMPKα) and an anti-autophagic mammalian target of rapamycin (mTOR). Consistent with HDAC6 knockdown, western blot analysis showed that the levels of acetyl-tubulin (Lys40) were increased in HDAC6 KD SCC1 cells. Interestingly, HDAC6 knockdown enhanced mTOR phosphorylation induced by BTZ as determined by anti-phospho-mTOR (Ser2448) antibodies (Fig. 1-7A). In contrast, HDAC6 KD negligibly affected AMPKα phosphorylation in SCC1 cells (Data not shown). Since mTOR activation could disrupt interaction between ULK1 and AMPK, we examined whether HDAC6 knockdown affected ULK1 phosphorylation mediated by AMPK. Western blot analysis showed that BTZ-induced ULK1 phosphorylation (Ser317) mediated by AMPK was partially inhibited. Consistently, we found that HDAC6 KD also reduced Beclin1 phosphorylation (Ser93/96) induced by BTZ in SCC1 cells. In addition, western blot analysis showed that the restoration of HDAC6 rescued the BTZ-induced autophagy activation by attenuating mTOR phosphorylation, ruling out off-target effect of shRNA (Fig. 1-7B). Furthermore, knockdown of ULK1 using shRNA in SCC1 cells inhibited autophagy activation and BTZ-induced apoptosis (Fig. 1-8A, 1-8B, and 1-8C). Consistently, ULK1 knockdown using siRNA (ULK1si) also significantly enhanced BTZ induced apoptosis in SCC1 cells (Data not shown). HDAC6 KD also inhibited heat shock protein 70
(HSP70) expression induced by BTZ-treatment in SCC1 cells (Fig. 1-8D) and SCC23 cells (Data not shown). HSP70 is one of the well-known HDAC6-regulated chaperone genes. HSP70 has been reported to express when there is accumulation of unfolded or misfolded proteins in the cytoplasm to restore ER homeostasis by stabilizing and preventing these unfolded proteins from forming toxic aggregates.

Similar to our HDAC6 KD data, BTZ or/and TSA treatment on SCC1 cells also altered the phosphorylation status of mTOR (Fig. 1-9A). Western blot analysis showed that the concurrent BTZ and TSA treatment on SCC1 cells increased acetylation of α-tubulin, indicating TSA treatment on SCC1 cells attenuate HDAC6 activity. TSA treatment also restored mTOR phosphorylation level, which was repressed by BTZ treatment alone. Interestingly, in addition to mTOR phosphorylation regulation, TSA treatment inhibited BTZ-induced AMPK phosphorylation level to inhibit ULK1 and Beclin1 activation and subsequent autophagy induction. The simultaneous treatment of TSA and BTZ also inhibited HSP70 expression in SCC1 (Fig. 1-9B) and SCC23 (Data not shown). These data together indicate that TSA enhances the cytotoxicity of BTZ by mainly inhibiting HDAC6 activity, which promotes mTOR activation to inhibit pro-survival autophagy induction through regulation of ULK1 in HNSCC cells.
FIGURE 1-7. The knockdown of HDAC6 attenuates BTZ-induced autophagy initiation in HNSCC cells. **A**, Western blotting of acetyl-α-tubulin (Lys40), α-tubulin, p-mTOR (Ser 2448), mTOR, p-ULK1 (Ser317), ULK1, p-Beclin1 (Ser93/96), Beclin1, and GAPDH in SCC1 cells following Btz treatment. **B**, Western blotting of Flag, acetyl-α-tubulin (Lys40), tubulin, p-mTOR (Ser 2448), mTOR, LC3, and GAPDH in HDAC6KD SCC1 cells following BTZ treatment.
FIGURE 1-8. The inhibition of ULK1 restores the cytotoxic effect of BTZ, and HDAC6 knockdown represses HSP70 expression in HNSCC cells. A, Western blotting of ULK1 and LC3 in scramble and ULK1 knockdown SCC1 cells following BTZ treatment. Scramble, SCC1 cells infected with viruses expressing scramble shRNA; ULK1KD, SCC1 cells infected with lentiviruses expressing ULK1 shRNA. B, The knockdown of ULK1 enhanced BTZ-induced cell death in SCC1 cells. Values are means ± S.D; ** p < 0.01. C, The ULK1KD enhanced BTZ-induced apoptosis in SCC1 cells. D, Real-time RT-PCR showing the mRNA level of HSP70 in SCC1 cells infected with lentiviruses expressing HDAC6 shRNA vector or scramble after BTZ treatment for 24hrs. ** p < 0.01.
**FIGURE 1-9.** BTZ and TSA together attenuates BTZ-induced autophagy initiation and inhibits HSP70 expression in HNSCC cells. 

A. Western blotting of acetyl-α-tubulin (Lys40), α-tubulin, p-mTOR (Ser 2448), mTOR, p-AMPK (Thr 172), AMPK, p-ULK1 (Ser317), ULK1, p-Beclin1 (Ser93/96), Beclin1, and GAPDH in SCC1 cells following DMSO, TSA, and/or BTZ treatment for 24hrs. 

B. Real-time RT-PCR showing the mRNA level of *HSP70* in SCC1 cells treated with DMSO, TSA, and/or BTZ for 24hrs. **p < 0.01.**
3.5 Discussion

Although BTZ was proposed as a potential alternative therapeutic regimen for HNSCC, clinical trials on BTZ-based regimens demonstrated that BTZ treatment alone exerts minimal antitumor effects in HNSCC patients. In this study, we showed that HDAC6-dependent autophagy induced by BTZ might be one of the potential mechanisms of chemoresistance. The inhibition of HDAC6 could enhance BTZ-induced apoptosis. Mechanistically, we found that the inhibition of HDAC6 affected mTOR phosphorylation and ULk1 activation in the early stage of autophagy initiation. Previously, we have shown that TSA potentiates BTZ-mediated apoptosis by inducing the expression of apoptotic genes. Our new results suggest that TSA might also sensitize HNSCC cells to BTZ by inhibiting autophagy.

In normal mammalian cells, protein synthesis is tightly controlled and balanced with the capacity of the cellular ubiquitin-proteasome system to degrade and prevent accumulation of unfolded or misfolded proteins, which can form toxic aggregates in the cytoplasm and result in activation of stress-induced apoptosis. However, in cancer cells, protein synthesis might be highly increased due to activated oncogenes promoting expression of mutant proteins and/or expression of excess proteins during tumorigenesis and tumor progress. This imbalance between the unfolded or misfolded protein load and ubiquitin-proteasome system is managed by overexpressing components of the UPR in multiple cancers to prevent cell death. UPR is an ER-mediated pro-survival mechanism network associated with numerous intracellular signal transduction pathways to regulate and maintain ER homeostasis in response to accumulation of unfolded proteins in its lumen. UPR can modulate expression of chaperones such as HSPs and/or activate autophagy machinery to remove large quantities of accumulated unfolded proteins before they can form toxic protein aggregates. Thus, UPR and autophagy have become a key
component in cancer cell survival and a critical complication to overcome resistance against chemotherapeutic agents such as proteasome inhibitors in various cancers. Previously, we found that BTZ treatment activated the cytoprotective ER transmembrane stress-sending kinase (PERK)-mediated UPR in HNSCC cells, resulting in upregulation of activating transcription factor 4 (ATF4)\(^{53,96}\). Along with our findings, recent articles revealed that increased PERK and ATF4 activation can subsequently upregulate transcription of LC3 in HNSCC cells\(^{53}\). In the present study, we were able to show that BTZ activated autophagy and the disruption of autophagy resulted in increased levels of apoptosis.

Interestingly, our previous study found that a combination regimen of TSA and BTZ synergistically induced ER-stress apoptosis by increasing Noxa expression in HNSCC cells and reducing tumor growth \textit{in vivo}\(^{53}\). Our data demonstrates that concomitant treatment of TSA and BTZ disrupts aggresome formation and inhibits autophagy, allowing CPAs to remain in the cytoplasm to apply persistent ER stress to induce apoptosis. Due to continued stress, UPR might switch from a cytoprotective role to a catabolic role, contributing to the enhanced apoptosis. Since HDAC6 plays a critical role in autophagy activation, we then analyzed the effect of HDAC6 knockdown on BTZ-induced apoptosis. Consistently, we found that knockdown of HDAC6 also enhanced BTZ-induced apoptosis by inhibition of autophagy and UPR.

Involvement of HDAC6 in aggresome formation and UPR-mediated autophagy is well established in many neurodegenerative disease studies and has recently been implicated in chemoresistance\(^{34,54,56,58}\). Concomitantly, our data also shows a critical role for HDAC6 in aggresome formation upon accumulation of unfolded/misfolded proteins followed by the BTZ treatment in HNSCC cells. Our data also suggests that HDAC6 might be involved in the activation of mTOR, which regulates autophagy initiation under ER-stress in HNSCC cells in addition to its
known roles of controlling autophagosome maturation and fusion of autophagosomes to lysosomes. Recently, Zhu et al. reported that HDAC6 is involved in modulating phosphoinositide 3-kinase (PI3K)-protein kinase B (Akt)-mTOR pathway in cerebral cortex neurons prepared from neonatal mice. mTOR and AMPK are known to directly regulate activation of ULK1, the initiating kinase for autophagosome formation and progression of autophagy. mTOR inhibits autophagy by phosphorylating ULK1 serine 757 while AMPK promote autophagy by phosphorylating ULK1 at serine 317, 555, and 777. Our data demonstrates that ablation of HDAC6 activity enhances phosphorylation of mTOR, resulting in decreased phosphorylation of ULK1 induced by AMPK and autophagy induction. However, since HDAC6 activity is inseparable from aggresome formation, we cannot determine whether HDAC6 alone specifically regulates autophagy initiation or aggresome formation. For instance, our TSA and/or BTZ treatment showed that TSA treatment alters mTOR phosphorylation as well as AMPK phosphorylation, which was not observed in HDAC6 KD SCC cells. Since TSA is a broad HDAC inhibitor, it may promote inhibition of BTZ-induce autophagy mainly by inhibiting HDAC6 activity; however, it may also inhibit other HDACs that have autophagy-related activity. Moreover, it will be interesting to examine how HDAC6 interacts with mTOR signaling. HDAC6 has been found to be highly expressed in HNSCC tissues. Based on our results, it is possible that HDAC6 might be a critical regulator of the cell protective response mediating the molecular network between ER stress, autophagy, and apoptosis to develop resistance to chemotherapy in HNSCC. Our results suggest that targeting HDAC6 might help to overcome BTZ-chemoresistance in HNSCC.
4 EPGENETIC REGULATION OF HNSCC CISPLATIN-RESISTANCE BY KDM3A AND KDM6B

4.1 Cisplatin-resistant HNSCC cell lines

Drug resistance is a critical problem in HNSCC treatment and has been attributed to the high recurrence rate and a low 5-year survival rate of patients for the last three decades. Thus, understanding the mechanisms underlying resistance to chemotherapy might have a profound impact in the survival of HNSCC patients. To evaluate the effect of cisplatin in HNSCC, our lab generated cisplatin resistant cell lines from two well-established HNSCC cell lines, UM-SCC1 (SCC1-R) and UM-SCC23 (SCC22B-R), by exposing the HNSCC cells to increasing concentrations of cisplatin over a long period of time. Also, we obtained another cisplatin-resistant cell line, UM-SCC-22BCis (SCC22B-R), and its parental cell line, UM-SCC22B (SCC22B), from Dr. Nor Jacques’s lab in University of Michigan to conduct experiments. All three cisplatin-resistant cell lines exhibited decreased cisplatin cytotoxicity compared to their parental cell lines, respectively, in a dosage dependent manner (Fig. 2-1A). Western blot analysis confirmed that cisplatin-induced apoptosis is also significantly inhibited in cisplatin-resistant cell lines compared to their parental cell lines (Fig. 2-1B). Additionally, we also examined the cytotoxic effect of cisplatin on both parental and cisplatin-resistant cell lines using a low dosage (3-5 μM), to determine the maintenance dosage for cisplatin-resistant cell lines during culture (Fig. 2-1C), and a high dosage (50 μM), to determine whether cisplatin-resistant cell lines can tolerate high dosages for a longer period of time compared to the parental cell lines (Fig. 2-1D). Further, we determined the half maximal inhibitory concentration (IC50) of cisplatin for both parental and cisplatin-resistant cell lines. We found that there was at least a 3-fold IC50 value difference between parental and resistant cell lines (Fig. 2-1E).
FIGURE 2-1. Establishment of cisplatin resistant HNSCC cell lines. **A**, Cisplatin-resistant cell lines (SCC1-R, SCC23-R, and SCC22B-R) are resistant to cisplatin in a dose dependent manner. Both parental and resistant cells were treated with 0, 5, 10, 20, and 50 μM of cisplatin for 24hrs. The cell viability assay results are representative of three independent experiments. Values are means ± S.D.; * p< 0.05; ** p < 0.01. **B**, Western blotting showed that caspase-3 and -9 activation was significantly inhibited in cisplatin-resistant cell lines. Both parental and resistant Cells were treated with DMSO (control) or 30 μM of cisplatin for 0, 1, 3, and 5 days. **C**, Maintenance dosage for cisplatin-resistant cell lines were examined. Both parental and resistant cells were treated with DMSO or 3 μM of cisplatin for 0, 3, 5, and 7 days. Values are means ± S.D.; * p< 0.05; ** p < 0.01. **D**, Cisplatin-resistant cell lines can tolerate high dosage cisplatin treatment for at least 3 days. Both parental and resistant cells were treated with DMSO or 50 μM of cisplatin for 0, 1, 2, and 3 days. Values are means ± S.D.; * p< 0.05; ** p < 0.01. **E**, The IC50 values were obtained from both parental (SCC1, SCC23, and SCC22B) and cisplatin-resistant cells. Cells were treated with 0.1, 0.4, 2, 10, 50, or 250 μM of cisplatin for at least 48 hours. Cell proliferation rate was measured with absorbance at 420-480 nm, with the reference wavelength more than 600 nm.

4.2 Characterization of cisplatin-resistant HNSCC cell lines

To further investigate the effect of cisplatin in HNSCC, we evaluated and characterized cisplatin-resistant cells, comparing to their respective parental HNSCC cells. First, we obtained gene expression profile of histone demethylases and attained the gene expression ratio between the parental (SCC1) and the cisplatin-resistant cells (SCC1-R) to identify the potential histone demethylases associated with cisplatin resistance (Fig. 2-2A). From this data, we observed that KDM3A (also known as JMJD1A), KDM6B (also known as JMJD3), and JHDM1D (also known as KDM7A) are highly expressed in SCC1-R cells compared to parental SCC1 cells. The results were further confirmed by real time RT-PCR in SCC1/SCC1-R and SCC23/SCC23-R cells and western blot analysis (Fig. 2-2B).
CSCs are resilient cells that has been shown to play a major role in resistance to chemotherapy and radiotherapy in various tumors including HNSCC\textsuperscript{31,71,97}. Previously, our lab and others isolated the population of CSCs in HNSCC\textsuperscript{31,71}, which are identified with a double staining of aldehyde dehydrogenase (ALDH) activity and cluster of differentiation 44 (CD44). CSCs isolated form HNSCC typically exhibit high ALDH activity level and CD44 expression (ALDH\textsuperscript{High}CD44\textsuperscript{High}) compared to other non-stem like HNSCC cells (ALDH\textsuperscript{Low}CD44\textsuperscript{Low} or High). We observed a significant increase in the fraction of ALDH\textsuperscript{High}CD44\textsuperscript{High} CSCs in cisplatin-resistant cell lines compared to their respective parental cell lines, indicating that the increased fraction of CSCs may contribute and enhance the cisplatin-resistance in these cisplatin-resistant cell-lines (Fig. 2-2C). In consistent with our previous findings on HNSCC CSCs, the expression of “stemness” genes, Bmi1 and Sox2, were highly elevated in ALDH\textsuperscript{High}CD44\textsuperscript{High} cells collected from both SCC1 and SCC1-R cell lines (Fig. 2-2D). Additionally, ALDH\textsuperscript{High}CD44\textsuperscript{High} cells, from SCC1 or SCC1-R cell lines, expressed significantly higher level of KDM3A and KDM6B compared to the ALDH\textsuperscript{Low}CD44\textsuperscript{Low} cells. To further explore whether KDM3A or KDM6B plays a critical role in maintaining CSC-like properties in cisplatin-resistant cells, we transiently depleted KDM3A or KDM6B using small interfering RNA (siRNA) in CSCs isolated from SCC1-R. The tumorsphere formation assay was utilized as a surrogate for CSC-like self-renewal \textit{in vitro}. Notably, while the ALDH\textsuperscript{High}CD44\textsuperscript{High} cells transfected with the control siRNA (Scr si) effectively formed sphere-like colonies as opposed to ALDH\textsuperscript{Low}CD44\textsuperscript{Low} cells, knockdown of KDM6B dramatically abolished the tumorsphere formation ability of ALDH\textsuperscript{High}CD44\textsuperscript{High} cells from SCC1-R (Fig. 2-2E and 2-2F). However, the depletion of KDM3A in ALDH\textsuperscript{High}CD44\textsuperscript{High} cells did not have a significant effect on the tumorsphere formation ability. We also obtained similar results in CSCs collected from SCC23-R and SCC22B-R (Data not shown). Further, we found that KDM6B
is highly expressed in human recurrent HNSCC tissue compared to the primary tumor, suggesting that KDM6B might promote HNSCC recurrence in human patients by mediating chemoresistance (fig. 2-2G).

Recent studies on cisplatin resistance in HNSCC reported that NF-κB mediates cisplatin-resistance in HNSCC cells. Our studies on resistant cell lines demonstrated that phosphorylation level of NF-κB subunit protein p65/RELA (p65) is significantly elevated in cisplatin-resistant cells compared to their parental cells (Fig. 2-3A). In addition, cisplatin promoted phosphorylation of p65 in a time dependent manner in SCC1 cells (Fig. 2-3B). The simultaneous treatment of cisplatin and IKKβ inhibitor, which blocks NF-κB activation, significantly increased cell death in both SCC1 and SCC-1R, suggesting a critical role of NF-κB in promoting cisplatin resistance in HNSCC and maintaining the survival of established resistant-cells (Fig. 2-3C). Furthermore, we conducted NF-κB target gene screening in SCC1 and SCC1-R and found that numerous NF-κB downstream genes, such as Bcl2A1, Mcl1, cIAP2 (also known as Birc3), Cox2, IL-8, IL-6, etc, were elevated in SCC1-R compared to SCC1 and SCC23-R (Fig. 2-3D). Bcl2A1, Mcl1, and cIAP2 are well-known NF-κB regulated anti-apoptotic molecules that interacts with the BCL-2 family proteins. Thus, we decided to further examine the gene regulation of Bcl2A1, Mcl1, and cIAP2 in relation to cisplatin-resistance. Taken together, our data exhibited that NF-κB and histone demethylases KDM3A and KDM6B may play a significant role in maintaining survival of cisplatin-resistant cells in HNSCC.
Gene Expression Ratio (scc1-cis/scc1)

Relative mRNA levels of kdm3a

Relative mRNA levels of kdm6b

SCC1 SCC1-R

KDM6B

KDM3A

TBP
C

**SCC1**

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D

**Relative mRNA levels of Bmi1**

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**Relative mRNA levels of Kdm3a**

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**Relative mRNA levels of Kdm6b**

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**Relative mRNA levels of Sox2**

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FIGURE 2-2. KDM3A and KDM6B expressions are elevated in cisplatin resistant HNSCC cells. A, Real time RT-PCR analysis of the histone demethylases family in parental SCC1 cells and cisplatin-resistant SCC1-R cells. Gene expression ratio of histone demethylases was obtained for SCC1-R over SCC1 cells. B, Real-time RT-PCR showing the mRNA level of KDM3A and KDM6B in SCC1, SCC23, SCC1-R, and SCC23-R cells. ** p < 0.01. Western blotting of KDM6B and KDM3A in SCC1 and SCC1-R. TBP was used as the loading control. C, Flow cytometry analysis of CSC fraction in the parental (SCC1, SCC23, and SCC22B) and the cisplatin resistant (SCC1-R, SCC23-R, and SCC22B-R). CSCs were isolated based on ALDH activity (x-axis) and CD44 expression (y-axis). D, mRNA levels of KDM3A (top left graph), KDM6B (top right graph), Bmi1 (bottom left graph), and Sox2 (bottom right graph) were obtained from ALDH Low CD44 Low and ALDH High CD44 High cells, which were isolated form SCC1 and SCC1-R cell lines. E, Microscopic images of tumorsphere formation. F, Knockdown of KDM6B using siRNA (KDM6B si) significantly inhibited tumorsphere formation of ALDH High CD44 High cells. The number of spheres were counted from five different fields and averaged. The results represent mean ± S.D. from three independent experiments; * p< 0.05; ** p < 0.01. G, Histochemical detection of KDM6B abundance in human primary HNSCC tissue and the recurrent HNSCC tissue.
### A

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### C

![Image](image40.png)
FIGURE 2-3. NF-κB pathway is highly activated in cisplatin resistant HNSCC cells. A, Western blotting of the whole cell lysate obtained from parental (SCC1, SCC23, and SCC22B) and cisplatin resistant (SCC1-R, SCC23-R, and SCC22B-R) cells using anti-phosphor-p65 (Ser536), p65, and tubulin. B, Western blotting of SCC1 cells treated with 30 μM cisplatin for 0, 0.5, 1, 2, 4, 8, 12, 16, and 24 hrs using phosphor-p65, p65, and tubulin antibodies. C, SCC1 cells were treated with DMSO, Ikkβ inhibitor, and/or cisplatin for 24 hrs. Values are means ± S.D; * p < 0.05; ** p < 0.01. D, real time RT-PCR analysis on NF-κB targeted genes in parental (SCC1 and SCC23) and cisplatin resistant (SCC1-R and SCC23-R) cells.
4.3 **KDM3A and KDM6B are required for the survival of cisplatin-resistant cells**

To further determine whether KDM3A and KDM6B promotes cisplatin-resistance in HNSCC cells via regulating pro-survival NF-κB target genes that were elevated in cisplatin-resistant cells, we knocked down KDM3A and/or KDM6B in SCC1-R using siRNA. The knockdown efficiency of KDM3A and/or KDM6B in SCC-1R was confirmed by real time RT-PCR (Fig. 2-4A) and western blot analysis (Fig. 2-4B). Apoptosis was significantly enhanced in SCC1-R cells that were transfected with either KDM3A and/or KDM6B siRNA compared to the control SCC1-R as well as SCC1 cells treated with cisplatin (Fig. 2-4C). Western blotting and real time RT-PCR analysis showed that caspase-3 and -9 activation was elevated while anti-apoptotic MCL1, cIAP2, and *BCL2A1* expression were downregulated in KDM3A and KDM6B double knockdown SCC1-R cells that were treated with cisplatin (Fig. 2-4D). Interestingly, although caspase-3 and -9 activation was slightly increased in SCC1-R cells depleted with KDM3A siRNA, it did not efficiently inhibit MCL1, cIAP2, and *BCL2A1* expression upon cisplatin treatment compared to KDM6B knockdown alone or KDM3A and KDM6B double knockdown SCC1-R cells. According to our data, the level of caspase activation and inhibition of MCL1, cIAP2, and Bcl2A1 expression was the highest when only KDM6B knockdown was established in SCC1-R cells. We also obtained similar results in SCC22B-R and SCC23-R cells that were transfected with KDM3A and/or KDM6B siRNA (Data not shown). Thus, we decided to focus and further examine the role of KDM6B in mediating cisplatin-resistance in HNSCC. Further mechanistic studies will be performed to obtain a detailed understanding of the molecular association between NF-κB and KDM6B to develop effective therapy to overcome cisplatin resistance in HNSCC.
Relative mRNA levels of kdm3a

Relative mRNA levels of kdm6b

B

Cytosol

Nuclear

KDM3A

KDM6B

TBP

Tubulin

C

SCC1

SCC1- R

DMSO

Cisp

FITC-Annexin V

FITC-Annexin V

0.0%

0.1%

6.1%

0.8%

6.1%

0.8%

5.6%

8.9%

5.6%

8.9%

6.1%

0.8%

6.1%

0.8%

10.8%

17.4%

11.4%

21.0%
FIGURE 2-4. KDM6B is required for the survival of cisplatin resistant HNSCC cells. A, real time RT-PCR analysis on KDM3A and/or KDM6B knock out SCC1-R cells using siRNAs. B, Western blotting of KDM3A, KDM6B, TBP (nuclear protein loading control), and Tubulin (cytosol protein loading control) on cytosol and nuclear fraction of SCC1-R cells transfected with KDM3A and/or KDM6B siRNAs. C, Apoptosis detection with FITC-Annexin V and propidium iodied (PI) using flow cytometry for KDM3A or KDM6B KD SCC1 cells treated with 30 μM cisplatin for 24 hrs. D, Western blotting and real-time RT-PCR analysis on expression of MCL1, cIAP2, BCL2A1, cleaved caspase-3, and cleaved caspase-9. ** p < 0.01.
4.4 Role of KDM6B in acquiring cisplatin-resistance in SCC cells

The role of histone demethylases KDM3A and KDM6B in the acquisition process of cisplatin-resistance in HNSCC were also assessed using parental SCC cell lines. Both KDM3A and KDM6B expressions were elevated in various HNSCC cell lines that were treated with cisplatin in a time-dependent manner (Fig. 2-5A). To further explore the role of KDM3A and KDM6B in HNSCC chemoresistance, we transiently inhibited the expression of KDM3A and/or KDM6B in SCC1 cells using the smartpool siRNAs. The knockdown efficiency of KDM3A and/or KDM6B was evaluated by real time RT-PCR (Fig. 2-5B). In consistent with the results obtained from SCC1-R cells, KDM3A and/or KDM6B knockdown notably decreased the cell viability and increased apoptosis upon cisplatin treatment (Fig. 2-5C). The KDM3A and/or KDM6B knockdown SCC1 cells also increased caspase-3 and -9 cleavage by increasing expression of pro-apoptotic Bid and Bik while inhibiting expression of anti-apoptotic MCL1 and cIAP2 when treated with cisplatin (Fig. 2-5D). Further, the rescue analysis assay revealed that increased cytotoxicity of cisplatin on SCC1 due to KDM3A and/or KDM6B knockdown was reverted when KDM3A and/or KDM6B levels were restored in these knockdown cells, indicating that KDM3A and KDM6B are directly related to cisplatin-resistance in HNSCC (Fig. 2-5E). Similar to the findings from the cisplatin-resistant cells, our data also showed that the effect of KDM6B alone on cisplatin-resistance of SCC1 cells is greater than KDM3A alone or KDM3A and KDM6B together. Thus, further future studies will be more focusing on the role of KDM6B in HNSCC chemoresistance. In addition, we also obtained similar results in SCC23, SCC22B, and Fadu cell lines, which further confirms our findings in SCC1 cells (Data not shown). In addition, we overexpressed KDM3A and/or KDM6B in SCC1 cells to further analyze their roles in HNSCC chemoresistance. The overexpression of KDM6B in SCC1 cells showed no changes in Mcl1 mRNA level upon cisplatin
treatment and decreased the percentage of overall cell death induced by cisplatin (Fig. 2-5F), suggesting the important role of KDM6B in promoting cisplatin-resistance in HNSCC.

To determine whether KDM6B epigenetically stimulated the expression of MCL1 and cIAP2 in SCC1 cells treated with cisplatin, we examined whether KDM6B was present on the promoters of MCL1 and cIAP2 using chromatin immunoprecipitation (ChIP) assays. ChIP assays revealed that both p65 and KDM6B were presented on the promoters of MCL1 and cIAP2 upon cisplatin treatment (Fig. 2-5G and 2-5H). Consistently, KDM6B binding to the promoter region of MCL1 and cIAP2 decreased the abundance of its substrate, H3K27me3, which is often a marker of gene silencing, suggesting that the histone demethylase ability of KDM6B might be required for the transcription of MCL1 and cIAP2 mediated by NF-κB. However, further experiments and ChIP assays are required to precisely assess the role of KDM6B on NF-κB regulated MCL1 and cIAP2 transcription, which is associated with cisplatin resistance in HNSCC.
Gene Expression

**KDM3A**

SCC1

SCC23

SCC22B

Fadu

cisplatin: 0h 8h 16h 24h
FIGURE 2-5. KDM6B is required for the acquisition of cisplatin resistance in HNSCC cells. 
A, real time RT-PCR analysis on KDM3A and KDM6B expression in SCC1, SCC23, SCC22B, and Fadu cells after 0, 8, 16, and 24 hours of cisplatin treatment. * p < 0.05; ** p < 0.01. B, real time RT-PCR analysis of KDM3A and KDM6B expression in KDM3A and/or KDM6B knockdown SCC1 cells. ** p < 0.01. C, KDM3A and/or KDM6B knockdown restores cisplatin cytotoxicity in SCC1 cells. Values are means ± S.D from three independent studies. ** p < 0.01. D, Western blotting of cIAP2, MCL1, cleaved caspase-3, and cleaved caspase-9 in KDM3A and/or KDM6B knockdown SCC1 cells, which were treated with 30 μM cisplatin for 24 hrs, and real-
time RT-PCR analysis of pro-apoptotic Bik and Bid expression. * p < 0.05; ** p < 0.01. E, Restoring KDM6B expression in KDM6B knockdown SCC1 cells reinstates diminished cisplatin cytotoxicity. F, Overexpression of KDM6B in SCC1 cells maintains high MCL1 expression even with cisplatin treatment and prevents cell death induced by cisplatin. G, ChIP assays of KDM6B, p65, and H3K27me3 abundance on the MCL1 promoter in SCC1 cells treated with cisplatin for at least 16 hrs. H, ChIP assays of KDM6B, p65, and H3K27me3 abundance on the cIAP2/Birc3 promoter in SCC1 cells treated with cisplatin for at least 16 hrs.

4.5 Discussion

Cisplatin is the most commonly used chemotherapeutic agent for HNSCC patients\textsuperscript{104,105}. However, numerous studies reported that the acquisition of cisplatin resistance in HNSCC is one of the main factors leading to disease relapse\textsuperscript{106,107}. In this study, we propose a putative explanation for a mechanism of cisplatin resistance mediated by histone demethylases using both cisplatin-responsive (parental) and cisplatin-resistant HNSCC cell lines. While this is still an ongoing study, our data indicate that histone demethylase KDM6B may play an essential role in promoting cisplatin resistance in HNSCC.

Tumors are heterogeneous and constituted by many subtypes of tumor cells\textsuperscript{108,109}. CSCs are rare subpopulation of cancer cells that has been recently identified in various tumors to promote tumor initiation, maintenance, progression, metastasis, and resistance to chemotherapy\textsuperscript{29,33,109}. CSCs are characterized as less differentiated, multipotent, and self-renewing compared to other types of tumor cells\textsuperscript{32,71,108,110}. Since cisplatin specifically targets high proliferating cells, cisplatin treatment may spare the CSCs, which undergo relatively slow proliferation rates compared to other subtypes of cancer cells, and promote tumor recurrence-mediated by CSCs. This suggests that a novel therapeutic strategy targeting CSCs, used in combination with conventional therapy, needs to be developed to treat HNSCC patients. Previously, our lab isolated CSCs in HNSCC and showed that CSCs highly express Bmi1, an important stem cell self-renewal factor\textsuperscript{31,67}. Consequently, targeting these Bmi1\textsuperscript{High} CSCs overcame chemoresistance and inhibited metastasis. In this study,
we identified that the fraction of CSCs is distinctly elevated in cisplatin-resistant cell lines compared to their parental HNSCC cell lines, indicating that cisplatin-resistant cells possess considerably more potent capacity to resist cisplatin. Additionally, we found that the expression of the histone demethylase, KDM6B, was enhanced in CSCs isolated from cisplatin-resistant HNSCC cells. Inhibition of KDM6B in these cells repressed the self-renewal capacity and the survival of CSCs in vitro. Altogether, our data indicate that KDM6B plays an important role in the self-renewal and chemoresistance properties of CSCs and therefore may be an attractive therapeutic target.

Recently, Almeida et al. reported that NF-κB signaling pathway is highly activated in the cisplatin resistant HNSCC cell lines: HN13, UMSCC74A, and Cal27, and it promotes chemoresistance by inhibiting the levels of the DNA repair molecule, BRCA1, through histone deacetylation. Consistently, we also observed that NF-κB signaling pathway is distinctly activated in the cisplatin resistant HNSCC cell lines. In addition, cisplatin treatment in non-resistant (parental) cells also induced NF-κB activation, indicating that NF-κB pathway may play a key role in acquiring cisplatin-resistance in HNSCC as well as promoting survival of these resistant cells leading to the relapse of the disease in the patients. In addition, Nor et al. revealed that CSCs reside in perivascular niches and surrounding tumor-associated endothelial cells express high levels of IL-6. They also demonstrated that IL-6 contributes to cisplatin-induced CSC stemness in HNSCC. It is well established that IL-6 signaling pathway, leading to the phosphorylation of signal transducer and activator of transcription 3 (STAT3), is employed by tumor cells to circumvent apoptosis-induced by chemotherapeutic drugs and maintain proliferation and neoplastic growth. Additionally, IL-6 signaling has been shown to mediate the expansion of the CSC population in transtuzumab-treated breast cancer. IL-6 is one of the well-known NF-
κB regulated genes\textsuperscript{113,114}. In our study, we showed that IL-6 expression is significantly enhanced in SCC1-R and SCC23-R cells, which correlates with the increased NF-κB activation in these cells. These data suggest that both the observed increase in the fraction of CSCs in cisplatin-resistant HNSCC cell lines as well as the maintenance of CSC stemness may be positively associated with NF-κB-mediated IL-6 expression in cisplatin-resistant cells. This ultimately enables the survival of CSCs and the re-population of the HNSCC following cisplatin treatment.

Here, we identified that the histone demethylase KDM6B plays an important role in promoting cisplatin-resistance by epigenetically modulating NF-κB pathway. Our data demonstrate that KDM6B regulates the expression of anti-apoptotic NF-κB targeted genes such as \textit{Mcl1}, \textit{cIAP2 (Birc3)}, and \textit{Bcl2A1} in both cisplatin-resistant cells and parental cells to promote survival of HNSCC upon cisplatin treatment. Mechanistically, KDM6B might be involved in \textit{Mcl1} and \textit{cIAP2} transcription by recruiting NF-κB to their promoter region and by removing repressive methyl groups from the histone H3. Future work will be focused on further validated association between KDM6B and NF-κB in HNSCC chemoresistance. Taken together, our study proposes that KDM6B is a critical target for inhibiting cisplatin-resistance in HNSCC patients.
5 ROLE OF KDM6B IN HNSCC INVASION AND METASTASIS

5.1 KDM6B and oncogenic MET-induced HNSCC invasion

Increased abundance of MET expression is frequently found in human HNSCC and has been reported to promote tumor metastasis in various cancers such as lung, prostate, and breast cancers\textsuperscript{76,115-117}. Previously, we showed that the activation of MET promoted cell scattering, invasion, and metastasis of HNSCC \textit{in vitro} and \textit{in vivo} using human SCC23 cells (SCC23) treated with HGF and SCC23 cells overexpressing constitutively activated MET (SCC23/MET).\textsuperscript{83} SCC23/MET cells were previously established in our lab to mimic robust cellular model system for screening genes associated with MET-induced invasion and metastasis by overexpressing oncogenic TPR-MET proteins\textsuperscript{83}. We found that mitogen-activated protein kinase (MAPK) pathway was constitutively activated in SCC23/MET cells, especially extracellular signal-regulated kinase (ERK) and the Akt kinase, in which the phosphorylation of ERK and Akt level was similar to SCC23 cells induced by HGF\textsuperscript{83}. The expression profile of histone demethylases in SCC23 cells treated with HGF and SCC23/MET cells were obtained (Fig. 3-1A). Our initial screening found that KDM4A, KDM6B, JMJD7, and KDM7A were strongly induced in both cell lines. Based on these data, we focused our work on KDM6B because the studies on MET-induced HNSCC metastasis associated with KDM4A were previously conducted in our lab\textsuperscript{83}, and precise mechanisms for the demethylases encoded by JMJD7 and KDM7A were not clear. The western analysis confirmed that KDM6B was indeed elevated in SCC23/MET cells compared to the control cell overexpressing the empty vector (SCC23/EV) (Fig. 3-1B). The elevation of KDM6B expression was also observed in various HNSCC cells treated with HGF in a time dependent manner (Fig. 3-1C). We also found that epidermal growth factor (EGF) promotes KDM6B expression in HNSCC, indicating that the role of KDM6B in HNSCC invasion and metastasis may not be only limited to MET signaling pathway (Fig. 3-1D).
To further explore the role of KDM6B in HNSCC invasion and metastasis, we generated two lentivirus-based short hairpin RNAs (shRNAs; sh1 and sh2), that target two different KDM6B sequences in SCC23 cells (SCC236Bsh1 and SCC236Bsh2) or SCC23/MET cells (SCC23/MET/6Bsh1 and SCC23/MET/6Bsh2). Knockdown efficiency of SCC236Bsh1 and SCC236Bsh2 in SCC23 cells treated with HGF and SCC23/MET/6Bsh1 and SCC23/MET/6Bsh2 in SCC23/MET cells were evaluated (Fig. 3-2A). Depletion of KDM6B in these cells notably inhibited invasion induced by HGF (Fig. 3-2B) or the oncogenic MET (Fig. 3-2C). However, we found that there is no significant difference in the *in vitro* cell proliferation rates between SCC23/MET/EV and SCC23/MET/6Bsh1 and SCC23/MET/6Bsh2 (Fig. 3-2D). In addition, we observed that the inhibition of the MAPK/ERK pathway using a selective MEK-1,2 inhibitor, U0126, significantly reduced the expression of KDM6B induced by HGF in SCC23 cells.

Since KDM6B knockdown drastically suppressed in MET-mediated invasion *in vitro*, we decided to further examine whether KDM6B knockdown also inhibited SCC23/MET cell metastasis *in vivo* using an orthotropic mouse model of HNSCC that were submucosally inoculated with SCC23/MET/EV or SCC23/MET/6Bsh1 cells at the floor of the mouth near the base of the tongue. We determined lymph node metastasis of HNSCC using both *in vivo* and *ex vivo* bioluminescence imaging. Typically, *in vivo* bioluminescence imaging clearly displays the location of cervical lymph node or distant metastasis sites that is distinct from the primary tumor site. However, due to the small size of the mouse head, our *in vivo* bioluminescence imaging of the cervical lymph node metastasis was masked by the presence of primary tumors that were closely located to the cervical lymph nodes (Fig. 3-3A). Thus, cervical lymph node metastasis was determined via *ex vivo* bioluminescence imaging using lymph nodes that were immediately isolated after sacrificing of the mice (Fig. 3-3B). Both the control group mice (n=8) and the
KDM6B knockdown group mice (n=8) were sacrificed for isolation of cervical lymph nodes at 20 days after inoculation. Six out of eight mice with the tumors derived from SCC23/MET/EV showed luciferase light emission, indicating lymph node metastasis, while only three of eight mice with tumors derived from SCC23/MET/6Bsh1 showed luciferase light emission (Fig. 3-3C). It is possible that we could not detect lymph node metastasis in the remaining two control mice (SCC23/MET/EV) because they died at day 14 due to their tumor burdens in the oral cavity. In consistent with in vitro findings, the knockdown of KDM6B exhibited no effect on the primary HNSCC tumor growth and size in an orthotopic mouse model of HNSCC (Fig. 3-3D). Hematoxylin and eosin (H&E) staining and pan-cytokeratin (PCK) immunostaining, a marker for HNSCC, on ex vivo cervical lymph nodes also showed marked HNSCC metastasis in SCC/MET/EV compared to SCC/MET/6Bsh1 (Fig. 3-3E). Taken together, our data suggests KDM6B as an important epigenetic factor that promotes HNSCC invasion and metastasis.
FIGURE 3-1. KDM6B expressions are elevated in MET-activated HNSCC cells and plays a role in invasion. A, Real time RT-PCR analysis of the expression of histone demethylases family in SCC23 cells treated with HGF and SCC23/MET cells. * p< 0.05; ** p < 0.01. B, Western blotting of KDM6B in SCC23/EV (control) and SCC23/MET cells. TBP, nuclear protein loading control. C, Western blotting of KDM6B in SCC23, SCC1, and Fadu cells that were treated with 20ng/ml of recombinant human HGF (rhHGF or HGF) for 0, 4, and 8 hours. 24 hours of pre-serum starvation was indicated before HGF treatment. D, KDM6B expression was elevated with EGF treatment in HNSCC cells.
FIGURE 3-2. KDM6B knockdown dramatically inhibits HNSCC invasion. A, Western blotting of KDM6B in SCC23/MET/Scr (control), SCC23/MET/6Bsh1, and SCC23/MET/6Bsh2 cells and also SCC23/Scramble (control), SCC23/6Bsh1 and SCC23/6Bsh2 cells with 0, 4, 8 hrs of HGF treatment. B, Analysis and images of invasion assay on SCC23/Scr, SCC23/6Bsh1, and SCC23/6Bsh2 cells treated with HGF. Data are means ± S.D. of three independent experiments. ** p < 0.01, unpaired two-tailed student’s t-test. Scale bar, 50 μm. C, Invasion assay analysis of SCC23/MET/Scr, SCC23/MET/6Bsh1, and SCC23/MET/6Bsh2. D, Cell proliferation analysis of SCC23/MET/Scr, SCC23/MET/6Bsh1, and SCC23/MET/6Bsh2 for 1-6 days. E, real time RT-PCR analysis of KDM6B expression in SCC23 cells treated with ERK inhibitor U0126.
FIGURE 3-3. KDM6B is required for MET-driven HNSCC metastasis in vivo. A, Xenograft tumors derived from SCC/MET/Scr cells (n=8) or SCC23/MET/6Bsh1 cells (n=8) in the roal cavity of mice were assessed by bioluminescence imaging for 20 days after inoculation. Data are representative of two independent experiments. B, Lymph node metastasis were assessed ex vivo by bioluminescence imaging. C, Number of mice with lymph node metastasis from xenograft tumors. D, Histochemical detection of HNSCC lymph node metastases using antibody against pan-keratin. H&E staining was used for histological examination. Images are representative of six metastatic samples derived from SCC23/MET/Scr cells and five non-metastatic samples from SCC23/MET/KDM6Bsh1 cells. Scale bar, 50 μm. E, Growth of xenograft tumors derived from either SCC23/MET/Scr or SCC23/MET/6Bsh1 cells assessed by bioluminescence imaging in mice. Data are means ± S.D. from a representative experiment (n=2 independent experiments). Unpaired two-tailed student’s t-test was used for statistical analysis.

5.2 KDM6B regulated metastasis associated genes

To determine how KDM6B epigenetically promoted HNSCC invasion and metastasis, we conducted gene profiling to screen for invasive genes that were affected by KDM6B knockdown. Gene enrichment analysis revealed that the knockdown of KDM6B significantly decreased MAPK/ERK target genes associated with metastasis, including HMGA2 (high mobility group AT-hook 2), ETS1 (ETS proto-oncogene 1), ARHGAP32 (Rho GTPase-activating protein 32, also known as p250GAP), BCL2 (B-cell lymphoma 2), BCL2L1 (Bcl2 like 1), ITGB8 (integrin subunit beta 8), MMP14 (matrix metallopeptidase 14), and SPOCK2 (SPARC/osteonectin, cwcv and kazal like domains proteoglycan 2) (Fig. 3-4A), indicating that KDM6B is highly associated with MET-MAPK/ERK signal transduction leading to HNSCC invasion and metastasis. To confirm the gene enrichment analysis results, we first examined whether HGF induced their expression in SCC23 cells. We found that ETS1 and HMGA2 are highly expressed in a time dependent manner (Fig. 3-4B). Since HMGA2 is DNA binding protein that regulates chromatin structure and typically reside in nucleus, we measured HMGA2 expression extracted from nucleus (Fig. 3-4C). We also observed increased expression of ETS and HMGA2 in SCC1 and Fadu cells that were treated with HGF (Data not shown). Thus, further experiments were focused on KDM6B regulation of the
transcription factors ETS1 and HMGA2 and their role in HNSCC invasion and metastasis. Real

time RT-PCR and western blot analysis also confirmed that KDM6B knockdown significantly
inhibited the expression of ETS1 and HMGA2 induced by HGF (Fig. 3-4D). Similarly, ETS1 and
HMGA2 were highly expressed in SCC23/MET/EV compared to SCC23/MET/6Bsh1 and
SCC23/MET/6Bsh2 cells (Fig. 3-4E and 3-4F). To exclude off-target effects, we also restored
KDM6B in SCC236Bsh1 cells using an shRNA-resistant construct (Fig. 3-4G). Western blot
analysis showed that the restoration of wild-type KDM6B rescued the expression of ETS1 and
HMGA2; however, overexpression of mutant KDM6B-H1390A could not reinstate the expression
of ETS1 and HMGA2, indicating that the histone demethylase activity of KDM6B is required for
the MET-induced expression of ETS1 and HMGA2 in HNSCC (Fig. 3-4H).

To further explore the role of ETS1 and HMGA2 in HNSCC invasion and metastasis, we
transiently knocked down ETS1 and/or HMGA2 using siRNA in parental SCC23 cells (Fig. 3-4I).
Inhibition of ETS1 or HMGA2 in SCC23 cells decreased MET-promoted invasion, and the
inhibition of invasion was further enhanced when ETS1 and HMGA2 were blocked together (Fig.
3-4J). We also obtained the similar results in SCC1 cells (Data not shown). The real time RT-PCR
demonstrated that inhibition of ETS1 and/or HMGA2 results in reduction of \textit{Mmp3} and \textit{Mmp9}
expression induced by MET (Fig. 3-4K). Matrix metalloproteinases 3 (MMP3) and MMP9 are
well-known players in the tumor dissemination process to promote invasion and metastasis in
various tumors. Previously, our lab demonstrated that KDM4A (also known as JMJD2A) is a key
epigenetic player in MET-driven HNSCC invasion and metastasis. Thus, we examined to identify
whether KDM4A also regulates expression of MET-induced ETS1 and HMGA2 expression
controlled by KDM6B in HNSCC cells. The knockdown efficiency of KDM4A was determined
by western blot analysis and also further confirmed by evaluating the mRNA expression of
KDM4A target gene c-FOS. Interestingly, knockdown of KDM4A in SCC23 cells did not alter expression of KDM6B, ETS1, and HMGA2, indicating that MET-driven invasion and metastasis promoted by expression ETS1 and HMGA2 is exclusively regulated by KDM6B (Fig. 3-4L).
FIGURE 3-4. KDM6B regulates ETS1 and HMGA2 expression to promote HNSCC invasion and metastasis. A, Gene expression profiling of KDM6B target genes in SCC23/MET/EV or SCC23/MET/6Bsh1. Red indicates increased expression, whereas green indicates decreased expression, with magnitude indicated by lighter shades of green. B, Real-time RT-PCR and western blotting result showing that ETS1 and HMGA2 are expressed under MET activation in SCC23 cells treated with HGF. * p < 0.05; ** p < 0.01. C, Western blotting of the nuclear extraction using HMGA2 antibodies. D, ETS and/or HMGA2 expression is decreased in MET-activated SCC23KDM6Bsh1 cells compared to the control cells. E, Western blotting result showing that ETS1 and HMGA2 are highly expressed in SCC23/MET cells comparing to SCC23/MET/6Bsh1 or sh2 cells. F, Real time RT-PCR analysis of the expression of ETS1 and HMGA2 in SCC23/MET cells and SCC23/MET/6Bsh1 or sh2 cells. ** p < 0.01. G, Western blotting of KDM6B in 293T cells infected with the retroviruses expressing pLKO (control), KDM6B, or mutant KDM6B (H1390A). H, Western blot analysis of the abundance of ETS1 and HMGA2 in SCC23KDM6Bsh1 cells overexpressing either wild-type (KDM6Bsh1+KDM6B) or enzyme-inactive mutant HA-tagged KDM6B (KDM6Bsh1+KDM6B Mut). I, Western blotting of ETS1 and HMGA2 after the transfection with siRNAs in SCC23 cells treated with HGF for 8 hrs. J, Images and analysis of invasion assay of ETS1 and/or HMGA2 knockdown SCC23 cells. Data are means ± S.D. of three independent experiments. ** p < 0.01, unpaired two-tailed student’s t-test. Scale bar, 50 μm. K, Real-time RT-PCR result showing decreased mmp3 or mmp9 mRNA expression in ETS1 and/or HMGA2 knockdown SCC23 cells compared to the control cells when treated with HGF. * p < 0.05; ** p < 0.01. L, Western blot analysis and real time RT-PCR result showing that KDM6B, ETS1, HMGA2 expressions unaltered in KDM4A knockdown SCC23 cells.
5.3 MET-activated ELK1 regulates KDM6B target gene expression

The ERK pathway is one of the major signaling cascades of the MAPK signaling pathway\textsuperscript{118-120}. The activation of the ERK1/2 cascade is initiated by membrane receptors such as receptor tyrosine kinases (RTKs) including MET, G protein-coupled receptors (GPCRs), and others. ERK1/2 are responsible for stimulating the downstream substrates either in the cytosol or in the nucleus including transcription factors such as ETS domain-containing protein (ELK1), c-FOS, and c-JUN to promote cell motility, transformation, and cell-cycle progression\textsuperscript{118,119,121}. Since our data showed that ERK inhibition by U0126 decreased KDM6B expression in HNSCC cells, we sought to identify which one of the ERK-targeted transcription factors is closely associated with ETS1 and HMGA2 expression induced by MET in HNSCC. Our compelling data demonstrated that the transient knockdown of c-JUN and c-FOS, which dimerize to form AP-1 transcription factor when activated by MET signaling, had no significant effect on ETS1 and HMGA2 expression (Fig. 3-5A and 3-5B). However, knockdown of ELK1 using siRNAs in SCC23 cells treated with HGF (Fig. 3-5C) showed that both ETS1 and HMGA2 expression were significantly inhibited, indicating transcription of ETS1 and HMGA2 is regulated by ELK1 (Fig. 3-5D). ELK1 expression was also highly expressed in SCC23/MET/EV cells, and knockdown of ELK1 in SCC23/MET cells decreased the expression of ETS1 and HMGA2 (Fig. 3-5E). Furthermore, our western blot analysis found that phosphorylation of ELK1-induced by MET activation was dramatically decreased in KDM6B knockdown SCC23 cells compared to the control, but the total ELK1 level was unaffected (Fig. 3-5F), suggesting that activation of ELK1 may require expression of MET-induced KDM6B to facilitate ETS1 and HMGA2 transcription to promote HNSCC invasion and metastasis.
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**Relative mRNA level of HMGA2**

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FIGURE 3-5. MET-activated transcription factor ELK1 regulates ETS1 and HMGA2 expression. A, Real-time RT-PCR and western blot analysis showed that c-JUN knockdown or B, c-FOS knockdown in SCC23 cells did not change MET-induced ETS1 and HMGA2 expression. C, Knockdown efficiency of ELK1 by siRNAs are determined by real-time RT-PCR analysis. D, Western blotting using anti-ELK1, anti-ETS1, anti-HMGA2, and anti-tubulin revealed that both ETS1 and HMGA2 expressions were significantly decreased in ELK1 knockdown SCC23 cells. E, ELK1 expression is also highly expressed in SCC23/MET/EV cells and inhibiting ELK1 expression using siRNAs decreased ETS1 and HMGA2 expression. F, Western blot analysis on phosphorylated ELK1 (p-ELK1; Ser383), ELK1, and tubulin showed that MET activation induces ELK1 phosphorylation, and the phosphorylation level of ELK1 decreases when KDM6B expression is attenuated in SCC23 cells.

5.4 Histone demethylation by KDM6B enables ELK1 binding to the promoter

To determine whether KDM6B epigenetically stimulated the expression of ETS1 and HMGA2, we examined whether KDM6B and ELK1 were present on the promoters of ETS1 and HMGA2 using chromatin immunoprecipitation (ChIP) assays. The promoters of ETS1 and HMGA2 contain both ELK1 binding site (5’-TTCC-3’) and the consensus sequence (5’-GGAA/T-3’) recognized by ETS family members. ChIP assays revealed that both KDM6B and ELK1 levels were enriched on the promoters of ETS1 in SCC23/MET/EV cells and that, as expected, KDM6B knockdown significantly reduced the binding of KDM6B and ELK1 to the promoters of ETS1 in SCC23/MET/6Bsh1 cells (Fig. 3-6A). Consistently, decreased binding of KDM6B to the ETS1 promoter region was associated with increased abundance of its substrate H3K27me3, a marker of
gene silencing. As a control, we could not detect KDM6B and ELK1 occupancy at 8 kb upstream of the transcription start sites in either SCC23/MET/EV or SCC23/MET/6Bsh1 cells, and the knockdown of KDM6B also did not affect the level of H3K27me3 level in that region. Similarly, HGF treatment also promoted the recruitment of KDM6B and ELK1 to the ETS1 promoter region while suppressing KDM6B substrate H3K27me3 level in SCC23 cells and vice versa in SCC23KDM6Bsh1 cells (Fig. 3-6B). ChIP assays on HMGA2 promoter region also showed a similar trend of KDM6B redundancy and its substrate H3K27me3 levels in SCC23/MET/EV and SCC23/MET/6Bsh1 cells (Fig. 3-6C). Specifically, there were high levels of KDM6B occupancy coupled with low H3K27me3 levels in SCC23/MET/EV cells and decreased level of KDM6B occupancy and high H3K27me3 level in SCC23/MET/6Bsh1 cells. However, ELK1 did not appear to present on the promoter of HMGA2 in both SCC23/MET/EV and SCC23/MET/6Bsh1 cells. Consistently, HGF treatment also did not facilitate the recruitment of ELK1 to the promoter of HMGA2 in both SCC23 and SCC23KDM6Bsh1 cells (Fig. 3-6D). Taken together our data shows that the demethylase activity of KDM6B is required for the MET-induced expression of ETS1 and HMGA2 to promote invasion and metastasis in HNSCC.
B

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Antibody:
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**ETS-1**

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**ETS-1**

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HGF:
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Antibody:
- IgG ELK1

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85
FIGURE 3-6. KDM6B binds to the promoters of ETS1 and HMGA2 and demethylates H3K27me3 marks to recruit transcription factor ELK1. A, ChIP assays determined the abundance of KDM6B, H3K27me3, and ELK1 on the ETS1 promoter at the ELK1 binding site or 8 kb upstream of the promoter region (negative control) in SCC23/MET/6Bsh1 cells compared to SCC23/MET/EV (control) cells. ** p < 0.01, unpaired two-tailed Student’s t-test (n=3). B, ChIP assays of KDM6B, H3K27me3, and ELK1 on the ETS1 promoter at the ELK1 binding site or 8 kb upstream of the promoter region in SCC23/Scramble (control) and SCC23/KDM6Bsh1 cells treated with 20 ng/ml HGF for 2 hours. ** p < 0.01, C, ChIP assays of KDM6B, H3K27me3, and ELK1 on the HMGA2 promoter at the ELK1 binding site or 8 kb upstream of the promoter region in SCC23/MET/EV (control) and SCC23/MET/6Bsh1 cells treated with 20 ng/ml HGF for 2 hours. ** p < 0.01. D, ChIP assays of KDM6B, H3K27me3, and ELK1 on the HMGA2 promoter at the ELK1 binding site or 8 kb upstream of the promoter region in SCC23/Scramble (control) and SCC23/KDM6Bsh1 cells treated with 20 ng/ml HGF for 2 hours. ** p < 0.01.

5.5 **In vivo human SCC lymph node metastasis**

To further determine whether KDM6B activation promotes human HNSCC progression and metastasis, we compared KDM6B expression between human primary HNSCC tissues collected from patients without lymph node metastasis (HNSCC without metastasis), primary HNSCC tissues from patients with lymph node metastasis (HNSCC with metastasis), and the adjacent normal epithelial tissues (NT). Although KDM6B expression was slightly elevated in HNSCC without metastasis tissues comparing to NT tissues, KDM6B abundance was most
significantly upregulated in HNSCC with metastasis tissues (Fig. 3-7A and Table 4). Further, we also examined the KDM6B expression in the lymph nodes (HNSCC LN) collected from the same patients who had HNSCC with metastasis, comparing to normal lymph node tissues (NT LN). The presence of HNSCC metastasis in the lymph nodes were evaluated by using anti-PCK antibodies. As expected, KDM6B was also highly elevated in HNSCC LN (Fig. 3-7B and Table 4). Together, these data suggest that KDM6B may promote HNSCC metastasis in human patients.

**FIGURE 3-7. KDM6B is highly expressed in human HNSCC with lymph node metastasis.** A, Histochemical detection of KDM6B expression in normal and human HNSCC tissues with or...
without lymph node (LN) metastasis. Images are representative of 34 (NT), 64 (HNSCC), and 33 (LN) samples. B, Histochemical detection of HNSCC metastasis using PCK (Keratin) antibody and the expression of KDM6B in the lymph nodes collected from HNSCC patients with lymph node metastasis. H&E staining was used for histological examination.

Table 4. KDM6B is highly expressed in human HNSCC with lymph node metastasis. Normal human adjacent epithelial tissues (NT; n=34), human primary HNSCC without metastasis (HNSCC without metastasis; n=31), human primary HNSCC with metastasis (HNSCC with metastasis; n=33), and lymph nodes obtained from HNSCC with metastasis patients (HNSCC LN; n=33) were stained for KDM6B. The staining intensity was scored as following: 0, negative staining; +, weak staining; ++, moderate staining; ++++, strong staining. * p < 0.05; ** p < 0.01. Wilcoxon rank sum test.

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<td>9/31 (29.0%)</td>
<td>13/31 (41.9%)</td>
<td>5/31 (16.1%)</td>
<td>4/31 (12.9%)</td>
</tr>
<tr>
<td>HNSCC with LN Metastasis</td>
<td>1/33 (3.1%)</td>
<td>3/33 (9.1%)</td>
<td>11/33 (33.3%)</td>
<td>18/33 (54.5%)</td>
</tr>
<tr>
<td>HNSCC LN</td>
<td>3/33 (9.1%)</td>
<td>5/33 (15.2%)</td>
<td>11/33 (33.3%)</td>
<td>14/33 (42.4%)</td>
</tr>
</tbody>
</table>

5.6 Discussion

The findings presented in this study revealed that KDM6B plays key role in promoting HGF/MET-mediated HNSCC invasion and metastasis by epigenetically regulating the transcription of metastasis-related genes such as ETS1 and HMGA2 through interaction with the ERK-targeted transcription factor, ELK1. HNSCC metastasis is promoted by a variety of growth factors and subsequent signaling pathways. Here, we demonstrated that KDM6B expression is regulated by MET-activated MAPK/ERK pathway; however, our data indicated that KDM6B could also be induced by different growth factors such as EGF in various HNSCC cells, suggesting that this KDM6B-regulated HNSCC invasion and metastasis is not only exclusive to MET signaling pathway. In addition, numerous studies revealed that activation of EGF rector (EGFR)
family members can trans-activate MET in cells that express both MET and EGFR in the absence of HGF by forming a heterodimeric receptor complex\textsuperscript{75,79,127,128}. The crosstalk between these two pathways results in activation of MAPK pathway and may contribute to high expression of KDM6B in human HNSCC cells, which typically overexpresses both activated EGFR and MET\textsuperscript{74,75}. To mimic this dynamic cellular system in our \textit{in vitro} studies, we have established and utilized SCC23/MET cells overexpressing oncogenic TPR-MET fusion proteins to constitutively activate MET and overexpress KDM6B in the absence of HGF. Further, we found that KDM6B-regulated invasion and metastasis is distinct from our previous findings on KDM4A mediated MET-induced HNSCC invasion and metastasis, which epigenetically control \textit{JUN} and \textit{FOSL1} transcription to establish a positive feedback loop that maintains activation of transcription factor AP1. Thus, our findings provide a novel insight on epigenetic regulations involved in MET-induced invasion and metastasis and highlights that KDM6B might serve as a therapeutic target for effective treatment of advanced HNSCC patients.

ELK1 is a member of the E-26 (ETS) family of transcription factors and known to be elevated in various cancers, including as breast cancer and HNSCC, promoting tumor progression, invasion, and metastasis\textsuperscript{129,130}. Our study showed first time that MET/ERK-induced ELK1 activation is regulated by KDM6B and requires KDM6B demethylase activity for transcription of target genes, ETS1 and HMGA2. ETS1 is also a member of the ETS family and often found overexpressed in advanced tumors. Studies reported that ETS1 is phosphorylated at the threonine 38 residue via MAPK/ERK signaling pathway and regulates gene transcription to promote cell motility and scattering, cell cycle progression, transformation, etc\textsuperscript{129-132}. It is also widely known that ETS1 contains ELK1 binding motif and work together with ELK1 as a complex to transcribe genes such as cancerous inhibitor of protein phosphatase 2A (CIP2A), a proto-oncogene that is
overexpressed in various types of human cancer\textsuperscript{129}. Our ChIP analysis data showed that both KDM6B and ELK1 are presented during \textit{ETS1} transcription; however, further experiments such as re-ChIP assay needs to be conducted to find out whether KDM6B and ELK1 co-occupy the \textit{ETS1} promoter during gene transcription. HMGA2 is a member of high mobility group (HMG) family that can alter the chromatin structure by directly binding to DNA without associating with histones to affect transcription, replication, recombination, and repair\textsuperscript{97,133-136}. Recently, HMGA2 has been recognized as a driver of human epithelial cancer metastasis by inducing epithelial-mesenchymal transition (EMT), which has been implicated as metastatic behavior of tumor cells\textsuperscript{134}. Also, HMGA2 was found to involve in CSC self-renewal in HNSCC and proposed as a HNSCC prognosis marker\textsuperscript{97,135}. In our study, we found that HMGA2 expression is induced by MET and epigenetically regulated by KDM6B. We also showed that the depletion of ELK1 led to decreased MET-induced expression of HMGA2 in HNSCC, suggesting HMGA2 expression is also regulated by ELK1. However, the ChIP assay result demonstrated that only KDM6B was enriched at the \textit{HMGA2} promoter site and directly regulated \textit{HMGA2} transcription. This could be due to several reasons: 1) Although ELK1 has been identified as one of the transcription factors binding to the promoter of \textit{HMGA2} gene in ENCODE ChIP-seq data set that was obtained from HeLa. S3 cells, ELK1 may not be a direct transcriptional regulator of HMGA2 in MET-mediated HNSCC invasion and metastasis. Activation of MEK/ERK signaling pathway has been identified in various cancers to control the expression of EMT factors such as HMGA2 by modulating its transcriptional regulators\textsuperscript{137}. Since ELK1 is a downstream target activated by MET-MAPK-ERK pathway and our data presented that ELK1 knockdown subsequently repressed the expression of HMGA2, ELK1 could still potentially regulate the expression HMGA2 indirectly; 2) ELK1 might still directly involve in HMGA2 expression in MET-activated HNSCC cells, but ELK1 could be bound
to one of the transcriptional regulators to form a complex and do not directly bind to the promoter. In this case, ChIP analysis cannot detect the presence of ELK1 during HMGA2 transcription. Thus, further experiments such as immunoprecipitation or re-ChIP assay need to be conducted; 3) It is also possible that ELK1 and KDM6B do not share same promoter sites on the HMGA2 promoter region.; and 4) There also could be a time delay between KDM6B and ELK1 occupancy on the promoter region of HMGA2. Thus, further experiments are required to determine how KDM6B and ELK1 together regulate HMGA2 expression during the transcription. Furthermore, we revealed that KDM6B abundance was highly increased in the tissues obtained from HNSCC patients with cervical lymph node metastasis, implicating that increased expression of KDM6B might be associated with HNSCC progression and inhibiting KDM6B may prevent HNSCC invasion and metastasis in patients. Taken together, our study unraveled the complex molecular network regulated by KDM6B through epigenetic regulation and proposed KDM6B as a critical therapeutic target to improve tumor burden and survival of HNSCC patients.
6 SUMMARY AND CONCLUSION

Resistance to the conventional chemotherapy and metastatic progression of cancer are important factors involved in the high mortality and recurrence rate of HNSCC and they remain as major challenges in HNSCC treatment. In this study, we explored the influence of epigenetic regulators on HNSCC chemoresistance, invasion, and metastasis and identified HDAC6 and KDM6B as key epigenetic regulators through three different studies.

The first study focused on unraveling the molecular mechanism behind the acquisition of BTZ resistance through autophagy activation in HNSCC and proposed a combination regimen of BTZ and TSA as an effective treatment to overcome BTZ and cisplatin resistance in HNSCC. We found that the depletion of HDAC6 inhibited autophagy activation and enhanced Bortezomib-induced apoptosis. Additionally, TSA synergistically enhanced the antitumor effects of Bortezomib in HNSCC cells. During Bortezomib treatment, HDAC6 mediated the activation of autophagy by modulating the activation of protein kinases, such as ULK 1, to promote clearing of large quantities of cytotoxic, unfolded protein aggregates induced by Bortezomib. Our findings provide the first insight that HDAC6 is a key epigenetic regulator of autophagy to promote chemoresistance in HNSCC, and targeting HDAC6 in combination with traditional therapeutic agents is a novel strategy to improve chemotherapeutic efficacy and decrease tumor burden of HNSCC patients through its synergistic cytotoxic effects.

The second study assessed the role of histone demethylase KDM6B in acquisition of cisplatin resistance in HNSCC and maintenance of cisplatin resistance utilizing three cisplatin-resistance HNSCC cell lines: SCC1-R, SCC23-R, and SCC22B-R. We revealed that both KDM6B and NF-κB are highly expressed and activated in these resistant cell lines and closely associated with both parental and resistant cell survival. Through a NF-κB target gene screening, we showed that anti-apoptotic and pro-survival genes such as MCL1, BCL2A1, and cIAP2 were enhanced in
cisplatin resistant cells, and their expression was regulated by KDM6B. We also found that the fraction of CSCs was significantly elevated. NF-κB and isolated CSCs from cisplatin-resistant cells overexpress KDM6B. KDM6B was required to maintain the self-renewal capacity of CSC. Since this is an ongoing study, further experiments are needed to obtain detailed mechanism of cisplatin resistance involving epigenetic alteration of KDM6B in HNSCC and to implicate KDM6B as an effective target to overcome cisplatin resistance in HNSCC patients.

The last study involved KDM6B and activated MET signaling, which is frequently overexpressed and highly associated with HNSCC invasion and metastasis. We identified KDM6B as a key epigenetic regulator of MET-driven HNSCC metastasis. KDM6B was highly expressed in both the growth factor-induced and TPR-MET expressing HNSCC cells. KDM6B knockdown significantly decreased the HNSCC invasion and metastasis by regulating the expression of ETS1 and HMGA2 genes, known as drivers of metastasis. Consequently, knockdown of ETS1 and HMGA2 decreased the HNSCC invasion by regulating MMP-3 and -9 expression. Mechanistically, histone demethylation by KDM6B facilitated the binding of the transcription factor ELK1, a downstream target of c-MET signaling pathway, to the promoters of ETS1 and HMGA2. In a mouse model, KDM6B knockdown inhibited lymph node metastasis. Moreover, KDM6B expression was highly elevated in human SCC tissues with metastasis compared to both normal and SCC without lymph node metastasis. KDM6B expression was also increased in human lymph node metastases tissue. Our study provides insight into the epigenetic regulation of HNSCC invasion and metastasis and suggests that KDM6B could be an important therapeutic target for inhibiting invasive tumor growth and metastasis.
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