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Intron 1-Mediated Regulation of *EGFR* Expression In EGFR-Dependent Malignancies

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

in

Biomedical Sciences

by

Nathan M. Jameson

Committee in charge:

Professor Frank Furnari, Chair
Professor Xiang-Dong Fu
Professor Paul Mischel
Professor Bing Ren
Professor Robert Wechsler-Reya

2019

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The dissertation of Nathan M. Jameson is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2019

DEDICATION

To:

My mother, Wendy Jameson, and my father, Patric Jameson, who's support, encouragement, and love allowed me to be who and where I am today. To my wife, Rachel Jameson, and my daughter, Avery Jameson, who's love, smiles, and unwavering support helped me enjoy every day no matter the challenges.

EPIGRAPH

The Road goes ever on and on
Down from the door where it began.
Now far ahead the Road has gone,
And I must follow, if I can,
Pursuing it with eager feet,
Until it joins some larger way
Where many paths and errands meet.
And whither then? I cannot say
—J.R.R. Tolkien

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LIST OF ABBREVIATIONS

3C	Chromosome Conformation Capture
4C	Circular Chromosome Conformation Capture
5-aza	5-Azacytidine
5hmC	5-Hydroxymethylcytosine
5mC	5-Methylcytosine
a-KG	Alpha-Ketoglutarate
ACH	Active Chromatin Hub
aKD	Asymmetric Kinase Domain
AML	Acute Myeloid Leukemia
AP-1	Activating Protein 1
ATAC-Seq	Transposase-Accessible Chromatin with Sequencing
ATP	Adenosine Triphosphate
BAC	Bacterial Artificial Chromosome
BET	Bromodomain and Extraterminal Domain
BRAF	v-Raf Murine Viral Oncogenes Homolog B1
BRD4	Bromodomain-Containing Protein 4
Cas9	*Streptococcus pyogenes* Cas9 (spCas9)
Cas9	CRISPR-Associated
CE	Constituent Enhancer
CE1	Constituent Enhancer 1
CE2	Constituent Enhancer 2
ChIP-seq	Chromatin Immunoprecipitation with Sequencing
CHORI	Childrens Hospital Oakland Research Institute
CNS	Central Nervous System
CRISPR	Clustered Regulatory Interspaced Short Palindromic Repeats
CRISPRa	CRISPR Activation
CRISPRi	CRISPR Interference
CTCF	CCCTC-Binding Factor
DBD	DNA Binding Domain
DM	Double Minute
DNase I	Deoxyribonuclease I
DNMT	DNA Methyltransferase
DSB	Double Strand Break
ECD	Extracellular Domain
ecDNA	Extrachromosomal DNA
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ENCODE	Encyclopedia of DNA Elements
eRNA	Enhancer RNA

ESC	Embryonic Stem Cell
EZH2	Enhancer of Zeste 2
FDA	Food and Drug Administration
FPKM	Fragments Per Kilobase of Transcript Per Million
GBM	Glioblastoma
GFP	Green Fluorescent Protein
GOF	Gain of Function
GR	Glucocorticoid Receptor
gRNA	Guide RNA
GSC	Glioblastoma Stem Cell
GTF	General Transcription Factor
GWAS	Genome Wide Association Study
H3K27Ac	Histone 3 Lysine 27 Acetylation
H3K27me3	Histone 3 Lysine 27 Trimethylation
H3K4me1	Histone 3 Lysine 4 Monomethylation
H3K4me3	Histone 3 Lysine 4 Trimethylation
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HDR	Homology Driven Repair
HIV	Human Immunodeficiency Virus
HMT	Histone Methyltransferase
HNSCC	Head and Neck Squamous Cell Carcinoma
HOMER	Hypergeometric Optimization of Motif EnRichment
HPV	Human Papilloma Virus
HRP	Horesradish Peroxidase
HSR	Homogeneously Staining Region
IDH	Isocitrate Dehydrogenase
IGV	Integrative Genomics Viewer
IHC	Immunohistochemistry
Indel	Insertion or Deletion
IR	Insulin Receptor
JunDN	c-Jun Dominant Negative
KRAB	Krüppel-Associated Box
LCR	Locus Control Region
LGG	Low Grade Glioma
LOF	Loss of Function
MACS2	Model-based Analysis of ChIP-Seq
MAPK	Mitogen-activated Protein Kinase
MBD	Methylated-CpG-Binding Domain
MED1	Mediator Complex
MGMT	O6-Methylguanine-DNA Methyltransferase

MMAF	Monomethyl Auristatin F
mTOR	Mechanistic Target of Rapamycin
ncRNA	Non-Coding RNA
NGS	Next Generation Sequencing
NHEJ	Non-homologous End Joining
NMD	Nonsense-Mediated Decay
NSCLC	Non-Small Cell Lung Cancer
O-T	Off-Target
OS	Overall Survival
PAM	Protospacer-Adjacent Motif
PI3K	Phosphatidylinositol 3-Kinase
PIC	Pre-Initiation Complex
PIP3	Phosphatidylinositol 3,4,5-Triphosphate
PKB/Akt	Protein Kinase B
Pol II	RNA Polymerase II
PRC3	Polycomb Repressive Complex 2
Raf	Rapidly Accelerated Fibrosarcoma
Ras	Rat Sarcoma
RNA-Seq	RNA Sequencing
ROSE	Rank Ordering of Super Enhancers
RTK	Receptor Tyrosine Kinase
SAM	S-Adenosylmethionine
SCLC	Small Cell Lung Cancer
SE	Super Enhancer
SH2	Src Homology 2
sKD	Symmetric Kinase Domain
SNP	Single Nucleotide Polymorphism
STARR-Seq	Self-Transcribing Active Regulatory Region Sequencing
T-ALL	T-Cell Acute Lymphoblastic Leukemia
TAD	Topologically Associating Domain
TCGA	The Cancer Genome Atlas
TDG	Thymine DNA Glycosylase
TET1	Ten-eleven Translocation Methylcytosine Dioxygenase 1
TF	Transcription Factor
TKI	Tyrosine Kinase Inhibitor
TRE	TPA-Response Element
TSS	Transcription Start Site
WHO	World Health Organization
wtEGFR	Wild-Type EGFR

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ABSTRACT OF THE DISSERTATION

Intron 1-Mediated Regulation of *EGFR* Expression In EGFR-Dependent Malignancies

by

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Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2019

Professor Frank Furnari, Chair

The epidermal growth factor receptor is known to be overexpressed in numerous solid tumor types and has been the subject of extensive therapeutic development efforts. Much of the research on EGFR is focused on protein dynamics and downstream signaling, however few studies have explored how the gene is regulated transcriptionally. Here, we identified two novel enhancers (CE1 and CE2) present within the first intron of the *EGFR* gene in models of glioblastoma (GBM) and head and neck squamous cell carcinoma (HNSCC). CE1 and CE2 contain open chromatin and H3K27Ac histone marks, functionally enhance transcription in reporter assays, and interact with the *EGFR* promoter. Genetic deletion of CE1 and CE2 by CRISPR/Cas9 editing significantly reduces *EGFR* transcript levels, with double deletion exercising an additive

effect. Similarly, targeted repression of CE1 and CE2 by dCas9-KRAB targeting demonstrates repression of transcription similar to that of genomic deletion. We identify AP-1 transcription factor family members in concert with BET bromodomain proteins as candidate modulators of CE1 and CE2 activity in HNSCC and GBM through *de novo* motif identification and validate their presence in these enhancers. Genetic inhibition of AP-1 or pharmacologic disruption of BET/AP-1 binding results in downregulated EGFR protein and transcript levels, further confirming a role for these factors in CE1 and CE2. Our results identify and characterize these novel enhancers, shedding light on the role that epigenetic mechanisms play in regulating *EGFR* transcription in EGFR-dependent cancer types.

Chapter 1

Introduction to the oncogene EGFR and its implications in cancer

1.1 EGFR Function

The epidermal growth factor receptor (EGFR) was the first receptor tyrosine kinase (RTK) to be discovered [1] and has served as the basis for discoveries of the principles and paradigms that underlie the action of other RTKs [2]. EGFR is a member of the ErbB family of RTKs, and is also known as ErbB1 or HER1. This family consists of four members, including ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4). Like all RTKs, EGFR is composed of an extracellular ligand binding domain, a single transmembrane domain, and a cytoplasmic domain which includes the protein tyrosine kinase core. With the exception of the insulin receptor (IR) family of RTKs, all known RTKs exist as monomers in the cell membrane. Canonical EGFR activation is mediated by binding of the epidermal growth factor (EGF) [3], however there are at least six growth factors which can bind to and activate EGFR [4–8]. For EGFR, as with all RTKs, activation is stimulated by ligand binding, inducing dimerization of two monomers [9] and autophosphorylation of multiple tyrosine residues in the cytoplasmic tail which serve as binding sites for signaling proteins containing Src homology 2 (SH2) domains [10]. Unlike some other RTKs which form homodimers exclusively (PDGFR, VEGFR), EGFR can form both homodimers

and heterodimers with other members of the ErbB family and other RTKs [11, 12]. ErbB2 is the preferred dimerization partner of all the ErbB receptors [13], and the heterodimer of EGFR and ErbB2 demonstrates robust signaling activity [14]. EGFR is involved in a number of downstream signaling pathways, and heterodimerization with different ErbB partners and other RTKs, including c-Met, modulates the phosphorylation of the cytoplasmic tail, expanding its downstream signaling potential [11,12].

Many critical signaling pathways are induced by the activation of EGFR. One such pathway is the Ras (Rat Sarcoma)/Raf (Rapidly Accelerated Fibrosarcoma)/MAPK (Mitogen-activated protein kinase) pathway. Following EGFR phosphorylation, the adaptor protein Grb2 binds directly to specific docking sites on the receptor [15]. This interaction recruits Sos and activates Ras, which in turn activates Raf1, which is then able to phosphorylate MAPK1/2 (also known as ERK1/2) [16, 17]. These activated MAPKs are translocated into the nucleus where they phosphorylate transcription factors which promote cell proliferation [18]. In addition to growth, Ras downstream effectors include proteins involved in migration, adhesion, cytoskeletal integrity, survival and differentiation [19]. The importance of this pathway is underscored by the prevalence of alterations in various members of the pathway in many solid tumors. Gain-of-function mutations in the three Ras family members in humans (KRas, HRas and NRas) together are found in up to 30% of all human tumors [20]. The frequency of alterations in this pathway decreases as one moves further downstream, however mutations in v-raf murine sarcoma viral oncogenes homolog B1 (BRAF), including $\text{BRAF}^{\text{V600E}}$, in human cancers is estimated at approximately 7% [21].

Another critical signaling pathway downstream of EGFR activation is the Phosphatidylinositol 3-kinase (PI3K)/Protein kinase B (PKB/Akt) pathway. PI3K contains two subunits: p85 which is responsible for the anchorage to the receptor docking sites, and p110 which is a catalytic subunit that generates phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 is responsible for activation of the Akt kinase through recruitment to the plasma membrane, leading to Akt activation by phosphorylation at two different sites [22]. Akt is an essential kinase that has been shown to interact with well over 100 substrates involved in a variety of cellular functions includ-

ing survival, growth, proliferation, angiogenesis, metabolism, and migration [23]. The activity of PIP3 is negatively regulated by dephosphorylation by the tumor suppressor Phosphatase and tensin homolog (PTEN) [24]. Again highlighting the importance of this pathway is the prevalence of alterations, however unlike the Ras/Raf/MAPK pathway, alterations in all the major elements of this pathway have been found mutated or amplified in a broad range of cancers. For example, the p110 catalytic subunit of PI3K has been found to be mutated in up to 30% of breast cancers, and between 10-30% of endometrial, colorectal, urinary tract and ovarian cancers [20]. Loss-of-function mutations or deletion of the tumor suppressor PTEN is also common across a variety of cancers, including approximately 45% of glioblastomas (GBM) [25, 26].

1.2 EGFR in Cancer

Aberrant EGFR signaling is implicated in many human diseases, including many different types of solid tumors. Across all the samples in the The Cancer Genome Atlas (TCGA), which includes over 9,000 tumors and 33 cancer types, the prevalence of EGFR alterations is approximately 4%, making it the third most common alteration in the RTK-RAS pathway [27]. In that same cohort, EGFR alterations were most common in glioblastoma (GBM), low grade glioma (LGG), head and neck cancer (HNSCC), lung adenocarcinoma, and esophagogastric squamous carcinoma, present in 50%, 52%, 13%, 13%, and 14% of these tumors respectively [27]. Alterations within EGFR can take many forms, including amplification, in-frame deletions, and kinase domain mutations. Universally activating, these mutations have variable downstream effects based on different factors including tumor type, intensity of amplification, type of mutation, and previous exposure to EGFR targeted therapy.

1.3 EGFR in Glioblastoma

From 2011-2015, malignant gliomas of the brain including glioblastoma, astrocytoma and oligodendroglioma, accounted for approximately 86,000 of the 110,000 diagnosed tumors of neuroepithelial tissue. This represents approximately 21.7% of all tumors diagnosed in this

time period [28]. Malignant gliomas are among the deadliest of human cancers because they are highly invasive and neurologically destructive [29]. The median survival of patients with the most common and most aggressive of these, grade IV glioblastoma (GBM), is 12- 15 months, with a 5-year survival rate that remains at less than 5% despite the use of intensive treatments [29]. The World Health Organization (WHO) classification of tumors of the Central Nervous System (CNS) distinguishes gliomas based on their histological appearances and molecular compositions, where the grade indicates the level of malignancy [30]. Gliomas, including both high grade GBM and LGG, are the prototypical tumor type which possess *EGFR* alterations at a high level. *EGFR* amplification occurs in approximately 60% of primary GBMs compared to 8% of secondary GBM patients [26] and is a hallmark of the classical subtype, found in approximately 95% of tumors classified in this manner. In contrast, the other molecular subtypes are associated with reduced rates of *EGFR* amplifications at 29% (mesenchymal), 67% (neural), and 17% (proneural) [31]. GBM possesses a strong correlation between *EGFR* copy number and expression. Over 90% of tumors which contain *EGFR* mutation and/or amplification overexpress the protein [32]. *EGFR* amplification in GBM occurs almost exclusively in the form of circularized double-stranded extrachromosomal DNA fragments known as double minutes (DM) [33]. The absence of a centromere in DMs results in a random segregation between daughter cells [34], resulting in highly variable numbers of DMs in a population of GBM cells which can drive tumor evolution and heterogeneity [35].

EGFR Extracellular Domain Deletion Mutations in GBM.

In GBM there are five common deletion mutants, EGFR variants I-V (EGFRvI-EGFRvV). Mutants EGFRvII, EGFRvIII and EGFRvIV have entire exons deleted, while EGFRvI and EGFRvV have NH2-terminal and COOH-terminal truncations, respectively [36]. EGFRvI and EGFRvIV are rare, while EGFRvII and EGFRvV are marginally more common, each accounting for about 10% of all GBM-associated *EGFR* mutations [37]. The most common deletion mutant of EGFR is EGFRvIII. EGFRvIII is a highly tumor specific [38] extracellular domain mutation which shows constitutive tyrosine kinase activity [39], conferring increased tumorigenicity [40]. EGFRvIII results from in-frame deletion of 801 base pairs spanning exons 2-7

of the coding sequence, removing 267 amino acids from the extracellular domain and creating a junction site between exons 1 and 8 and a new glycine residue [41, 42]. Prevalence of EGFRvIII in GBM is in the range of 30-60% depending on the method of identification [43, 44], with recent large-scale genomic analysis indicating an overall prevalence of approximately 40% [26]. EGFRvIII protein has been identified in other tumor types including HNSCC [45], although genomic deletion of exons 2-7 has not been detected. Though EGFRvIII cannot bind ligand and exhibits lower levels of tyrosine kinase activity than activated wild-type EGFR [39], it is able to evade Cbl-mediated receptor internalization [46], rendering the protein highly stable at the cell surface and drastically increasing its tumorigenicity [40]. EGFRvIII is always amplified and exclusively present on ecDNA [47] and exhibits dynamic regulation by integrating and dissociating from the chromosome in response to anti-EGFR therapies [48]. Interestingly, EGFRvIII is heterogeneously expressed among cells within a given tumor, being detected at both very high and very low protein levels in GBM [49]. Previous work from our lab has demonstrated that interclonal cooperation between populations of tumor cells is a predominant mechanism by which GBM aggressive growth is maintained [50]. Mutant EGFRvIII was shown to drive this process via a cytokine circuit to the less aggressive neighboring cells, the majority of which expressed amplified wild-type EGFR (wtEGFR) [50].

Targeting EGFR with Monoclonal Antibodies.

Because of the high prevalence of wtEGFR/EGFRvIII amplification, often present within the same tumor, targeting EGFR and its variants has been a focus in the GBM field. One approach to inhibit EGFR-mediated signaling is to disrupt receptor-activating ligand binding [51]. Monoclonal antibodies, both unconjugated and conjugated, directed towards wtEGFR and EGFRvIII have been developed for therapeutic use in GBM. The most developed of the unconjugated antibodies is cetuximab which functions to prevent EGFR-mediated signal transduction by interfering with ligand binding and EGFR extracellular dimerization [51]. Additionally, cetuximab is believed to trigger EGFR receptor internalization and destruction [52]. Mouse xenograft studies demonstrated that treatment with cetuximab decreases tumor proliferation and increases cell death and overall survival [53], however in clinical trials cetuximab

failed to demonstrate efficacy either as a single agent for recurrent glioblastoma [54], in combination with other reagents [55], or combined with radiation [56]. Some monoclonal antibodies have been engineered to specifically target EGFRvIII, with the hypothesis that these antibodies will be highly tumor specific and avoid off-target side effects. One such antibody is mAb806, which attenuates receptor autophosphorylation by binding to the short cysteine loop of the extracellular domain that is always exposed in EGFRvIII, but may also weakly target amplified wtEGFR, which transiently exposes this epitope during the switch from the inactive to the ligand-activated conformation [57, 58]. Pre-clinical data showed that mAb806 strongly inhibits the growth of tumor xenografts that express EGFRvIII and as expected, more weakly those that express wtEGFR [59]. To enhance the anti-tumor effect of this antibody, a microtubule depolymerization agent monomethyl auristatin F (MMAF) conjugated version was developed and termed ABT-414 [60]. Though it showed initial promise in phase I/II clinical trials [61, 62], use as a monotherapy ultimately failed phase III clinical trials for subjects with newly diagnosed GBM with EGFR amplification [63].

Targeting EGFR with Tyrosine Kinase Inhibitors.

The most clinically advanced, yet altogether disappointing, strategy for targeting amplified *EGFR* in GBM is through the use of tyrosine kinase inhibitors (TKI). Many agents have been developed to target the kinase domain of EGFR, and development has gone through multiple classes of drugs over approximately two decades. Mechanistically, these inhibitors compete with adenosine triphosphate (ATP) for binding to the tyrosine kinase domain of EGFR [64] inhibiting activity and downstream signaling [65]. The leading TKI representatives for GBM include erlotinib, gefitinib, and lapatinib, all of which have shown to be ineffective as both single agents and in combination. Erlotinib showed no efficacy and significant side effects as a single agent in newly diagnosed GBM [66] and also failed in combination with mechanistic target of rapamycin (mTOR) inhibitors [67] or anti-angiogenesis drugs (bevacizumab) [68]. Similarly, gefitinib did not improve overall survival after radiation therapy [69] or administered concurrently in combination with radiation [70] in newly diagnosed GBM. Additionally, although lapatinib is also able to inhibit HER2 [71], it has shown limited efficacy both as a single agent [72] or in

combination with anti-angiogenic agents [73]. Many factors are hypothesized to contribute to the limited efficacy of these TKIs. Evidence supports lack of brain penetrance as a major obstacle to EGFR TKI therapy, with studies showing in vivo EGFR phosphorylation is not significantly blocked in patients receiving these inhibitors [74, 75]. Tumors which are exposed to sufficient doses of TKI therapy are also often able to become resistant through mechanisms including inactivation of PTEN [76], co-activation of other RTKs [77], and signaling pathways such as the TNF-JNK-Axl-ERK pathway [78]. Designing smaller molecules which can more easily pass the blood brain barrier (BBB) [79,80] and rational drug therapy combinations represent novel angles for treating EGFR-positive glioma, however the lack of clinical success thus far by these strategies underscores the challenges in treating GBM by small molecule.

EGFR Extracellular Domain Missense Mutations.

In addition to structural variation at the *EGFR* locus, extracellular domain (ECD) missense mutations also represent a significant proportion of *EGFR* mutations in GBM. Approximately 10-15% of EGFR-altered tumors have these ECD mutations [26, 81] and they confer a significant negative survival effect, lowering the median overall survival (OS) and 2 year survival rate from 15 months and 21% to 6 months and 12% respectively [82]. Although there are many ECD mutations which have been identified, the most common and clinically relevant mutations are A289D/T/V, R108G/K, and G598V which were found in 6%, 3% and 2% of a cohort of 411 GBM cases [82]. Expression of these mutants is associated with increased downstream signaling [81] through ligand independent constitutive signaling [81,83], leading to a more invasive and proliferative phenotype [82]. Importantly, ECD mutation is also highly correlated with *EGFR* amplification [26]. One critical characteristic of the ECD mutations is their ability to induce conformational changes in EGFR on the cell surface. Crystal structures of the EGFR catalytic domain have identified different receptor conformations [84] which significantly affect how these receptors can be targeted. In the presence of ECD mutations, the receptor adopts an inactive symmetric kinase domain (sKD) state in contrast to ligand activated wtEGFR which adopts an asymmetric kinase domain (aKD) [75]. GBMs expressing ECD mutations have been shown to respond to TKIs, however the responses are more robust when using class II TKIs, including

lapatinib, due to preferential binding to the inactive, type II conformation [75,85]. Recent data also indicates the importance of this conformation change in the response to treatments using targeted monoclonal antibodies. In addition to the aKD and sKD conformational states, research using the mAb806 indicates a third ECD transitional state, characterized by exposure of a cryptic epitope [86]. Surprisingly, mAb806 was also found to bind wtEGFR when it is present at high levels on the cell surface [87], however the epitope is buried when the receptor is bound by EGF [88]. Previous research from our lab has shown the enhanced ability of mAb806 to bind to GBM cells expressing $EGFR^{A289V}$ when compared to wtEGFR, nearly to the same degree as it binds EGFRvIII. This binding efficiency led to a significant reduction in both tumor growth and significant enhancement of survival in a mouse xenograft model [82]. These results indicate additional patient stratification may further enhance the clinical potential of the weaponized mAb806 antibody ABT-414.

1.4 EGFR in Non-Small Cell Lung Cancer

Lung cancer is the most prevalent malignancy in the world with an estimated global incidence of approximately 1.6 million and 1.4 million deaths from this disease annually [89]. Lung cancers are classified clinically into two major groups: non-small cell lung cancer (NSCLC), which accounts for about 85% of all lung cancers, and small-cell lung cancer (SCLC) which accounts for the remainder [90]. The most common types of NSCLC include squamous cell carcinoma, large cell carcinoma, and adenocarcinoma which are responsible for approximately 35%, 10% and 45% of NSCLC respectively [91]. Alterations in EGFR are present in a large subset of lung cancers, occurring almost exclusively in NSCLC and primarily those which are of the adenocarcinoma histological subtype [90]. Interestingly, there are significant differences in EGFR alteration prevalence in adenocarcinomas of different populations with Western populations exhibiting approximately 19% EGFR-altered tumors, while Asian populations exhibited EGFR-altered tumors with as much as 48% frequency [92]. Because of this high prevalence of EGFR alteration and the observation that as much as 60% of NSCLCs overexpress EGFR as measured by immunohistochemistry (IHC) [90] anti-EGFR therapies were quickly applied to the

treatment of these tumors. Today, multiple EGFR TKIs are approved by the Food and Drug Administration (FDA) for the treatment of EGFR-positive lung tumors, the first of which was Gefitinib in 2003. Gefitinib was approved as a monotherapy for locally advanced or metastatic NSCLC after failure of both platinum-based and docetaxel chemotherapies, and had an observed objective response rate in about 10% of patients [93]. Interestingly, there was no correlation between EGFR expression and response to gefitinib [94] so to stratify patients more accurately responders and non-responders were compared. Three independent groups identified EGFR kinase domain mutations as the primary predictive marker of response to EGFR TKI including both gefitinib and erlotinib [95–97].

EGFR Kinase Domain Mutations.

Although over 200 EGFR mutations have been described in NSCLC [98], approximately 90% of the activating mutations are one of two mutations: in-frame deletion of five exon-19 residues (exon 19 deletion) and the exon-21 substitution of an arginine for leucine (L858R) [99]. These mutations increase the kinase activity of EGFR, leading to the hyperactivation of downstream pro-survival and growth pathways [100]. Though these mutations have been shown to activate EGFR to a similar degree [101], exon 19 deletion mutations seem to respond better to gefitinib and erlotinib than tumors with the L858R mutation [102]. Perplexingly, the predictive value of EGFR activating mutations is controversial, as approximately 10-20% of patients who show a partial response to gefitinib do not have identifiable EGFR mutations, indicating they are not the sole determinants of TKI response [103]. In contrast to GBM, the role of amplification of *EGFR* in response to TKI therapy is unclear in NSCLC in part due to inconsistencies in EGFR copy number prediction [103]. The frequency of use and initial effectiveness of EGFR TKI therapies in NSCLC spawn inevitable resistance mutations which arise after a median duration of 10-13 months [104]. The most common resistance mechanism by which NSCLC tumors are able to overcome EGFR TKI is the T790M mutation in exon 20 which occurs in 50-60% of patients whose tumors progress on TKI therapy [105]. This mutation replaces threonine with the larger methionine residue near the ATP-binding site, rendering ATP able to compete more effectively with TKIs for binding and decreasing the inhibitory effect of the drugs [106]. To over-

come this common resistance mechanism, mutant-selective third generation EGFR TKIs such as rociletinib [107] and osimertinib [108] were developed. FDA approval through the Breakthrough Therapy designation of osimertinib for patients with metastatic NSCLC whose disease has progressed on EGFR targeted therapy and harbor a T790M mutation speaks to the power of these third generation drugs, however even tumors which initially respond to these treatments eventually develop resistance through additional mutations in exon 20 including C797S. This mutation abolishes the covalent bonding of osimertinib to EGFR [109] and has a prevalence range between 22-40% in patients who have progressed on osimertinib [110]. The ability of NSCLC to adapt and overcome targeted therapy is a testament to the determination of cancer to continue to persevere and the subsequent perseverance of researchers continuing to develop personalized medicines.

1.5 EGFR in Head and Neck Squamous Cell Carcinoma

Although commonly referred to as head and neck cancer, squamous cell carcinomas of the head and neck (HNSCC) represent a diverse set of tumors from various regions of the body including the larynx, throat, lips, mouth, nose, and salivary glands. Worldwide, HNSCC is the seventh most common cancer type with a yearly incidence rate of approximately 600,000 cases, with 40-50% mortality [111]. Although the tumors originate in the epithelial cells of the mucosal linings of the upper airway and food passages, the disease is remarkably heterogeneous at the molecular level [112]. The major known risk factors for development of HNSCC are environmental exposures to tobacco and alcohol, as well as infection with human papillomaviruses (HPV) which is now recognized as the strongest prognostic marker [113]. Because of the heterogeneous nature of this cancer type, significant efforts have been made to identify clinically actionable molecular subtypes and biomarkers [114,115]. Interestingly, these studies have identified distinct genetic signatures between HPV$^-$ and HPV$^+$ tumors whereby the driver genes of HPV$^+$ tumors are largely a mystery while HPV$^-$ tumors have high rates of known oncogenic mutations, including EGFR [116]. One of the largest and most comprehensive datasets of both HPV$^-$ and HPV$^+$ HNSCC is the molecular profiling of 279 tumors by the TCGA [117]. Based on this analysis, between 50 and 100 genes are indicated to be substantially mutated and are considered candidate

cancer driver genes. Many of the genes implicated as drivers for GBM are also frequently altered in HNSCC, including TP53 (84%), CDKN2A (57%), PIK3CA (34%), and EGFR (15%) [117]. Paradoxically, while amplification of EGFR is relatively uncommon in comparison to other driver genes, studies have identified protein overexpression in 47-84% of HNSCC tumors [118–120]. This range may be due to differences in expression of EGFR in different sites of HNSCC [121]. In contrast to both GBM and NSCLC, alteration of EGFR in the form of either KD or ECD mutations is rare. Although high rates of EGFRvIII have been reported in HNSCC [45], updated molecular profiling by TCGA identified only 1 of 279 tumors with EGFRvIII by RNA-seq (0.4%) [117]. Additionally, kinase domain mutations were only identified in 117 out of 4122 patients (2.8%) with missense mutations in exons 18-21 occurring in 73% of the 117 [122].

EGFR Targeted Therapies in HNSCC.

Of the myriad anti-EGFR therapies discussed, only cetuximab has been approved for the treatment of advanced HNSCC as either a single agent [123], in combination with radiation [124], or in combination with platinum based chemotherapeutics [125]. Although it was able to significantly improve survival statistics, response rates as a single agent remain low at approximately 13% [124]. This relatively low response rate does not seem to be due to lack of EGFR expression in these tumors, rather investigation into the relation between EGFR expression and the outcome of HNSCC patients treated with cetuximab have shown that EGFR levels detected by IHC have no impact on response [126, 127]. However, other studies have reported a positive relationship between EGFR expression and anti-EGFR (cetuximab, nimotuzumab) therapy responses [128,129]. These disparities underscore the importance of tumor heterogeneity and careful patient curation in trials assessing the treatment of HNSCC tumors with anti-EGFR therapies. HNSCC tumors commonly escape anti-EGFR therapy by upregulating pathways which can compensate for reduced EGFR signaling and/or modulate EGFR-dependent signaling. One of the primary escape mechanisms in response to cetuximab therapy is upregulation of signaling by other growth factor receptors. The most extensively studied of these receptors in this capacity are HER2/ErbB2 and HER3/ErbB3. HER2 alterations have been directly linked to resistance to EGFR-targeted therapy, with expression of phospho-HER2 and HER2 expression

significantly correlating with protein expression of EGFR [130]. HER3 activity is dependent on EGFR and HER2, and the HER2/HER3 heterodimerization is one of the main forms of cetuximab resistance in HNSCC [131, 132]. In light of these observations, simultaneous blocking of EGFR with either HER2 or HER3 has been proposed. Indeed, combinatorial treatment of cetuximab with pertuzimab (HER2 monoclonal antibody) or seribantumab (HER3 monoclonal antibody) results in potent tumor suppression [131, 132]. Likewise, EGFR TKIs like lapatinib (EGFR/HER2) [133] or dacomitinib (pan-HER) [134] have shown some preclinical benefit. The lack of reliable biomarkers for response to anti-EGFR therapies underscores the need for novel avenues of research on the role of EGFR in HNSCC.

1.6 Summary

EGFR was the first RTK to be identified and labeled as an oncogene, and as such it has become one of the most thoroughly studied proteins in biomedical research. Canonical EGFR signaling is involved in many signaling pathways which are widely recognized as critical for the growth and survival of cancer cells, making EGFR itself and its downstream effectors attractive targets for therapy. Indeed, EGFR has been the target of a myriad of treatments including but not limited to monoclonal antibodies, TKIs, RNA interference [135], vaccines [136], and immune therapy [137]. While some successes have been achieved, including approvals for the monoclonal antibody Cetuximab in HNSCC [123] and TKIs Erlotinib [138] and Gefitinib [93] in NSCLC, the clinical outcomes for many patients with EGFR alterations, particularly those with GBM, remain dismal as evidenced by the most recent phase III failure of an EGFRvIII-specific monoclonal antibody therapy [63]. Thus, while knowledge of inherent genetic alterations is pertinent in determining rational therapeutic targets, the monotherapies that have emerged from this knowledge are inadequate for generating a durable response in many patients. These observations underscore the critical need for new paradigms of thinking for targeting EGFR-positive malignancies. Perhaps though the EGFR protein and its domains have been the focus for anti-EGFR therapies in the past, it is time to look to the EGFR gene for clues to guide the next generation of therapy.

Chapter 2

Introduction to epigenetics and its role in cancer

2.1 Epigenetics

Epigenetics as a term was first used to categorize all of the regulated developmental processes that, beginning with genetic material, lead to a mature organism [139]. The present definition of epigenetics better reflects the understanding that although DNA is essentially the same in all of an organisms somatic cells, patterns of gene expression differ greatly among different cell types, and these patterns can be inherited. Thus, a succinct definition of epigenetics was proposed as the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence [140]. Since the fully completed sequencing of the human genome in 2001, it is known that only approximately 2% of the human genome encodes for protein [141], however large scale functional mapping suggests that more than 80% of the human genome participates in at least one RNA- or chromatin-associated event in at least one cell type [142]. This is concrete evidence that what was once thought of as junk DNA is in fact playing a critical role in the function of the human genome.

2.2 The Nucleosome

The most fundamental unit of epigenetic gene regulation is the nucleosome. The nucleosome is made up of 147 DNA base pairs wrapped around a histone octamer composed of two subunits of each histone H2A, H2B, H3 and H4. The wrapping of histones by DNA allows for the packaging of DNA into highly compacted structures, folding the DNA further into tighter and tighter structures, which when taken together comprise the entire DNA/protein structure within the cell known as chromatin [143]. Nucleosomes are powerful regulatory units due to their ability to be modified at specific residues within the unstructured N-terminal tails of core histones. To date, there are 16 identified classes of histone modifications [144], with the covalent modifications acetylation, methylation, and phosphorylation encompassing most of the known functions. These modifications can alter chromatin structure by modifying noncovalent interactions within and between nucleosomes. They also serve as docking sites for specialized proteins with unique domains that specifically recognize these modifications, which then recruit additional chromatin modifiers and remodeling enzymes, serving as effectors of the modifications [145]. These histone modifications are regulated as part of a highly dynamic process, as their deposition and removal at specific genomic regions is constantly changing through the activity of large classes of specialized histone modifier proteins [143]. The fluidity of this process is underscored by the observation that all of these modifications are reversible [146].

2.3 DNA Methylation

The DNA around the histone is also subject to modifications. To date, four different DNA modifications have been identified [147], however methylation of the 5-carbon on cytosine residues (5mC) in CpG dinucleotides was the first described covalent modification of DNA and is perhaps the most extensively characterized modification of chromatin [148]. To date there are three DNA methyltransferases (DNMT) that have been identified to methylate DNA directly. DNMT1 recognizes DNA bases whose partners have yet to be methylated during DNA synthesis (hemimethylated), and methylates those newly synthesized CpG dinucleotides [149]. DNMT3a

and DNMT3b are also capable of recognizing hemimethylated DNA, however their primary functions are as *de novo* methyltransferases to establish DNA methylation during embryogenesis [150]. Importantly, there are no known mammalian direct DNA de-methylases, however demethylation of mammalian DNA can occur both passively and actively. Passive DNA demethylation can occur through successive cycles of DNA replication in the absence of functional DNMT1, therefore diluting the total number of methylated cytosines [151]. In contrast, active demethylation requires multiple steps and the activities of several proteins. A key intermediate of the demethylation process, 5-hydroxymethylcytosine (5hmC), was discovered by Tahiliani and colleagues [152]. 5hmC occurs through the activity of Ten-eleven translocation methylcytosine dioxygenase 1 (TET1), which oxidizes the methyl group of 5mC. This oxidation process was also discovered to be iterative, yielding further oxidized products such as 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) [153]. These oxidized bases are recognized by thymine DNA glycosylase (TDG), excised from the DNA strand, and then repaired by base excision repair (BER) to the correct cytosine base [154]. Methylation represses transcription directly by inhibiting the binding of specific transcription factors, and by recruiting methylated-CpG-binding domain (MBD) proteins and their associated repressive chromatin remodeling activities [155]. MeCP2 is a well-known methyl-CpG binding domain protein, which contains an MBD domain and a transcriptional repression domain that acts to recruit the Sin3 HDAC complex. MeCP2 is capable of binding to a single methylated CpG and facilitates the methylation of lysine 9 in H3, possibly serving as a bridge between DNA methylation and histone methylation [156]. CpG dinucleotides are overall underrepresented in the genome, but are locally enriched in shorter stretches of DNA known as CpG islands [157]. An estimated 60% of human genes contain a CpG island, and these islands are most often associated with a transcriptional control region known as the promoter [158].

2.4 Non-coding Regulatory Elements

Complex organisms are made up of a wide variety of cell types, yet each of these cell types contains the same genome. This incredible diversity can be explained by the presence of non-coding regulatory elements which govern when, where, and to what level each gene will be

expressed.

Promoters.

Gene expression starts with transcription, a process which initiates at a defined position, known as the transcription start site (TSS), embedded within a core promoter sequence. The core promoter serves as a binding platform for the transcription machinery, comprising RNA polymerase II (Pol II) and other associated general transcription factors (GTF) [159]. Because of their critical role in the control of gene expression, the promoter is a tightly regulated region of DNA. As mentioned earlier, CpG islands are often found at gene promoters and are often hypomethylated [160], and the methylation of CpG islands results in stable silencing of gene expression [161]. In addition to promoter methylation as a mechanism of transcriptional control, the histone tails at promoter regions can also be modified and be indicative of activation or repression. Active core promoters are surprisingly known to be devoid of histones, and are instead comprised of non-canonical or partial nucleosomes [162], making them accessible and allowing for the assembly of the pre-initiation complex (PIC) [163]. It is instead the nucleosomes flanking the active promoter which are modified post-translationally.

Identification of these post-translational modifications was only recently applied genome-wide. The current gold-standard for identifying the binding positions of post-translationally modified histones is chromatin immunoprecipitation with sequencing (ChIP-seq) [164]. By crosslinking protein to DNA, shearing the crosslinked DNA by physical or chemical methods, and immunoprecipitating fragmented DNA using antibodies specific to a histone modification of interest one is left with a library of DNA fragments in direct contact with the factor of interest. Subjecting these DNA fragments to next generation sequencing methods allows for the identification of DNA regions which are enriched for the protein of interest. Active promoters are most often marked by tri-methylation of histone 3 at lysine 4 (H3K4me3) and acetylation of histone 3 at lysine 27 (H3K27Ac) [165]. Though these marks are strongly correlated with transcriptional activity, the mechanism is unclear. H3K4me3 is proposed to provide a memory of recent transcriptional activity, facilitating new rounds of transcription [166] while H3K27Ac appears to work through decreasing the affinity of DNA for nucleosomes, promoting open chromatin [167]

and providing binding sites for cofactors which bind acetylated lysines such as BRD4 [168]. The tri-methylation of histone 3 at lysine 27 (H3K27me3) is also often found at promoters, and is methylated by the Enhancer of Zeste 2 (EZH2) subunit of the polycomb repressive complex 2 (PRC2) [169]. H3K27me3 is know to have dual roles in the control of gene transcription. Broad enrichment of H3K27me3 in the gene body is known to be indicative of transcriptional repression [170], however bivalent presence of both H3K27me3 and H3K4me3 at the promoter is a marker of poised genes which can be rapidly transcribed in response to stimuli [171].

Enhancers.

While the promoter is immediately adjacent to the gene it affects, enhancers are additional key regulatory elements which can control cell-type-specific spatiotemporal gene expression irrespective of genomic location [172]. The initial discovery of these elements was a 72-bp sequence of the SV40 virus genome which could enhance transcription of a reporter gene by several hundred fold [173]. Subsequently, enhancers have been found to be highly abundant in the human genome, with some groups estimating >400,000 regions with enhancer-like features [142]. These regions are identified largely through the identification of enriched histone post-translational modifications including mono-methylation of histone H3 at lysine 4 (H3K4me1) and H3K27Ac [174]. Different combinations of these marks in combination with H3K27me3 define various enhancer-states. Neutral or intermediate enhancers are marked by H3K4me1 alone, while active enhancers are marked by H3K4me1 in combination with H3K27Ac [175]. Similarly to promoters, a poised state exists marked specifically by a combination of H3K4me1 and H3K27me3 [176]. Although these are widely used marks for identifying enhancers, there is no consensus on which histone marks should be used. This stems from the fact that none of the known histone modifications correlates perfectly with enhancer activity [177] and the functional roles for most of the chromatin modifications associated with active enhancers is unknown. Indeed, mutation of H3K4 or H3K27 such that they can no longer be modified are still compatible with gene transcription, suggesting these modifications are neither necessary, sufficient, or mechanistically involved in transcription [178]. Enhancer prediction based on these histone marks is not sufficient to ascribe functionality, so additional methods have been utilized to determine the activity of spe-

cific enhancers including enhancer activity assays [173], next generation sequencing (NGS)-based techniques [179], and CRISPR/Cas9-based techniques [180].

Another hypothesis for how enhancers can be identified and are able to influence transcription was proposed following the discovery of extragenic transcripts at the locus control regions (LCR), later understood to be enhancers, of a few genes [181, 182]. These transcripts were expressed in a cell type manner and correlated with LCR functionality, suggesting their transcription was linked to LCR activity [181]. Definitive evidence of enhancer transcription came with the discovery of pervasively transcribed putative enhancers, which produced largely non-polyadenylated non-coding RNAs (ncRNA) henceforth known as enhancer RNA (eRNA) [183, 184]. eRNA transcription appears to be a strong indicator of highly functional enhancers [185], however the specific function of eRNA has been the subject of much research. eRNAs generally display low stability and abundance [186] but are able to respond rapidly, often before promoters, to stimuli [187]. Studies of individual genes have ascribed direct consequences on transcription for specific eRNAs [188, 189], however the direct mechanism is still a mystery. Hypotheses for eRNA function include enhancing chromatin accessibility [186], formation and stabilization of chromatin organization [188], Pol II pause release [190], and stabilization of TF binding [191].

2.5 3D Genome Organization

DNA Interaction.

The intense compaction of DNA into chromatin fueled the hypothesis that a physical interaction between enhancers and promoters facilitated the transcriptional enhancement activity of enhancers. Recent evidence has solidified the validity of this hypothesis through forced chromatin looping between the mouse *Hbb* gene promoter and its enhancer, leading to strong transcriptional activation even in the absence of other transcriptional co-activators [192]. Development of chromosome conformation capture (3C) [193, 194] technologies has further strengthened our understanding of which enhancers and promoters interact. 3C and 3C derivatives coupled with NGS enabled the mapping of genome wide enhancer-promoter contacts [194] and

has facilitated the segmentation of the genome into distinct units such as active chromatins hubs (ACH) [195] and topologically associating domains (TAD) [196]. The discovery of the TAD as a unit of chromatin organization brought together many hypotheses on how enhancers and promoters are able to interact specifically with only a few genomic partners. TADs are megabase scale structures that function as a large DNA loop, pinched off at the base by CCCTC-binding factor (CTCF) and cohesion [197, 198], within which interactions are preferentially occurring and are largely independent of interaction with other TADs [196]. Within a single TAD can exist multiple protein coding genes and their associated promoters, enhancers, and associated transcriptional co-factors, forming an ACH [195, 199, 200]. Though significant evidence suggests genes within TADs form preferential contacts with each other, the boundary insulation is not absolute. High-resolution interaction profiling has shown about one-third of significant interactions crossed TAD boundaries [201] and significant interactions can occur between elements located across large genomic distances, often bypassing more proximally located genes [202]. These observations further demonstrate the highly complex nature of chromatin organization and underscore the need for functional validation of identified transcriptional control units.

Nucleosome Positioning and Open Chromatin.

The likelihood of a chromatin-binding protein mediating its effects on DNA or histones is drastically increased in open or accessible regions of the genome. The opening of chromatin through nucleosome eviction or destabilization at promoters and enhancers results from the binding of specific regulatory factors responsible for transcriptional activation [203]. Open or accessible regions of the genome are thus regarded as the primary positions for regulatory elements [204]. Transposase-accessible chromatin using sequencing (ATAC-seq) is the current gold-standard for assaying chromatin accessibility [205]. In the ATAC-seq protocol, unfixed nuclei are tagged *in vitro* with sequencing adapters by purified Tn5 transposase. Due to steric hindrance the majority of adapters are integrated into regions of accessible chromatin that are subsequently PCR amplified for library construction followed by paired-end NGS [205]. ATAC-seq data can be immensely valuable for identifying nucleosome-TF spacing patterns and TF occupancy [206], enabling identification of TFs within enhancer and promoter regions.

2.6 Alterations in Epigenetic Elements

Mutations in Chromatin Proteins.

Although cancer is typically considered a genetic disease, epigenetic alterations are nearly ubiquitous. Approximately 50% of human cancers harbor mutations in chromatin proteins [207] and many tumors exhibit markers of epigenetic reprogramming [208]. Non-neoplastic cells maintain chromatin homeostasis through the interplay of many different families of proteins repressing, activating, or remodeling chromatin at the appropriate time and in response to the appropriate signals. Recurrent mutations in the genes encoding these factors thus are likely to disrupt this homeostasis. As introduced earlier, the PRC2 complex is a critical mediator of gene repression through the activity of EZH2, a catalyzer of histone methylation. Dysregulation of EZH2 is frequent in several tumor types [209] and can occur in the form of gain-of-function (GOF) [210] and loss-of-function (LOF) mutations [211], overexpression [212], mutations in its demethylase UTX [213], and mutations in the SWI-SNF chromatin remodeling complex [214]. EZH2 can function as a potent oncogene, with forced expression causing neoplastic transformation of human breast epithelial cells [215]. The primary residue which is mutated in EZH2 across cancer types is Y641, a critical residue within the C-terminal catalytic SET domain, which leads to hypermethylation of H3K27me2 and a shifting of the steady state of H3K27 modification to trimethylation thus repressing polycomb targets [216]. Loss of UTX activity by mutation also has a phenotypically identical effect of EZH2 GOF mutations, as UTX catalyzes the removal of methyl groups from H3K27me3. Importantly, the SWI-SNF chromatin remodeling complex has been found to be mutated in approximately 20% of all human cancers [214], and performs a critical role in opposing the activity of PRC2. Mutations in critical SWI-SNF subunits, including SNF5/SMARCB1, are known driver mutations in malignant rhabdoid tumors [217] and can form synthetic lethal interactions with EZH2 in other SWI-SNF-mutant cancers [218]. Given the evidence for EZH2 enzymatic gain of function being a cancer driver, the development of EZH2-specific inhibitors has been an active area of investigation. Potent and selective inhibitors of EZH2 were first identified through high-throughput biochemical screens, the first iterations of which are S-adenosylmethionine (SAM)-competitive inhibiting compounds which bind wild-type and Y641

mutant EZH2 and inhibit EZH2-mediated H3K27me3 deposition [219]. SMARCB1-mutant preclinical models showed dose dependent regression [220], and EZH2 inhibitors in clinical trials and have shown some encouraging response in hematological malignancies [221]. Phase I clinical trial results in solid tumors is less robust, with initial reports showing tumor response in only 2 of 43 (5%) SMARCB1/SMARCA4-negative tumors [222]. As with many treatments, resistance emerges in the form of secondary mutations (Y111L and Y661D) following prolonged exposure to EZH2 inhibitors [223], underscoring the tumor dependency on EZH2 and the challenges involved in precision medicine.

Aberrant DNA Methylation.

Another mechanism of epigenetic alteration in cancer is aberrant DNA methylation, either in the form of hypo- or hyper-methylation depending on the tumor type and associated mutations. In hematological malignancies like acute myeloid leukemia (AML) and T cell acute lymphoblastic leukemia (T-ALL), LOF mutations of the methyltransferase DNMT3A are present at a frequency of approximately 25% [224, 225]. These mutations lead to hypomethylation of cancer-associated genes and aberrant activation of enhancers that drive oncogenic gene expression patterns [226, 227]. Interestingly, the opposite phenotype can also be tumorigenic. *De novo* methylation has been shown to occur at tumor suppressor genes in many tumor types [228] and may be a byproduct of the aging process [229], the most important demographic risk factor for cancer [230]. Some common genes are specifically methylated across tumor types. One example is the gene encoding the DNA repair enzyme O6-methylguanine-DNA methyltransferase (MGMT). MGMT is silenced by hypermethylation events in many cancers, and is often an early event in tumorigenesis [231]. The inactivation of MGMT is primarily epigenetic, evidenced by the higher percentage of MGMT-negative tumors with methylation than mutation [232, 233]. In central nervous system (CNS) malignancies, methylation is now being used to stratify tumor types and inform diagnosis and treatment [234]. In particular, MGMT promoter methylation status has been used to indicate the efficacy of treatment [235] and testing for promoter methylation is becoming part of the current standard of care [236]. Aside from prognostic value, direct inhibition of DNA methylation has shown some effectiveness for the treatment of cancer. The

classical demethylating agents comprise the analogs of deoxycytidine and were developed initially as cytotoxic drugs, however they were found to be effective DNA methylation inhibitors [237]. Incorporation of these nucleoside analogs, including 5-azacytidine (5-aza), inhibits methyltransferases at low doses and at high doses result in cell death [238]. Initial clinical trials with these drugs in solid tumors produced negligible effects [239], however subsequent trials have shown the demethylase activity to be effective in reactivating the expression of previously methylated tumor suppressor genes [240]. While promising, this reactivating effect has yet to demonstrate a clinical response either on its own or in combination with classical cytotoxic therapies [241].

Another example of common methylation-associated mutations are alterations in the genes encoding isocitrate dehydrogenase (IDH). GOF mutations in IDH are frequent initiating events in glioma, leukemia and other less common tumor types [242]. Normal IDH1 and IDH2 are located in the cytoplasm and peroxisomes respectively, and catalyze a redox reaction that converts isocitrate to α-ketoglutarate (α-KG) and reduces NADP to NADPH. NADPH supplies reducing power to key reactions in a number of macromolecular biosynthetic pathways, and to systems that defend against the oxidative stress imposed by reactive oxygen species (ROS). Therefore, mutations that disrupt the normal functions of IDH1 and IDH2 have significant consequences on cellular redox balance [242]. Additionally, mutant IDH was discovered to convert α-KG to 2-hydroxyglutarate (2-HG) in a reaction that consumes, rather than produces, NADPH [243]. 2-HG has received significant attention as an onco-metabolite as it has been shown to inhibit the TET proteins involved in demethylation of DNA, leading to a hypermethylation signature characteristic of stem/progenitor cells [244]. Low grade gliomas in particular are susceptible to IDH mutation, with mutation occurring in 70-90% of all adult grade 2/3 astrocytomas, oligodendrogliomas, and secondary gliomas, but only 5-15% of primary glioblastoma [245]. So strong is this association that IDH mutation is used in the WHO classification of gliomas [30], and is an effective favorable prognostic biomarker of survival when compared to IDH wild-type gliomas [246]. Targeting mutant IDH with specific inhibitors showed promise in preclinical models of glioma [247] and AML [248], and has recently led to FDA approval of IDH1 or IDH2 specific inhibitors for the treatment of newly diagnosed IDH-mutant AML [249].

Genomic Variation.

Beyond chemical modification of DNA or histones, genomic variation within regulatory elements and transcription factors provide a significant risk factor for the development of disease. Recent studies have shown that enhancer-like regions contain a high density of genomic variants [142, 250]. To date, hundreds of genome wide association studies (GWAS) have been conducted spanning diverse diseases and phenotypes, and the majority (~93%) of the identified disease- and trait-associated variants lie within noncoding sequence [251]. The exact mechanism for how non-coding variants can alter gene expression is unknown, however some studies have shown that putative causative single nucleotide polymorphisms (SNP) interfere with recognizable TF binding sites, however this only explains the mechanism behind 10-20% of the variants [252, 253]. Additional proposed mechanisms include post-transcriptional processes like mRNA splicing [254] and stability [255], altered DNA methylation [256], and changes in DNase hypersensitivity [257].

Mutations in genes encoding enhancer binding proteins and proteins involved in mediated enhancer-promoter specificity represent a second class of disease-associated variants. Beyond SNPs in regulatory regions, there are a few examples of direct causal variations in epigenetic-associated proteins in developmental disorders and cancer. Cornelia de Lange syndrome is most likely caused by defects in the subunits of the cohesion complex, leading to alterations in cohesins role in enhancer-promoter communication [258]. Similarly, Kabuki syndrome is caused by missense or nonsense mutations in MLL4, a member of the complex including the H3K27 demethylase UTX, or direct mutation in the *UTX* gene [259]. Gene fusions with MLL are known to be expressed in leukemias, and alter transcriptional elongation and proper H3K4 methylation [260]. Additionally, mutations in the genes encoding CBP or P300, major histone acetyltransferases (HAT), can cause the developmental disorder Rubinstein-Taybi syndrome [261]. Perhaps the most widely studied oncogenic transcription factor is c-Myc. *MYC* is the most frequently amplified oncogene, and the elevated expression of its gene product c-Myc can promote tumorigenesis in a wide range of tissues [262, 263]. Rather than binding and regulating a new set of genes when overexpressed, the transcription factor is known to accumulate in the promoter regions

of most active genes, causing transcriptional amplification of the existing gene expression program [262, 263]. These observations make c-Myc an enticing target for therapeutics, however direct targeting of c-Myc has proven exceedingly difficult. Indirect targeting strategies including disrupting stabilization [264], disruption of Pol II activity [265], and targeting of transcriptional activation partners [266] have shown some promise, however no FDA approved therapeutics have emerged.

2.7 Summary

Though the idea of epigenetics has been around for at least as long as we've known DNA is the carrier of genetic information, its functional application to the expression of genes is a relatively new field of study. In this relatively short time period, epigenetics has become one of the fundamental facets of biomedical research due to its clear role in the myriad of processes described above. The power in the study of epigenetics is that it is a fluid process, unlike the rigid genetic code of DNA. Through the dynamic deposition and removal of various DNA and histone modifications, huge families of proteins can associate, dissociate, and interact in every imaginable combination to produce the huge variety of cell types present in our bodies. The mobile nature of these modifications also provide a significant challenge for the study of epigenetics.

Most assays take a snapshot in time, a static picture of where modifications and proteins happened to be sitting at the arbitrary point in time we decided to fix them. It is therefore currently impossible to get a full understanding of the epigenetic changes undergoing in a cell during development or tumorigenesis. It is nonetheless important to study these snapshots to begin to understand the fundamental processes that underly gene expression in these important cell types. We can utilize the knowledge we gain to come up with hypotheses for rational treatment of diseases like cancer. The validity of this process is exemplified in the drugs that have already been developed to treat cancers like lymphoma [221] and leukemia [249]. Clearly alterations in DNA methylation, histone post-translational modification, and mutation in chromatin modifiers have significant effects on the progression of disease as discussed above, thus underscoring the rationale behind targeted therapies to reduce the malicious effects of these changes.

Continued study of the epigenetic drivers behind disease progression is an important endeavor, and will continue to produce significant breakthroughs as evidenced by the positive results thus far generated.

Chapter 3

Identification of putative active enhancers in the first intron of *EGFR*

3.1 Introduction

Recently, a new subset of enhancers was discovered when it was observed that there are unusual co-occurrences of clusters of multiple enhancers spread throughout the genome. These enhancer groups, initially called stretch enhancers and now commonly referred to as super enhancers (SE), have unique properties which distinguish them from common enhancers and justify their super moniker. The initial discovery of super enhancers stemmed from the observation that known master transcription factors OCT4, SOX2 and Nanog are critical for the embryonic stem cell (ESC) state, and they bind enhancers along with the Pol II co-activator Mediator [267]. ChIP-seq for these factors identified two groups of enhancers. First, small enhancers with low levels of Mediator, and much larger clusters of enhancers which were found within the same TADs as critical ESC identity genes which they dubbed super enhancers [267]. Specific knockdown of TFs enriched at these SEs had larger effects at genes nearby the SEs, indicating the increased role the SEs play in gene expression. Subsequent studies have laid out many themes

which distinguish SEs from other regulatory elements [267].

SEs are densely occupied by H3K27ac and H3K4me1 enhancer marks, the Mediator complex (MED1) and bromodomain-containing protein 4 (BRD4) [268, 269]. They contain cell type-specific TFs and constituent enhancers that exhibit an order-of-magnitude higher abundance of enhancer associated chromatin marks and TFs as compared to the composition of regular enhancers [267–269]. The genes driven by SEs are expressed at significantly higher levels than are genes under the control of regular enhancers [267–269]. Within a single SE, individual constituent enhancers are capable of increased transcriptional activation as compared to regular enhancers, and exhibit some functional redundancy but can also interact with additional genes independently of each other [268, 270–272]. Additionally, massively parallel constituent enhancer silencing identified only 1-2 constituent enhancers within a SE that can significantly affect gene expression [273]. Importantly, SEs are characterized by differential binding of tissue-specific or disease-specific TFs [269, 270]. For example, in many cancers SEs are marked by lineage-specific TFs at oncogenes including neuroblastoma [274], medulloblastoma [275], esophageal cancer [276], and melanoma [277]. These specific signatures open up the possibility of tumor-specific treatments which target SEs, and CDK inhibitors [278] or bromodomain inhibitors [274] have proven effective at inhibiting SE-specific gene expression. In contrast to the myriad of genetic and epigenetic changes that have been observed between tumor cells *in vitro* and *in vivo*, thus far established cancer cell lines have recapitulated the SE profiles identified in primary human tumors, supporting the hypothesis that SEs are key to the maintenance of tumor identity [278, 279] (Figure 3.2).

In spite of the prevalence of EGFR dependency in solid tumors, few studies have attempted to elucidate the mechanisms of transcriptional control of the gene. Early studies have primarily focused on the *EGFR* promoter, a 36bp element upstream of the AUG translation initiation codon [280]. The promoter does not contain TSS consensus sequences, such as TATA or CAAT box, thus transcription of the gene starts at multiple initiation sites [281]. Later studies attempted to identify other *cis*-acting elements which act in concert with the promoter to drive *EGFR* expression. These studies identified CA dinucleotide repeats [282–284], intron 1 DNase

I hypersensitive sites [285], and cooperative promoter-upstream and intron 1 enhancers [286] as important regulatory mechanisms. Lack of access to or utilization of NGS techniques limited the scope of these studies and argues for a larger breadth of analysis. Recently, *EGFR* super enhancers were identified in several cancer cell lines of different origins [287–291], however detailed mapping of these super enhancers was lacking. Additionally, while some of these studies identified TF binding to *EGFR* enhancers [287, 288] they either failed to demonstrate any functional effect of loss of these transcription factors on *EGFR* expression or did not include *EGFR* as part of their detailed analysis [289–291].

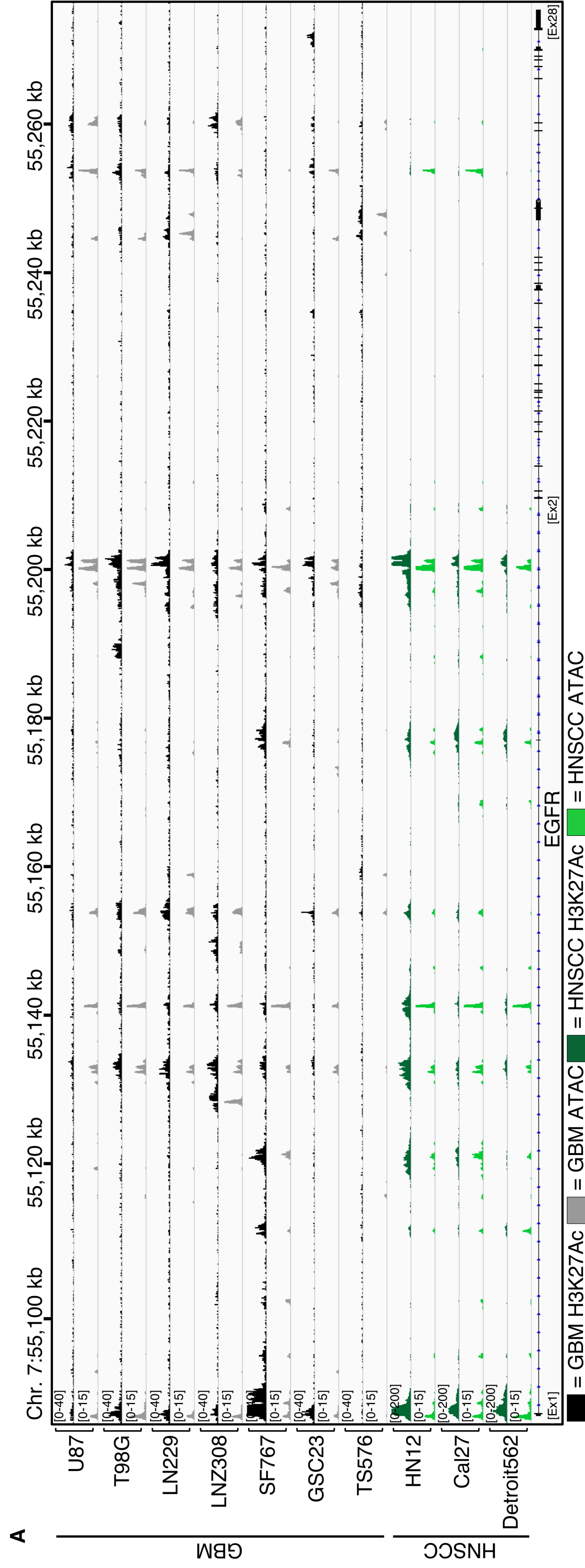
As described in Chapter 2, enhancer prediction based on histone marks is not sufficient to ascribe functionality. The gold standard for validating enhancers is the reporter assay. These assays rely on the ability of enhancers to increase the expression of a gene, independent of its orientation and flexible with respect to its position relative to the transcriptional start site [173,292]. The standard approach is to relocate the candidate enhancer sequence to a reporter vector, adjacent to a minimal promoter driving expression of a reporter gene, e.g. luciferase or green fluorescent protein (GFP). Many groups have attempted to functionally annotate predicted enhancers through a multitude of genome wide methods [180, 293]. The most comprehensive method for functionally testing predicted enhancers is self-transcribing active regulatory region sequencing (STARR-seq). This strategy was first validated in *Drosophila melanogaster* DNA, where initial enhancer prediction identified 50,000-100,000 enhancers. Brute force methods to validate these enhancers required analyzing fly embyos by *in situ* hybridization for 7,705 reporter constructs, requiring massive time and effort resulting in the identification of 3,557 active enhancers [294]. STARR-seq in these fly cells required only a single sequencing experiment to cover 96% of the non-repetitive genome and ascribe functionality to 5,499 regions which passed the threshold of activation to be considered enhancers. Validation of 77 randomly chosen peaks by reporter assays showed that 81% of the peaks could be functionally annotated, bringing the validated total strikingly close to low-throughput methods [179]. Ultimately as these studies have demonstrated, validation of enhancers still falls to the reporter assay for the final confirmation of activity.

3.2 Results

EGFR Intron 1 Contains Open Chromatin Regions Containing Histone Marks Indicative of Enhancers

To gain further insight into the mechanisms responsible for *EGFR* transcriptional control in HNSCC and GBM we performed ChIP-seq for the enhancer-marking histone modification H3K27Ac and ATAC-seq in 7 GBM and 3 HNSCC cell lines with non-amplified *EGFR* copy number and varying levels of *EGFR* activation. Cell lines with different *EGFR* expression levels were chosen to select for signatures which would preferentially identify regulatory elements which are responsible for driving *EGFR* expression. Overlay of IGV tracks of all 10 cell lines showed conservation of H3K27Ac intensity and open chromatin regions throughout intron 1, indicating the presence of enhancers in these regions (Figure 3.1A).

Figure 3.1: Chromatin landscape of wild-type *EGFR*. **A:** IGV snapshots showing H3K27Ac ChIP-seq (dark) and ATAC-seq (light) read densities at the *EGFR* locus in GBM and HNSCC cell lines. **B:** *EGFR* expression in 7 GBM and 3 HNSCC cell lines were analyzed by RT-qPCR. *EGFR* transcript level was first normalized to GAPDH and subsequently calculated as fold change relative to U87. **C:** Total aligned H3K27Ac ChIP-seq reads were calculated and plotted against the relative *EGFR* expression fold change. The relationship was analyzed using Spearmans rank order correlation.


A
GBM
HNSCC
U87
T98G
LN229
LNZ308
SF767
GSC23
TS576
HN12
Cal27
Detroit562
Chr. 7:55,100 kb
55,120 kb
55,140 kb
55,160 kb
55,180 kb
55,200 kb
55,220 kb
55,240 kb
55,260 kb
EGFR
[Ex1]
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[Ex28]
= GBM H3K27Ac
= GBM ATAC
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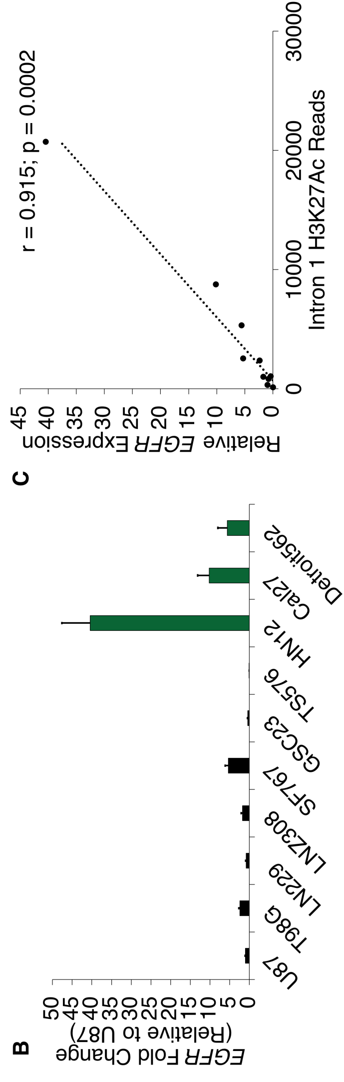

B
EGFR Fold Change
(Relative to U87)
50
45
40
35
30
25
20
15
10
5
0
U87
T98G
LN229
LNZ308
SF767
GSC23
TS576
HN12
Cal27
Detroit562
C
Relative EGFR Expression
r = 0.915; p = 0.0002
Intron 1 H3K27Ac Reads
0
10000
20000
30000

Since SEs are defined as large clusters of transcriptional enhancers that drive expression of genes that define cell identity, and are often found at oncogenes [268] we identified SEs using the ROSE (Rank Ordering of Super-Enhancers) algorithm [267, 269] (Figure 3.2A). The ROSE algorithm was specifically written to identify super enhancers utilizing sequencing data from enhancer marking histone modifications and TFs. ROSE requires two inputs: a file containing previously identified constituent enhancers and sequencing reads for the factor of interest with a control. From these data sets ROSE is able to rank the constituent enhancers based upon their specific enrichment of reads for the factor of interest over control, then stitch the highly ranking constituent enhancers together based upon given separation requirements. The default distance, and the distance used in this study, is 12.5kb. In the initial description of this algorithm, this distance was found to be optimal for stitching together the closely spaced enriched regions with very high signal while not being so large as to stitch together the more widely spaced regions with lower signal [267]. Finally, the constituent enhancers and their associated factor enrichment are plotted in relationship to each other, and the data is scaled such that the x and y axis are from 0-1. The x-axis point for which a line with a slope of 1 is tangent to the curve is identified, and enhancers above this point are defined as SEs, and enhancers below that point are defined as typical enhancers. Using this algorithm, we discovered cell-line specific SEs in the first intron of *EGFR*, many of which rank highly amongst all the identified SEs in several of our cell lines (Figure 3.2B). Interestingly, the location and size of these SEs varied and were dependent upon the local enrichment of H3K27Ac ChIP-seq signal (Figure 3.2A).

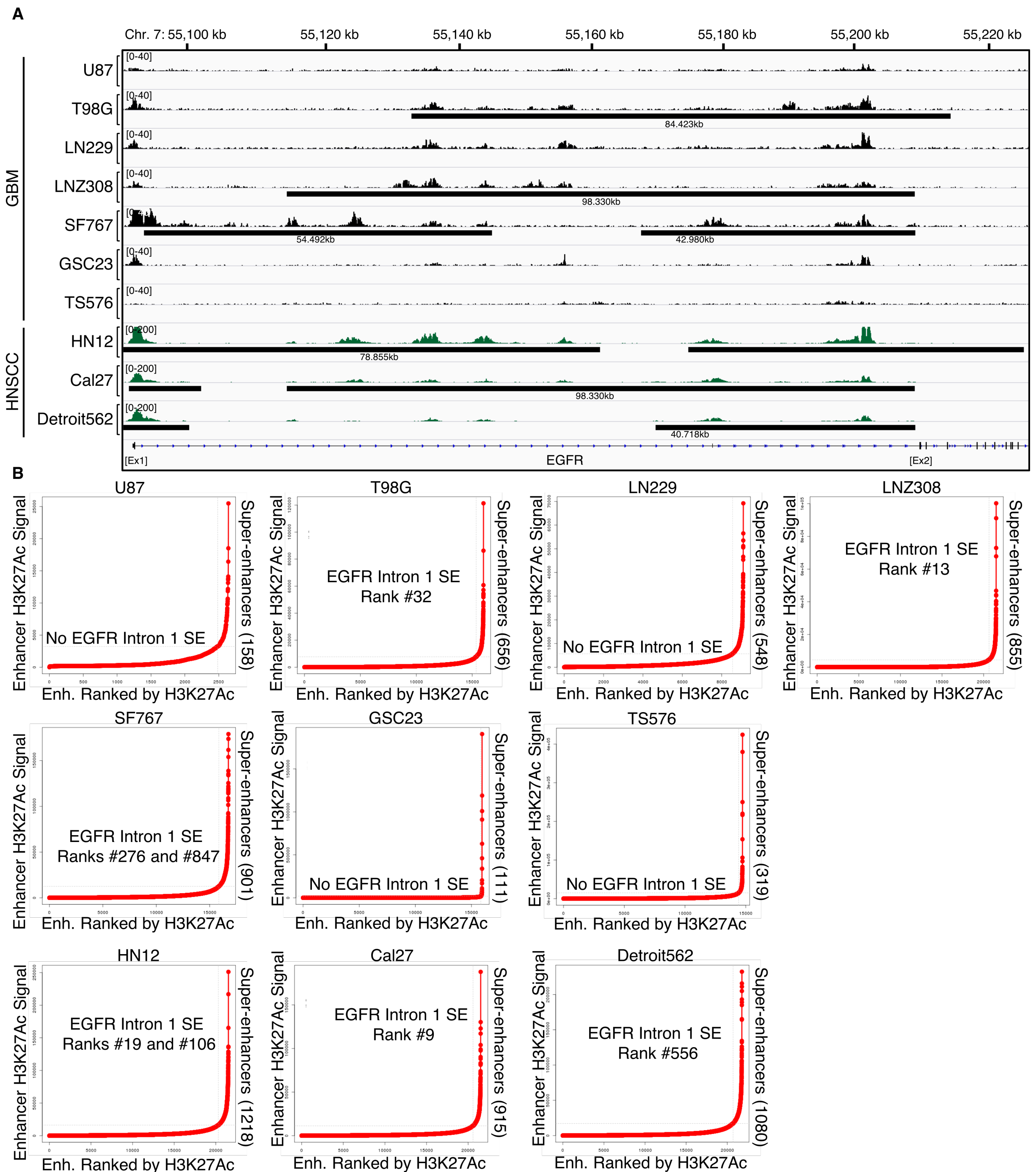


Figure 3.2: Identification of Super enhancers by ROSE. **A:** Super enhancer tracks in IGV overlaid with H3K27Ac tracks from measured cell lines at the EGFR gene. Super enhancers were identified by stitching together peaks of H3K27Ac histone marks with the ROSE software package. (Black bars) Super enhancers as called by ROSE. **B:** The EGFR intron 1 super enhancers were ranked based on H3K27Ac signal at the shown intron 1 super enhancers in the indicated GBM and HNSCC cell lines using the ROSE software package.

To determine if presence of putative enhancers in *EGFR* intron 1 was predictive of *EGFR* expression we first measured *EGFR* transcript levels in each GBM and HNSCC cell line (Figure 3.1B). Relative to the U87 glioma cell line, stark differences in *EGFR* expression were found between each measured cell line. Overall, measured glioma cell lines expressed significantly lower *EGFR* transcript than HNSCC cell lines. Among glioma cell lines, SF767 had the highest *EGFR* expression levels, while the HNSCC cell line HN12 exhibited the highest *EGFR* transcript amongst HNSCC lines and overall (Figure 3.1B). Because of these significant differences in *EGFR* transcript and the presence of *EGFR* SEs in intron 1 as identified by H3K27Ac enrichment, we hypothesized that *EGFR* transcript level may be correlated with SE-associated H3K27Ac enrichment. Due to cell line-specific presence and location of predicted *EGFR* super enhancers, we used total number of intron-1 mapped H3K27Ac ChIP-seq reads as a measure of enhancer presence and plotted these values against the relative fold change in *EGFR* expression. Analyzing the relationship by Spearman's correlation showed a highly significant correlation ($p = 0.0002$) (Figure 3.1C). To further confirm the conserved nature of these enhancers we utilized a recently published data set consisting of 44 patient-derived glioblastoma stem cells (GSCs) and 50 primary tumors [289]. The majority of the samples had gene expression, whole exome, copy number profiles, DNA methylomes and histone modification profiling completed, thus integration of these data sets was possible. We curated RNA sequencing (RNA-seq) and H3K27Ac ChIP-seq data for the 87 samples and ordered their acetylation tracks by the expression of *EGFR* as represented by fragments per kilobase of transcript per million (FPKM) (Figure 3.3). Those samples which expressed GBM to the highest degree are depicted at the top of the diagram, and maintain a consistent H3K27Ac signature reminiscent of the cell lines we have measured. As *EGFR* expression levels decrease, so too do intron 1 H3K27Ac reads (Figure 3.3). This extensive dataset further confirms the conservation of putative enhancers within *EGFR* intron 1 and argues for their regulatory potential both *in vivo* and *in vitro*. Together, these results identify regions containing characteristics of SEs in the first intron of *EGFR* and suggests that activity of these putative enhancers is important for high levels of *EGFR* transcript.

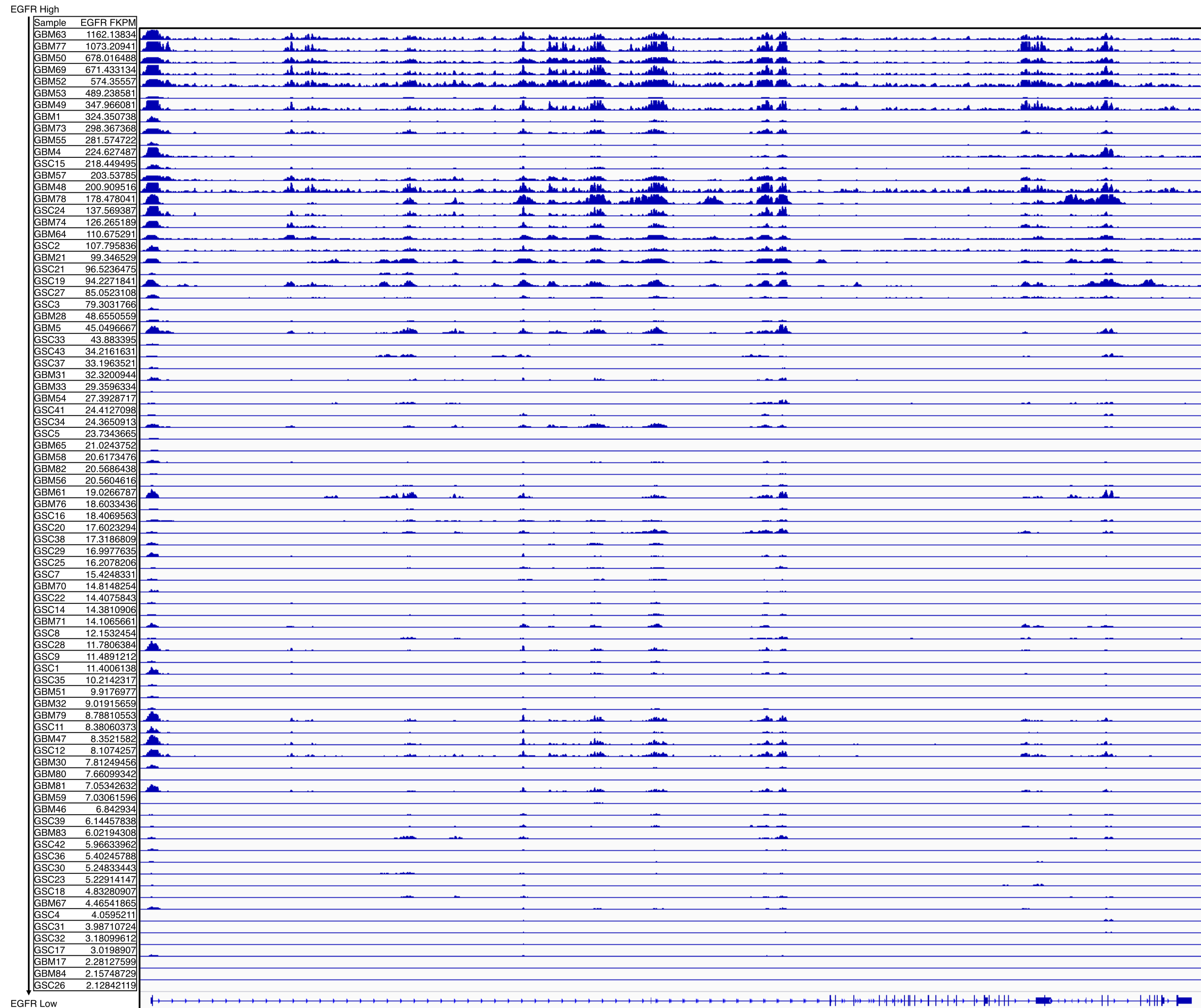


Figure 3.3: GSCs and GBM primary tumors confirm putative *EGFR* intron 1 enhancer presence. H3K27Ac ChIP-seq and RNA-seq from 87 *in vivo* and *in vitro* GBM samples were accessed from publicly available databases. ChIP-seq tracks were ordered from most to least *EGFR* expression as measured by FPKM determined by RNA-seq from matched tumor samples.

Two Critical Constituent Enhancers Reside in the First Intron of *EGFR*

As introduced earlier, it has been reported that SEs are congregations of active constituent enhancers (CE). To determine which CEs of the identified SEs are active, we performed a luciferase reporter assay utilizing regions which were identified as active by H3K27Ac. We segmented regions which exhibited highly conserved H3K27Ac ChIP-seq and ATAC-seq signals into 2kb segments (Figure 3.4A, Figure 3.5A). Each segment was then measured for enhancer activity by *in vitro* bioluminescence in each of the 10 cell lines (Figure 3.4B, Figure 3.5B). Measuring activity in each cell line is critical due to differential binding of tissue-specific or disease-specific TFs which may be active or inactive within each individual cell line [269, 270]. Regions which exhibited conserved luciferase expression included 1, 3, and 16-19 (Figure 3.4B, Figure 3.5B). Interestingly, activity of segments 8 and 9 were HNSCC specific (Figure 3.5B), while segments 1 and 3 were more GBM specific (Figure 3.4B). Segments 16-19 consistently enhanced luciferase activity in both tumor models (Figure 3.4B, Figure 3.5B). Each luciferase reporter segment resides in regions which have been identified as enhancers, yet some segments enhance luciferase expression to a larger extent than others. We hypothesized that the specific enrichment of H3K27Ac at an enhancer segment would correlate to luciferase expression in the matched cell line. Indeed, plotting the normalized luciferase intensity against the average H3K27Ac read intensity for each segment reveals a highly significant relationship ($p < 0.001$, Spearman's correlation) for both GBM and HNSCC cell lines (Figure 3.4C, Figure 3.5C). Combining H3K27Ac presence by ChIP-seq, open chromatin accessible regions by ATAC-seq, and functional activity as defined by our luciferase system in both tumor types, we define two distinct CEs. Specifically, we combined segments 1-3 into an approximately 6kb region which we have termed Constituent Enhancer 1 (CE1), and combined segments 16-19 into an approximately 8kb region which we have termed Constituent Enhancer 2 (CE2) (Figure 3.4A, Figure 3.5A). These results define two conserved putative CEs within *EGFR* intron 1 and establish a relationship between histone acetylation at these regions and enhancer activity as measured by *in vitro* bioluminescence.

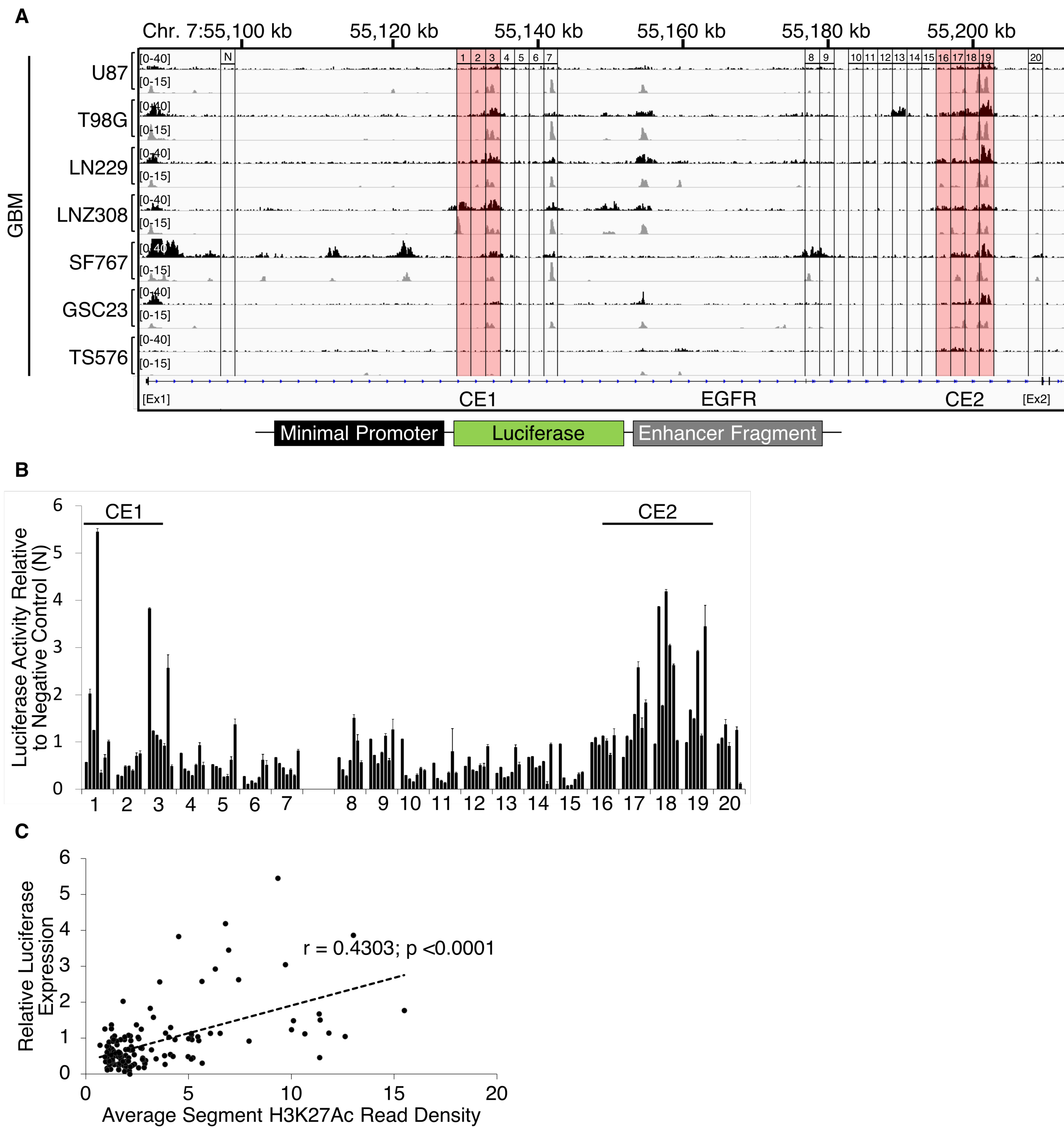


Figure 3.4: Identification of critical constituent enhancers in *EGFR* intron 1 of GBM. **A:** (Top) Schematic of positioning of enhancer segments 1-20 and the negative control region analyzed in the pGL4.24 vector. CE/CE2 (pink outline) are highlighted. (Bottom) Schematic of positioning of the enhancer segments in the pGL4.24 construct. **B:** Luciferase activity in GBM (cell lines left to right: U87, T98G, LN229, LNZ308, SF767, GSC23, TS576) cell lines after transfection with pGL4.24 constructs containing cloned fragments of *EGFR* intron 1. A negative control region 10kb downstream of the *EGFR* promoter was used for normalization. **C:** Relative luciferase expression for P1-20 was plotted against the average H3K27Ac read density for each individual segment. The relationship was analyzed using Spearman's rank order correlation.

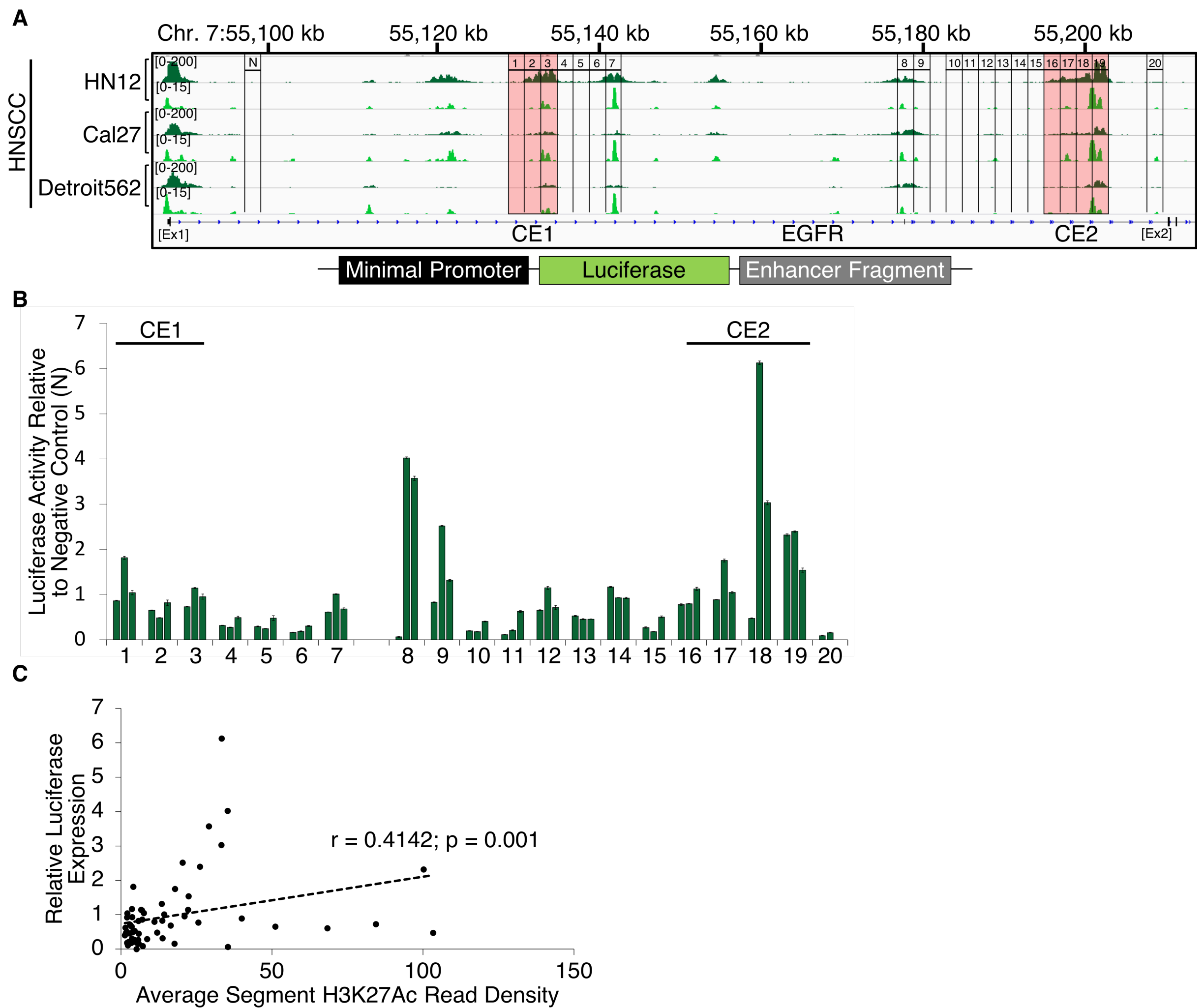


Figure 3.5: Identification of critical constituent enhancers in *EGFR* intron 1 of HNSCC. **A:** (Top) Schematic of positioning of enhancer segments 1-20 and the negative control region analyzed in the pGL4.24 vector. CE/CE2 (pink outline) are highlighted. (Bottom) Schematic of positioning of the enhancer segments in the pGL4.24 construct. **B:** Luciferase activity in HNSCC cell lines (left to right: HN12, Cal27, Detroit562) after transfection with pGL4.24 constructs containing cloned fragments of *EGFR* intron 1. A negative control region 10kb downstream of the *EGFR* promoter was used for normalization. **C:** Relative luciferase expression for P1-20 was plotted against the average H3K27Ac read density for each individual segment. The relationship was analyzed using Spearman's rank order correlation.

Significant Interactions Between CE1, CE2 and the *EGFR* Promoter

As introduced earlier, a critical characteristic of enhancers is their ability to make contact with one or more gene promoters through long-range interactions [268]. To test if the enhancer regions CE1 or CE2 interact with the *EGFR* promoter we performed a simple 3C assay. As opposed to high throughput methods which measure the interactions of a single region with the rest of the genome (circular chromosome conformation capture [4C] [295]) or of all regions of the genome with each other (Hi-C) [296], 3C measures the relative interaction frequency of two specific regions. 3C is based on formaldehyde crosslinking of interacting chromatin segments, followed by restriction digestion and ligation of crosslinked fragments. Ligation products are subsequently analyzed by PCR using primers specific for the restriction fragments of interest. Detection of a ligation product between two segments does not reveal much, instead to identify a chromatin loop between two segments, it needs to be demonstrated that they interact more frequently with each other than with neighboring DNA fragments [297]. To accurately identify 3D interactions between CE1 and CE2 and the *EGFR* promoter, qPCR primers were designed around the EcoRI sites in CE1 (Figure 3.6A, F1 to F5) and CE2 (Figure 3.6A, F6 to F10) and nearby the *EGFR* promoter. Since we hypothesized that an increased interaction frequency would correlate with increased transcript levels, we chose the cell lines with the highest *EGFR* expression levels in both tumor types and compared them against a cell line with no *EGFR* expression. Compared to TS576, SF767 cells exhibited low levels of interaction with CE1 and CE2 (Figure 3.6B); however, significantly stronger interactions were identified at F2 and F4 of CE1 and 4 out of 5 regions of CE2 in the HN12 cell line (Figure 3.6B). The primers which produced the strongest signals of interaction reside in highly acetylated regions in HN12 cells (Figure 3.6A), thus we hypothesized that H3K27Ac enrichment and 3C-interaction were correlated. Correspondingly, we identified a significant correlation between H3K27Ac peak intensity and interaction frequency at these measured regions (Figure 3.6C). Together, the data thus far indicates that CE1 and CE2 have characteristics ascribing them to active enhancers: surrounded by nucleosomes with high H3K27Ac, open chromatin, transcriptional enhancement, and interaction with a promoter.

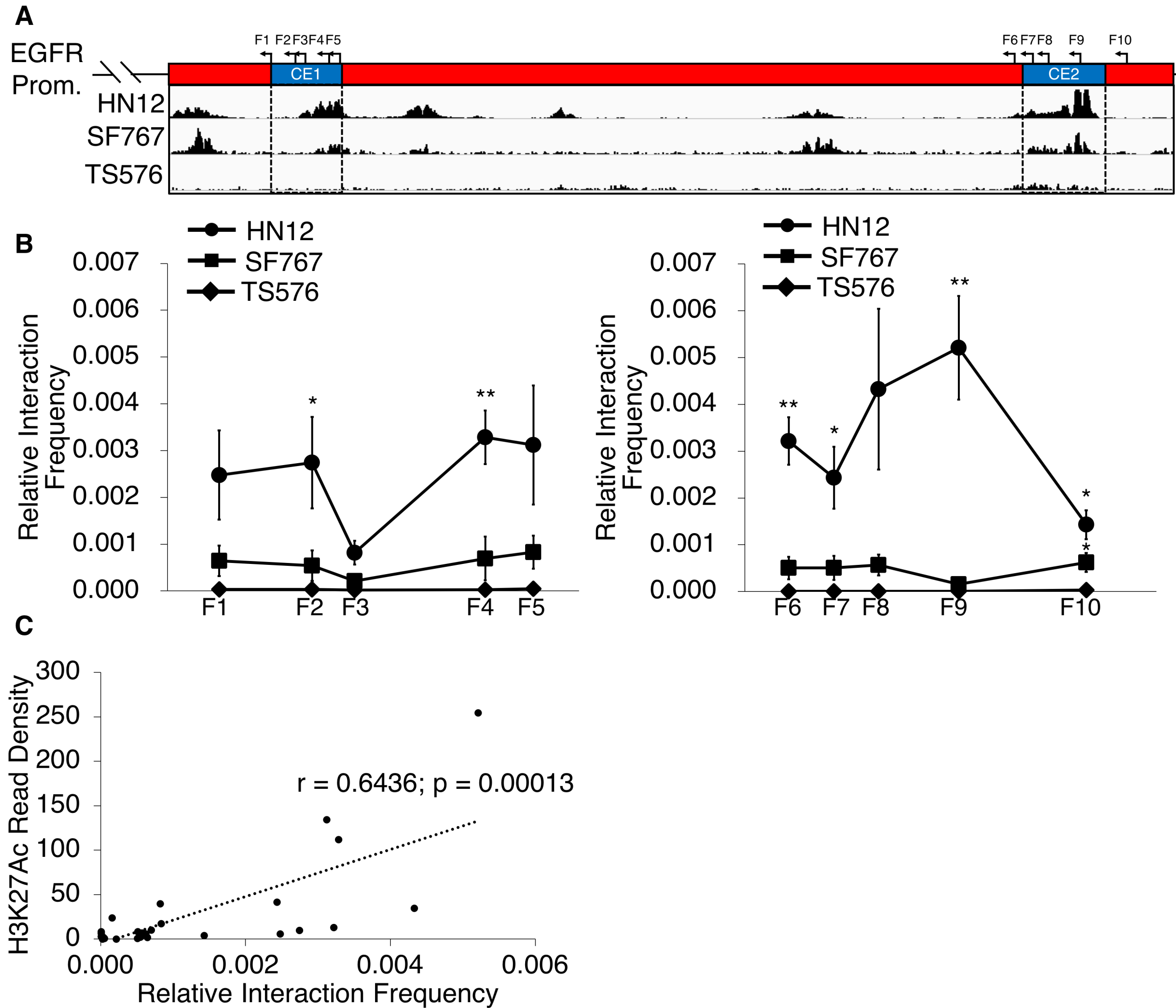


Figure 3.6: Enhancer-promoter interaction by chromatin conformation capture (3C). **A:** Schematic showing position of 3C primers relative to CE1 and CE2. 3C qPCR was done in combination with a forward primer in the *EGFR* promoter region. **B:** Relative interaction frequency of each restriction fragment (F1-10) was calculated as described in the experimental procedures and was plotted against genomic location of the cutting site of each fragment. Values reported were derived from three biological repeats (*$p < 0.05$, Student's t test). **C:** H3K27Ac read density at the primer site was plotted against the relative interaction frequency. The relationship was analyzed using Spearman's rank order correlation.

3.3 Discussion

The rules governing the location of an enhancer with respect to its target promoter has been a topic in which there is little agreement. Some studies indicate that the closer an enhancer is to a promoter, the more likely it is to interact with and enhance transcription of that gene. As such, many studies use the most proximally located gene to putatively identify the interaction partner of an enhancer in genome-wide analyses [183, 298]. Still other studies have shown no preference for proximity [299] and that interactions can occur across TAD boundaries [201], at vast distances [202], and even inter-chromosomally [300]. What is not up for debate, however, is the relationship between transcriptional enhancers and the first intron of a gene. A multitude of studies have identified enhancer elements within the first intron of a gene, and have shown them to be functionally relevant to the activity of the gene in which they are located [301–308]. Additionally, there appears to be a correlation between intron size and *cis*-regulatory DNA with the first intron often being the largest and most predisposed to having regulatory DNA contained within [309–312]. Though a direct relationship between intron size and gene expression could not be confirmed genome wide, 5' UTR length was positively correlated with expression in specific gene families [313], and as many as 35% of genes have introns in their 5' UTR [314]. Whole genome analysis of enhancer elements has further strengthened the relationship between introns and regulatory DNA, showing over 55% of functional enhancer elements in *Drosophila Melanogaster* are located in introns and over 37% in the first intron [179]. Thus, it is not surprising that there is evidence for potent enhancers in the first intron of *EGFR* in the data we have presented here.

The characterization of super enhancers that are critical for the expression of oncogenes or key development genes spawned a new appreciation for the impact of transcriptional enhancers, and rapidly expanded the research into drug development to target this small subset of enhancers. The identification of these SEs is far from standardized. The initial SE study used a combination of OCT4, SOX2, Nanog and Mediator [315] binding to define a highly enriched enhancer population in ESCs, however subsequent studies have shown other combinations of factors to be sufficient. Mediator and BRD4 plus H3K4me1 and H3K27Ac was applied widely to cancer cells

for SE identification [269], however the algorithms written to identify SEs only require input from a single factor (e.g. H3K27Ac) and SEs are still able to be robustly identified from this single factor [316]. This discordance in the field on the necessary factors makes defining a SE difficult and potentially diminishes the relevance of a study if the improper factor is used. Regardless, extensive functional validation is still required to ascribe a true function to a SE.

3.4 Materials and Methods

Cell Culture.

GBM cell lines U87 (RRID:CVCL_0022), T98G (RRID:CVCL_0556), and LN229 (RRID:CVCL_0393) were purchased from ATCC. LNZ308 (RRID:CVCL_0394) was provided by Dr. Erwin Van Meir (Emory University, Atlanta, GA). SF767 (RRID:CVCL_6950) was provided by Dr. Mitch Berger (UCSF Brain Tumor Center, San Francisco, CA). Head and neck cell lines HN12 (RRID:CVCL_5518), Cal27 (RRID:CVCL_1107) and Detroit562 (RRID:CVCL_1171) were provided by Dr. Silvio Gutkind (UCSD Moores Cancer Center, San Diego, CA). Adherent cell lines were maintained in DMEM (Hyclone, #SH30022.01) supplemented with 10% fetal bovine serum (Atlanta Biologicals, #S12450) and 1% penicillin-streptomycin (Gibco, #15140-122) and grown as adherent cultures. GBM neurosphere cell lines GSC23 (RRID:CVCL_DR59, Dr. Fred Lang, University of Texas MD Anderson Cancer Center, Houston, TX) and TS576 (Dr. Cameron Brennan, Memorial Sloan Kettering Cancer Center, New York, NY) were maintained in DMEM/F12 (Gibco, #11320-033) supplemented with B27 supplement (Gibco, #12504-044) and 1% penicillin-streptomycin and grown in suspension. Mycoplasma testing was performed with the PlasmoTest kit (InvivoGen, #rep-pt1) and found to be negative. All experiments are performed within 10 passages of the original frozen stock or post-manipulation.

Luciferase Reporter Assays.

DNA fragments tested in the luciferase reporter assay were cloned from human genomic DNA. PCR products were cloned downstream of firefly luciferase in the pGL4.24 minimal promoter vector (Promega, #E8421) using the SalI (NEB, #R3138S) site. Constructs were sequence

confirmed by Sanger sequencing using the pGL4.24-R primer. pMIEG3-JunDN was a gift from Alexander Dent (RRID: Addgene_40350). pMIEG3-Empty was created by removing the JunDN sequence by EcoRI digestion. For each transfection reaction, 100ng control plasmid expressing Renilla luciferase (Promega, #E2241) and 1µg Firefly luciferase construct were co-transfected with Lipofectamine 2000 (ThermoFisher, #11668030) into 2 x 10^5 cells in a 12-well plate well. After 24 hours, cells were collected in 1X PLB. Luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega, #E1910) on a Tecan Spark 10M with injection control. Transfection efficiency was controlled for by dividing Firefly luminescence by Renilla luminescence, and final activity was normalized to a negative control.

Quantitative real-time PCR.

RNA was extracted with the RNeasy Plus kit (Qiagen, #74134) according to the manufacturers instructions. Reverse transcription of mRNA was performed using 1µg RNA with the iScript Reverse Transcription Supermix (BioRad, #1708841). For real-time PCR analysis, 5µl of cDNA (50ng of starting RNA) was amplified per reaction using the iTaq Universal SYBR Green Supermix (Bio-Rad, #1725124) and the Bio-Rad CFX96 qPCR system. Fold change analysis was performed using the $2^{-\Delta\Delta Ct}$ method and normalized as indicated.

Chromatin Conformation Capture (3C).

The experiment was performed as described [297] with the following modifications. Nuclei were treated with 1000U EcoRI (NEB, #R3101S) at 37°C overnight. 100U T4 enzyme (NEB, #M0202S) was added to digested nuclei and incubated at 16°C for 4 hours. Another 100U T4 enzyme was added and nuclei were incubated with rotation at 4°C overnight. 150ng of ligated DNA was quantified in triplicate by TaqMan real-time PCR using the PrimeTime Gene Expression Supermix (IDT, #1055772). Control 3C template was generated by using two bacterial artificial chromosomes (BAC) encompassing the entire EGFR gene, RP11-159M24 and RP11-148P17, were purchased from the Childrens Hospital Oakland Research Institute (CHORI). Equimolar of the two BACs were digested with EcoRI and ligated. The ligation product from BAC control was used for normalization. The relative interaction frequency was calculated as:

$2^{\text{Ct (BAC)-Ct (3C)}}$.

Chromatin Immunoprecipitation.

Chromatin immunoprecipitation was performed as described previously [317] with the following modifications. Chromatin was sheared in diluted lysis buffer to 200-500bp using a Covaris M220 Focused-Ultrasonicator with the following parameters: 10 minutes, peak incident power 75, duty factor 10%, 200 cycles/burst. Antibodies for ChIP were obtained from commercially available sources: anti-H3K27Ac (Active Motif, #39133). 5% of the chromatin was not exposed to antibody and was used as control (input). For ChIP-seq samples, after DNA purification ChIP-seq DNA libraries were prepared with either the TruSeq ChiP Library Prep Kit (Illumina, #IP-202-1012) or the Accel-NGS 2S Plus DNA Library kit (Swift Bioscience, #21024) and sequenced using 75 bp single-end sequencing on an Illumina Hi-seq 4000.

ChIP-seq analysis.

Raw reads from Illumina Hi-seq 4000 were aligned to the human genome (hg19) using Bowtie2 software [318] with default parameters. Non-uniquely mapped reads were removed with awk command and unique reads were used for secondary analysis. Genome-wide read coverage was calculated by igvtools count and visualized using the Integrative Genomics Viewer (IGV). Peaks were called by first creating TagDirectories of H3K27Ac and Input samples using the makeTagDirectory command in the HOMER (Hypergeometric Optimization of Motif EnRichment) suite of tools [319]. Peaks were identified from the H3K27Ac tag directories using the input directories for normalization with the findPeaks command from HOMER with the following parameters: -style histone -size 250 -minDist 250.

Super enhancer identification.

The ROSE (Rank Ordering of Super-Enhancers) algorithm was downloaded and run from the command line [267, 269]. Bam files of H3K27Ac and Input ChIP-seq reads for each cell line were generated from raw files with bowtie2 and sorted with the samtools command samtools sort. GFF files were created from peak files for each cell line using the awk command. ROSE

was called using the command python ROSE_main.py with the following parameters: -g HG19 -i [GFF_File] -r [H3K27Ac_ChIP.sorted.bam] -o [Output_Folder] -c [Input.sorted.bam] -t 3000.

ATAC-seq.

Approximately 50,000 permeabilized nuclei were transposed using Tn5 transposase (Illumina, #FC-121-1030) as described previously [205]. Libraries were amplified using NEBNext High-Fidelity 2X PCR Master Mix (NEB, #M0541) with primer extension at 72°C for 5 min, denaturation at 98°C for 30s, followed by 8 cycles of denaturation at 98°C for 10s, annealing at 63°C for 30s and extension at 70°C for 60s. Each library was size selected and sequenced on an Illumina NextSeq500 or HiSeq4000 to a depth of $\geq$ 20 million usable reads pairs. Sequencing runs that did not meet the read pair threshold were sequenced again, and all replicates were pooled for analysis.

ATAC-seq analysis.

Nextera adapters were trimmed from the raw fastq files by using cutadapt [320] with parameters -m 5 -e 0.10 -a CTGTCTCTTATA -A CTGTCTCTTATA and then aligned to human reference genome hg38 using bowtie2 with parameters -X2000 –mm –local. Next, the improperly mapped, poorly mapped and unmatched reads were filtered from the resultant raw bam files using samtools view with parameters -F 1804 -f 2 -q30. Duplicates were marked with Picard with the command picard MarkDuplicate and removed by samtools view. Final bam files were generated after removing mitochondrial reads by awk command. Replicate Bam files were merged using samtools merge and converted to tagAlign format using the bedtools command bamtobed with parameters -bedpe -mate1 -I and awk. To account for the cutting offset of Tn5 transposase, mapping position was shifted using awk. Peaks were called from tagAlign files using the Model-based Analysis of ChIP-Seq (MACS2) [321] callpeak command with parameters -g hs -p 0.01 –nomodel –shift -75 –extsize 150 -B –SPMR –keep-dup all –call-summits. Bedgraph files output from MACS2 were converted to BigWig files using bedGraphToBigWig and visualized using IGV.

Data Access.

All raw and processed sequencing data generated in this study have been submitted to the NCBI Gene Expression Omnibus (GEO) under accession number GSE128275. Accession numbers for publicly available data accessed are as follows: ChIP-seq (GSE119755), RNA-seq (GSE119834).

3.5 Acknowledgements

Chapter 3 in part, has been submitted for publication of the material as it may appear in Molecular Cancer Research; **Jameson, N.M.**, Ma, J., Benitez, J., Izurieta, A., Han, J.Y., Mendez, R., Parisian, A., Furnari, F., AACR Publications, 2019. The dissertation author was the primary investigator and author of this paper.

Chapter 4

Genetic perturbation of *EGFR* intron 1 enhancers

4.1 Introduction

As has been thoroughly established previously, it is essential that putative transcriptional enhancers be extensively validated to determine their true function in a particular cell type. Many of the most powerful and versatile techniques utilize the relatively new discovery of the Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) mechanism and the CRISPR-associated (Cas) proteins. The mechanism by which CRISPR/Cas works was first described in the prokaryotic *Streptococcus thermophilus*. This study showed experimentally that CRISPR systems are prokaryotic adaptive immune responses which work by capturing small DNA sequences from invading viruses and bacteriophages, and use them to create DNA segments known as CRISPR arrays [322]. The CRISPR array serves as a memory bank within the bacteria, allowing it to produce RNA segments from the array which recognize and target invading virus DNA through double strand breaks (DSB) carried out by the nuclease ability of a Cas protein [323, 324]. While interesting in the context of bacterial immunity, it was the adaptation of CRISPR/Cas to eukaryotic cells which provided the platform for the CRISPR revolution in biomedical research. Two studies published at nearly the same time discovered the ability to

guide the *Streptococcus pyogenes* Cas9 (spCas9) protein to a genomic region using a CRISPR guide RNA (gRNA) [325], which recognizes a protospacer-adjacent motif (PAM) and cuts specifically at that PAM sequence [326, 327]. Targeting this system to a genomic region facilitated a double strand break at single nucleotide resolution, and resulted in DNA repair through either non-homologous end joining (NHEJ) or homology directed repair (HDR) [327].

CRISPR-mediated HDR or NHEJ.

The utilization of either of these repair pathways has advantages depending on the purpose of the experiment. In most eukaryotic cells, the NHEJ pathway generates insertions and deletions during DSB repair. However, in the presence of a DNA template with homology to the sequences flanking the DSB location, HDR can seal the DSB in an error-free manner [328]. The efficiency of HDR is determined by the concentration of donor DNA present at the time of repair, the length of the homology arms of the donor DNA, the cell cycle, and the activity of the endogenous repair systems [329]. HDR methods allow for very precise mutations, and shows incredible promise for introducing single base substitutions to correct disease phenotypes [330,331]. Though CRISPR/Cas9 editing is relatively easy and incredibly powerful, significant concerns have been raised about its use in a clinical setting due to the evidence of off-target effects [332, 333]. The ethics of using CRISPR in a human setting is under hot debate, and its misuse cannot be better represented than recent reports of so-called CRISPR-babies in China. Though based on science supporting the mutation of *CCR5* can promote resistance to human immunodeficiency virus (HIV) infection [334], editing of this gene in human children resulted in considerable backlash from the scientific community as being premature and irresponsible [335, 336]. Though not as precise, NHEJ-mediated repair is particularly useful when a precise mutation is not necessary as it is an error-prone repair mechanism that often leads to insertions or deletions (indel). These indels can cause frameshift mutations, premature stop codons, and/or nonsense-mediated decay (NMD) to the target gene, which results in loss-of-function. Additionally, NHEJ allows for multiple gRNAs to be introduced at the same time to make genomic deletions [337]. This process however is relatively inefficient, and there is an inverse relationship between deletion frequency and deletion size [338].

CRISPR/Cas9 for gene regulation.

In addition to its nuclease activity, Cas9 can serve as a unique platform to recruit protein and RNA factors to a targeted DNA site, and it has been engineered into powerful tools for sequence-specific gene regulation. To achieve this, transcriptional activators and repressors are fused to a catalytically dead Cas9 (dCas9) [339]. dCas9 maintains its ability to bind both the gRNA and targeted DNA, but it lacks nuclease activity and can thus serve as a sequence-specific RNA-guided DNA-binding platform. In bacterial cells, but less so in mammalian cells, dCas9 alone can efficiently inhibit the transcription of targeted genes through steric hindrance of transcriptional machinery in a process known as CRISPR interference (CRISPRi) [339]. Earlier studies using other DNA targeting proteins fused to transcription effector domains [340] showed proof-of-principle that targeting these domains could affect transcription either positively or negatively depending on the domain. Due to the ease of fusion of dCas9 to these effector domains, it was a natural progression to apply this technology to CRISPRi and CRISPR activation (CRISPRa). The first study to describe CRISPRi and CRISPRa utilized dCas9 fused to the Krüppel-associated box (KRAB) domain of Kox1 (dCas9-KRAB) to mediate gene repression, and dCas9 fused to four copies of the transcriptional activator VP16 (dCas9-VP64) to mediate gene activation [341]. Highly specific and stable activation or repression of the target gene was observed, and a linear relationship between the level of expression of the gRNA and the level of gene activation/repression was discovered [341]. Genome wide application of these techniques have also allowed for comprehensive mapping of complex pathways [342]. Taken together, the CRISPR toolkit represents a powerful methodology for performing functional genomics.

4.2 Results

Deletion of CE1 and CE2 by CRISPR/Cas9 Results in Reduced *EGFR* Expression

As introduced in Chapter 2, the functional testing of enhancers is critical for determining their phenotypic effects in a cell type of interest. Many groups have chosen to use the CRISPR/Cas9 system to carry out these functional testing assays. Examples of this strategy are abundant in both mouse and human models. The simultaneous use of two gRNAs to delete

defined genomic regions has been used, for example, to measure the regulatory contribution of enhancers near oncogenes [269, 315, 316], at the Sox2 locus in ESCs [343, 344], at the Myc locus [345], at the HER2 locus [346], at the MGMT locus [347] and at the androgen receptor locus [348]. To directly assess if CE1 and CE2 were essential for *EGFR* expression, we used the CRISPR/Cas9 system to delete CE1 (hg38, chr7: 55,060,994-55,066,815) and CE2 (hg38, chr7: 55,127,646-55,135,347). To minimize clonal effects, we selected at minimum 2 homozygous clones for each deletion and mixed them at equal numbers (Figure 4.1).

To test how enhancer loss effects cells with different *EGFR* expression levels we made these deletions in two GBM cell lines and two HNSCC cell lines which had either high (SF767, HN12) or low (U87, Cal27) relative *EGFR* expression. Single enhancer deletions were generated with a dual-guide deletion strategy (Figure 4.1) and the presence of editing at none, one, or both alleles was confirmed by genotyping PCR using a combination of primers within and outside the deleted region (Figure 4.1, Figure 4.2A). Compared to parental cell lines, *EGFR* transcript was significantly decreased in each deletion (Figure 4.2B). Interestingly, deletions of either CE1 or CE2 had different effects depending on the cell line. Though each cell line exhibited the strongest enrichment for H3K27Ac in CE2 (Figure 3.1A), SF767 and Cal27 showed significantly more repression of *EGFR* transcript with the loss of CE1. These cell-type specific differences in transcript levels between CE1 and CE2 indicate there may be differential utilization of either CE1 or CE2 in different cell lines.

Figure 4.1: CRISPR/Cas9-mediated deletion of CE1 and CE2. Schematic outlining the deletion strategy. Guides were designed flanking the intended deletion regions. PCR primers were designed inside and outside the deletion region to test for the presence of editing at neither, one, or both alleles.

Previous studies which deleted individual constituent enhancers within SEs revealed that enhancer activity is mostly dependent on a few constituents that activate transcription [270, 273, 316, 349]. Additionally, partial redundant control of a gene by multiple CEs within a single SE has been observed [349]. To evaluate if there was partially redundant control of *EGFR* transcription by either CE1 or CE2, we performed a second round of CRISPR/Cas9 editing on the homozygous edited populations. These deletions utilized the same set of gRNAs used for single deletion, and again a minimum of 2 homozygous clones for each deletion were isolated and mixed at equal numbers (Figure 4.3A). Compared to parental cell lines, *EGFR* transcript levels were most significantly decreased with loss of both enhancers (Figure 4.3B). Notably, the amplitude of *EGFR* transcript loss was greater in CE1$^{-/-}$ + CE2$^{-/-}$ when compared against CE1$^{-/-}$ or CE2$^{-/-}$ alone. Together, these results demonstrate both cell type-specific CE utilization as well as a cooperative relationship between the CE1 and CE2 whereby double enhancer deletion results in more significant deleterious effects than single deletions alone.

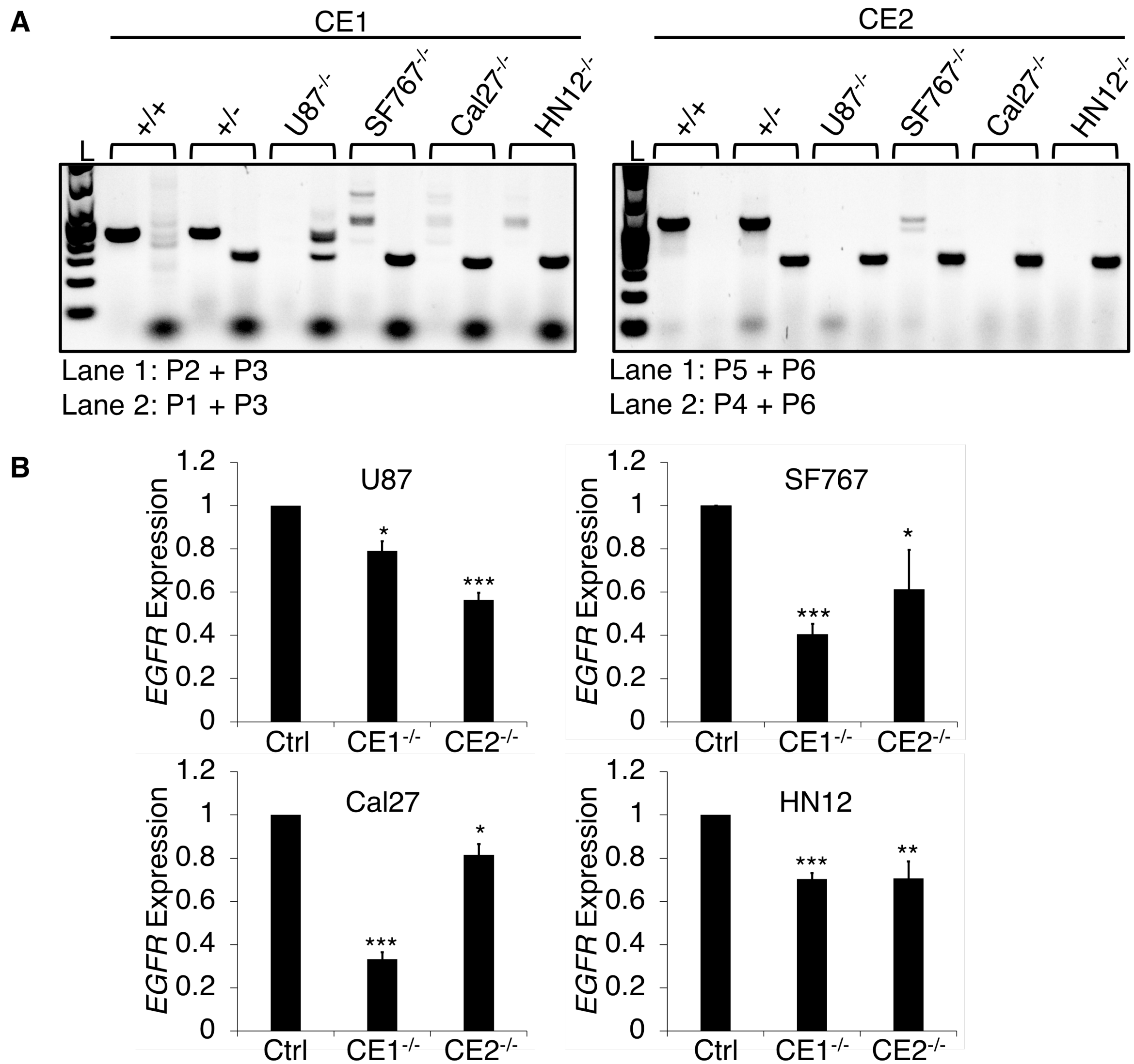


Figure 4.2: CRISPR/Cas9-mediated deletion of CE1 and CE2 in GBM and HNSCC cell lines reduces *EGFR* expression. **A:** Genotyping PCR for (left) CE1 and (right) CE2. Homozygous parental (lanes 1, 2) and heterozygous deleted (lanes 3, 4) are shown as PCR controls. Homozygous enhancer deletion (lanes 5-12) is shown for clone mixtures. **B:** *EGFR* expression in deleted cell lines were analyzed by RT-qPCR. *EGFR* transcript level was first normalized to *GAPDH* and subsequently calculated as fold change relative to parental. **B:** (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, $n = 3$ independent experiments, Students t test)

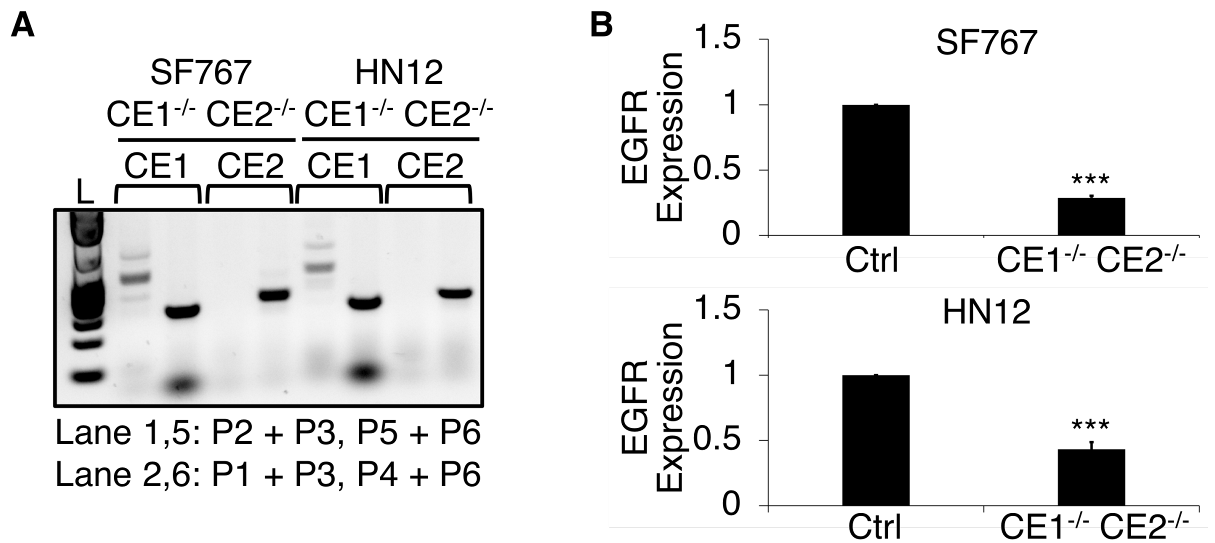


Figure 4.3: CRISPR/Cas9-mediated double deletion of CE1 and CE2 in GBM and HNSCC cell lines further reduces *EGFR* expression. **A:** Genotyping PCR for CE1 and CE2 deletion in HN12 and SF767 cells. Homozygous enhancer deletions are shown for clone mixtures. **B:** CE2 deletions were layered on top of CE1$^{-/-}$ cells in SF767 and HN12 cells. A minimum of 2 double-deleted homozygous clones were combined for downstream analysis. *EGFR* expression in double-deleted cell lines was analyzed by RT-qPCR. *EGFR* transcript level was first normalized to *GAPDH* and subsequently calculated as fold change relative to parental (*p < 0.05, **p < 0.005, ***p $<$ 0.0005, n = 3 independent experiments, Students *t* test).

Repression of H3K27Ac by dCas9-KRAB Decreases *EGFR* Expression

Recently, a mechanism known as enhancer hijacking has demonstrated that structural variation can significantly alter the enhancer-promoter contacts within a cell. For example, duplication, inversion or deletion of genomic regions containing oncogenes or regulatory elements in group 3 and 4 medulloblastoma rearranges the local chromatin structure such that oncogenes and enhancers that were once located distally are now located proximally to each other [350]. This phenomenon is further enforced by the identification of *de novo* 3D contact domains within the IGF2 gene following recurrent tandem duplications intersecting with a TAD boundary, mediating the formation of a novel interaction with a lineage-specific SE and facilitating high level gene activation [351]. To eliminate the possibility of structural variation being the root cause of *EGFR* expression loss in CRISPR/Cas9 deleted clones, we aimed to perform a CRISPRi experiment which would repress enhancer activity without genomic deletion. We hypothesized that histone de-acetylation would be sufficient for *EGFR* transcriptional repression due to the relationship between H3K27Ac and enhancer activity as demonstrated in Figure 3.1, Figure 3.4 and Figure 3.5. dCas9-KRAB is known to recruit endogenous chromatin modifying complexes to de-acetylate histones [352], therefore to test our hypothesis we targeted dCas9-KRAB to four regions within CE1 and five regions within CE2 with specific gRNAs in HN12 (Figure 4.4A) and SF767 (Figure 4.5A) cell lines. Additionally, we targeted a non-acetylated region within *EGFR* intron 1 as a negative control (Figure 4.4A, Figure 4.5A).

We first transduced each cell line with a lentivirus engineered to integrate dCas9-KRAB and a blasticidin selection gene [353]. Following selection, HN12 cells expressed high levels of dCas9 (Figure 4.4B), while SF767 cells expressed relatively less protein (Figure 4.5B). Because of the linear relationship between gRNA expression and target repression [341], we chose to utilize a lentivirus engineered to integrate a puromycin resistance gene as well as each specific gRNA driven by a U6 promoter [354]. Cell lines were selected for gRNA expressing cells with puromycin, creating cell lines with stable repression of the target region. To ensure successful targeting of dCas9-KRAB we confirmed de-acetylation at each region. Compared to an enhancer off-target (O-T) gRNA, the targeting of dCas9-KRAB resulted in significant decreases in H3K27Ac in

HN12 cells (Figure 4.4C). Interestingly, in HN12 cells compared to the O-T control, CE1.4 and CE2.4 gRNA targeted regions also had significantly decreased enrichment of H3K27Ac at the opposing targeted region (e.g. H3K27Ac loss at CE1.4 when 2.4 was targeted) (Figure 4.4C). This phenotype was not present in SF767 cells targeted with dCas9-KRAB. This cross-repression is likely not due to off-target effects or due to the large size of dCas9-KRAB protein complex [352], but may in fact be due to the cooperation between CE1 and CE2 at the *EGFR* promoter in the 3D chromosome, a hypothesis supported by the ACH hypothesis introduced in Chapter 3 [195]. To assess functional impact of de-acetylation at these regions, we measured *EGFR* transcript levels in each engineered cell line and observed significant decreases in transcript in 73% (8/11) of targeted regions in HN12 cells (Figure 4.4D). Significant repression of *EGFR* transcript levels was observed in 45% (5/11) of targeted regions in SF767 (Figure 4.5D), though less significantly, likely due to the decreased expression of dCas9-KRAB and subsequent reduction in repressive activity. Interestingly, in HN12 cells, targeting CE2 had an overall stronger repressive effect on *EGFR* transcript levels, with repression by gRNA targeting CE2.4 achieving a greater than 3-fold decrease in expression. In SF767 cells there was no observable preference for CE2. We performed double dCas9-KRAB repression by adding a second gRNA (CE1.4 + CE2.4) by a second round of lentiviral transduction and observed the most significant decrease in *EGFR* transcript levels, achieving an 8-fold decrease in expression compared to the off-target gRNA (Figure 4.4D). Similarly, in SF767 cells the strongest repression was observed by combining CE1.4 and CE2.4 (Figure 4.5D).

Finally, the effect of dCas9-KRAB-mediated *EGFR* repression on cell proliferation was assessed in HN12 cells by ATPlite assay. To enhance the effect of *EGFR* repression and demonstrate *EGFR* dependence, we chose to perform this assay at low serum. Serum is known to contain many growth factors which can activate other EGFR-parallel growth pathways and mask the effects of *EGFR* depletion. At low (0.5%) serum, the relative proliferation of all on-target *EGFR*-repressed cells was significantly inhibited (Figure 4.4E). Importantly, cell lines with stronger repression of *EGFR* exhibited the most significant negative effect on proliferation over time. These results indicate that *EGFR* transcriptional changes in enhancer deleted regions (Figure 4.2, Figure 4.3) are

not due solely to structural alteration within the first intron. Moreover, these results demonstrate that loss of H3K27Ac at the identified *EGFR* enhancers is sufficient for significant decreases in *EGFR* transcript levels.

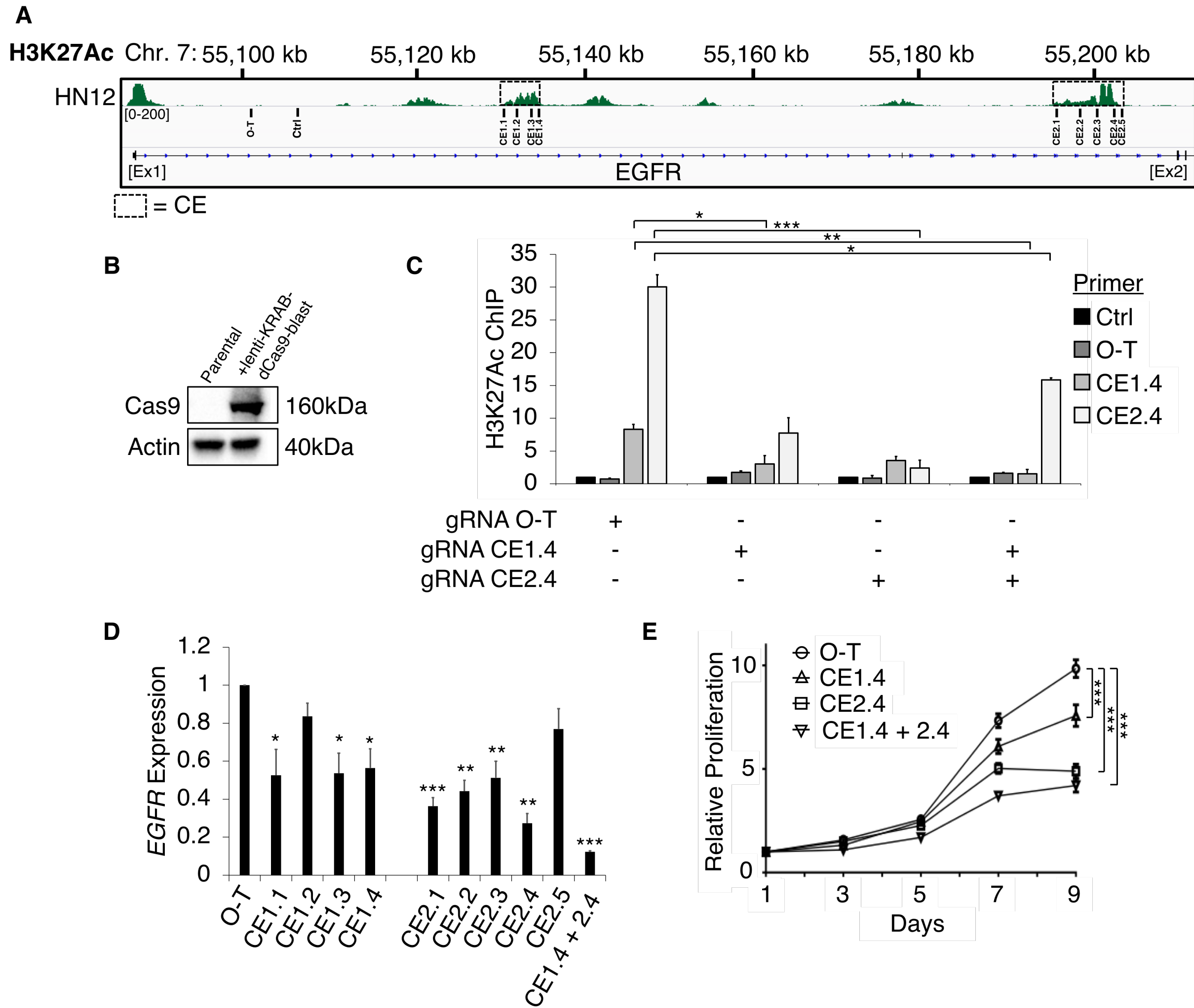


Figure 4.4: Targeting dCas9-KRAB to the CEs decreases *EGFR* gene transcription and reduces proliferation in HN12 cells. **A:** H3K27Ac IGV track of HN12 cells showing the position of gRNAs targeting the *EGFR* intron 1 enhancers and off-target (O-T) control. **B:** Western blot of dCas9-KRAB expression in HN12 cells after transduction with lenti-dCas9-KRAB-blast. β-Actin was used as a loading control. **C:** H3K27Ac enrichment at the targeted enhancer regions before and after dCas9-KRAB targeting was analyzed by ChIP-qPCR. Primers were designed around the targeted regions as well as a PCR negative control (Ctrl) from a H3K27Ac negative region of *EGFR* intron 1. A primer within a gene desert in chromosome 12 was used for normalization. **D:** *EGFR* expression in dCas9-KRAB expressing cell lines was analyzed by RT-qPCR. *EGFR* transcript level was first normalized to *GAPDH* and subsequently calculated as fold change relative to off-target control. **E:** Cell proliferation curves were generated by measuring ATP levels every two days over 9 days. Significance is measured relative to O-T. **C-E:** (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, $n = 3$ independent experiments, Students *t* test)

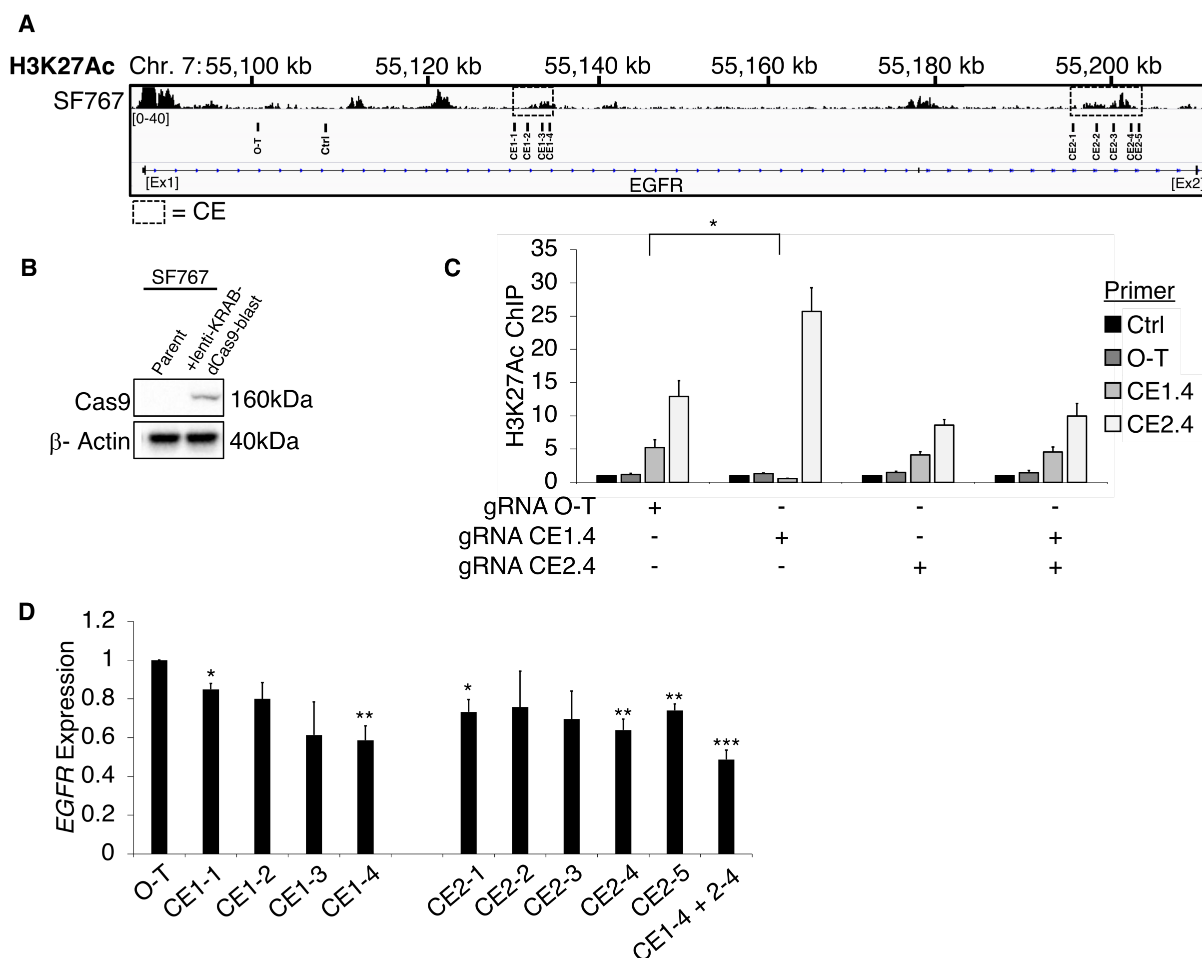


Figure 4.5: Targeting dCas9-KRAB to the CEs decreases *EGFR* gene transcription SF767 cells. **A:** H3K27Ac IGV track of HN12 cells showing the position of gRNAs targeting the *EGFR* intron 1 enhancers and off-target (O-T) control. **B:** Western blot of dCas9-KRAB expression in SF767 cells after transduction with lenti-dCas9-KRAB-blast. β-Actin was used as a loading control. **C:** H3K27Ac enrichment at the targeted enhancer regions before and after dCas9-KRAB targeting was analyzed by ChIP-qPCR. Primers were designed around the targeted regions as well as a PCR negative control (Ctrl) from a H3K27Ac negative region of *EGFR* intron 1. A primer within a gene desert in chromosome 12 was used for normalization. **D:** *EGFR* expression in dCas9-KRAB expressing cell lines was analyzed by RT-qPCR. *EGFR* transcript level was first normalized to *GAPDH* and subsequently calculated as fold change relative to off-target control. **C-D:** (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, $n = 3$ independent experiments, Students t test)

4.3 Summary

The discovery of CRISPR/Cas9 as a tool for genetic editing and functional genomics has revolutionized many fields of biomedical research, and the field of epigenetics has certainly benefitted from the tools which have been developed. In particular, the ability to functionally validate the activity of an enhancer has been expanded far beyond the simple reporter assay. Making a deletion of a putative enhancer, even one which has been validated by a reporter assay, allows for the native functional effect of that enhancer to be determined. Reporter assays suffer from many caveats including artificially high copy number, non-native cell contexts, and a lack of chromatin and DNA which surrounds the enhancer in the chromosome. CRISPR/Cas9 allows us to bypass many of these caveats. Through direct deletion, we can see how a regulatory element was influencing gene expression in the cell type in which it was deleted. Dose dependency of an enhancer cab be measured through incomplete (heterozygous) or complete (homozygous) editing. Enhancer-promoter contacts and how they are disrupted (or formed) can additionally be measured.

Although deletion holds a significant amount of power, the question remains whether structural variation affects local and distal gene expression control. Recent studies have identified methods such as enhancer hijacking which indicate that altering genomic regions through structural variation can have significant effects on the local chromatin structure, leading to aberrant gene expression [350, 351]. Additionally, these deletions are irreversible so further perturbation of other pathways can only inform you as to their effects in that particular context. For these reasons, the discovery of a mutation in Cas9 which destroys its nuclease function has been critical for creating other less permanent methods for targeted gene expression alterations [339]. dCas9-KRAB in particular has been widely utilized for repressing transcription as it has the ability to recruit repressive chromatin complexes, including histone deacetylase (HDAC) and histone methyltransferase (HMT) proteins [352].

In this study we performed both of these analyses on the *EGFR* gene in glioma and head and neck cancer cell lines which either express or do not express the gene. Single enhancer deletions through a dual gRNA strategy resulted in significant decreases in *EGFR* expression

in every case. Deletions of either CE1 or CE2 had different effects depending on the cell line. Though each cell line exhibited the strongest enrichment for H3K27Ac in CE2 (Figure 3.1A), SF767 and Cal27 showed significantly more repression of *EGFR* transcript with the loss of CE1. These cell-type specific differences in transcript levels between CE1 and CE2 indicate there may be differential utilization of either CE1 or CE2 in different cell lines. This suggests there may be a more complicated mechanism which determines which CE is used in each cell line, and may be due to differential expression of enhancer-specific transcription factors in those cell lines. Because partial redundant control of a gene by multiple CEs within a single SE has been observed [349], double enhancer deletion in these cells was tested. *EGFR* transcript levels were most significantly decreased with loss of both enhancers (Figure 4.3B) and the amplitude of loss was greater in $CE1^{-/-}$ + $CE2^{-/-}$ when compared against $CE1^{-/-}$ or $CE2^{-/-}$ alone. These data confirm the previous observations that enhancers can compensate for each other when necessary, and suggests that complete loss of enhancer-associated *EGFR* transcription would require targeting of at least these two, and potentially additional, enhancers.

To eliminate the possibility of structural variation being the root cause of *EGFR* transcriptional changes in response to CRISPR/Cas9 editing, we performed a dCas9-KRAB repressive experiment. Targeted repression, as confirmed by loss of H3K27Ac levels at the intended regions, was successful in significantly reducing *EGFR* expression in two EGFR-high cell lines. Interestingly, some important differences were observed between CRISPR/Cas9 deletion and dCas9-KRAB repression. First, in contrast to deletion in HN12 cells in which loss of either CE had equally deleterious effects (Figure 4.2B), there appeared to be a significant preference for repression of CE2 to downregulate *EGFR* transcript levels (Figure 4.4D). Our hypothesis is that the complete loss of CE2 in the deleted cells allowed for a restructuring of the 3D organization of the *EGFR* gene, leading to more efficient compensation of expression by CE1. In contrast, dCas9-KRAB repression of CE2 was able to silence this compensatory effect by DNA bending, bringing the repressive KRAB in close contact with CE1 and the *EGFR* promoter. CE1 deletion in (Figure 4.2B) perhaps indicates the true repressive effect of CE1 loss, as CE1 repression by dCas9-KRAB produces similar fold changes in *EGFR* transcript (Figure 4.4D). Although these

phenotypes are not maintained in SF767 cells (Figure 4.5), this may be due to poor expression of dCas9-KRAB protein (Figure 4.5B) or gRNA through inefficient transduction by lentivirus in these cells. However, as with our deletion approach, repression of both CE1 and CE2 in combination produced strong inhibition of *EGFR* expression (Figure 4.4D, Figure 4.5D), further confirming their cooperativity and strengthening the hypothesis that complete enhancer blockade would be critical for full *EGFR* repression.

4.4 Materials and Methods

Cell Culture.

GBM cell line SF767 (RRID:CVCL_6950) was provided by Dr. Mitch Berger (UCSF Brain Tumor Center, San Francisco, CA). Head and neck cell line HN12 (RRID:CVCL_5518) was provided by Dr. Silvio Gutkind (UCSD Moores Cancer Center, San Diego, CA). Cells were maintained in DMEM (Hyclone, #SH30022.01) supplemented with 10% fetal bovine serum (Atlanta Biologicals, #S12450) and 1% penicillin-streptomycin (Gibco, #15140-122) and grown as adherent cultures. Mycoplasma testing was performed with the PlasmoTest kit (InvivoGen, #rep-pt1) and found to be negative. All experiments are performed within 10 passages of the original frozen stock or post-manipulation.

Guide RNA design.

Guide RNAs were designed using the MIT CRISPR Design website (http://crispr.mit.edu). To minimize potential off-target effects of guides, only high-score guide RNAs (score >80) were used. Guide RNAs were annealed and diluted 1:200 in ddH2O and used for downstream applications.

CRISPR/Cas9-mediated Genomic Deletion.

Guide RNAs were cloned into pX330-BFP (from Dr. Tim Fenton) for upstream guides or pX458-GFP (Addgene, Plasmid #48138) for downstream guides. For pX330-BFP constructs, annealed gRNA duplexes were first cloned into pX335-U6-Chimeric_BB-CBh-hSpCas9n (D10A) (Addgene, #42335). pX335 was digested at 37°C for 1 hour with BbsI (NEB, #R0539S). Lin-

earized plasmid was run on a 1% agarose gel, excised and purified with the QIAquick Gel Extraction Kit (Qiagen, #28704). Annealed gRNA duplexes were ligated into the linearized plasmid with the DNA Ligation Kit Version 2.1 (TaKara, #6022). Ligation products were transformed into XL1-Blue *E. Coli* and single clones were purified with the QIAprep Spin Miniprep Kit (Qiagen, #27104) and sent for sequencing (IDT) with the hU6-F sequencing primer. Sequencing-confirmed PX335-gRNA and PX330-BFP-empty were double digested with KpnI-HF (NEB, #R3142S) and FspI (NEB, #R0135S) and run on a 1% agarose gel. The 1kb digested piece from pX335-gRNA was extracted and purified with the QIAquick Gel Extraction Kit. The larger digested construct from PX330-empty was extracted and purified as well. The DNA fragment containing the gRNA and the PX330-empty digested construct were ligated. Ligated constructs were transformed into XL1-Blue and single clones were mini-prepped. Presence of guides in the PX330-BFP constructs was confirmed by Sanger sequencing (IDT). Constructs were maxi prepped and co-transfected with Lipofectamine 3000 (ThermoFisher, #L3000015) into 4.5 x 10^5 of the indicated cell line in a 6-well plate well. After 24 hours cells were collected and the top 1% of BFP+/GFP+ cells were sorted using the SH800S Cell Sorter (Sony Biotechnology). Single cells were plated in 96 well plates and grown for 2-3 weeks. Single clones were screened using PCR with primers described in Figure 4.2. A minimum of 2 homozygous clones were mixed at equal ratios and used for downstream analysis.

Enhancer Silencing by CRISPR/dCas9-KRAB.

SF767 and HN12 cells were transduced with maxi-prepped Lenti-dCas9-KRAB-blast (from Dr. Paul Mischel) and selected with 10µg/ml blasticidin for 72 hours post transduction. Lentivirus was generated by transfecting 293T cells with the plasmid together with the packaging plasmids pVSVG and pΔ8.9 using Lipofectamine 3000. Supernatant containing virus was harvested 24 and 48 hours after transfection, filtered through a 40µm filter, and used to transduce the indicated cell lines. Viral transductions were performed in the presence of 4mg/ml polybrene. Medium was changed 24 hours after transduction and replaced with medium containing 10µg/ml blasticidin for 3 days. Guide RNAs were cloned into the lentiGuide-Puro vector (Addgene, Plasmid #52963). LentiGuide-Puro was first digested with BsmBI (NEB, #R0580S) and run on

a 1% agarose gel. The upper, larger band was excised and purified using the QIAquick Gel Extraction Kit. Purified products were added to a ligation reaction with the following components: 1µl BsmBI digested plasmid (50ng), 1µl diluted oligo duplex, 5µl 2X Quick Ligase Buffer (NEB), 3µl ddH2O, 1µl Quick Ligase (NEB, #M2200S) and incubated at room temperature for 10 minutes. 1uµl ligation product was transformed into Stbl3 bacteria. Individual colonies were mini-prepped and confirmed for gRNA sequence by Sanger sequencing (IDT) using the hU6-F sequencing primer. Confirmed constructs were maxi-prepped and made into virus as described above. Individual gRNA-expressing viruses were transduced into cells expressing dCas9-KRAB. Medium was changed 24 hours after transduction and replaced with medium containing 1µg/ml puromycin for 3 days. After assessing *EGFR* transcript levels by RT-qPCR, one highly effective CE1 guide was selected for double gRNA expression. One stable line expressing the highly effective CE1 guide was transduced a second time with the complementary CE2 guide. Enhancer activity was assessed by H3K27Ac ChIP-qPCR.

Western Blotting.

Protein samples were collected in SDS sample buffer, separated using gel electrophoresis and transferred via wet transfer onto a PVDF membrane. The membrane was blocked with 5% milk in TBST and probed with primary antibodies at 1:1000 dilution overnight at 4°C and secondary horeseradish peroxidase (HRP) antibodies at 1:2000 for 1 hour at room temperature. Signal was assessed via chemiluminescence with the SuperSignal West Pico PLUS substrate (ThermoFisher, #34580) and visualized on a ChemiDoc MP system (Bio-Rad). anti-β-actin (Sigma, #A3854) and Anti-Cas9 (Cell Signal, #14697) antibodies were used for analysis.

Quantitative real-time PCR.

RNA was extracted with the RNeasy Plus kit (Qiagen, #74134) according to the manufacturers instructions. Reverse transcription of mRNA was performed using 1µg RNA with the iScript Reverse Transcription Supermix (BioRad, #1708841). For real-time PCR analysis, 5µl of cDNA (50ng of starting RNA) was amplified per reaction using the iTaq Universal SYBR Green Supermix (Bio-Rad, #1725124) and the Bio-Rad CFX96 qPCR system. Fold change

analysis was performed using the $2^{-\Delta\Delta Ct}$ method and normalized as indicated.

Chromatin Immunoprecipitation.

Chromatin immunoprecipitation was performed as described previously [317] with the following modifications. Chromatin was sheared in diluted lysis buffer to 200-500bp using a Covaris M220 Focused-Ultrasonicator with the following parameters: 10 minutes, peak incident power 75, duty factor 10%, 200 cycles/burst. Antibodies for ChIP were obtained from commercially available sources: anti-H3K27Ac (Active Motif, #39133). 5% of the chromatin was not exposed to antibody and was used as control (input). For ChIP-qPCR analysis DNA quantity for each ChIP sample was normalized against input DNA.

Cell growth analysis.

$5x10^2$ HN12 cells expressing the indicated dCAs9-KRAB gRNA were seeded in black, clear bottom 96 well plate in 6 replicate wells in complete media. After 24 hours, complete media was removed and 100µl of 10µg/ml blasticidin and 1µg/ml puromycin in DMEM + 0.5% were added to each well. Baseline luminescence was measured at day 1 with the ATPlite 1step Luminescence Assay System (PerkinElmer, #6016731) on a Tecan Spark 10M. Luminescence measurements were obtained at every other day for 9 days and plotted using GraphPad Prism.

Data Access.

All raw and processed sequencing data generated in this study have been submitted to NCBI GEO under accession number GSE128275.

4.5 Acknowledgements

Chapter 4 in part, has been submitted for publication of the material as it may appear in Molecular Cancer Research; **Jameson, N.M.**, Ma, J., Benitez, J., Izurieta, A., Han, J.Y., Mendez, R., Parisian, A., Furnari, F., AACR Publications, 2019. The dissertation author was the primary investigator and author of this paper.

Chapter 5

Identification and characterization of transcription factors critical for intron 1-mediated *EGFR* expression

5.1 Introduction

Sequence-specific transcription factors (TF) interpret the signals encoded within regulatory DNA. Many TFs function as master regulators of specific cell types, including stem cells [355] or muscle cells [356], or signaling pathways like immune response [357]. Their power can be directly demonstrated through the ability to force terminally differentiated fibroblasts to a stem-cell state through only the expression of a small set of stem-cell-specific master TFs [358]. The initial discovery of human-specific TFs was aided by the discovery of Deoxyribonuclease I (DNase I) footprinting [359], a process which takes advantage of the remodeling of nucleosomes after the binding of TFs to regulatory DNA regions. This remodeling exposes DNA, normally protected by nucleosome interactions, to cleavage by DNase I. This cleavage is not uniform, as regulatory DNA currently bound by TFs is protected, leaving footprints that mark TF occupancy at high resolution [360]. These footprints are enriched with DNA sequences specific to particular TFs, known as motifs. Many TFs mediate their effects through direct binding to DNA via

these motifs, utilizing DNA binding domains (DBD) of which there are three major classes: zinc fingers, homeodomains, and helix-loop-helix domains [361]. TFs can have 1,000-fold or greater preference for these specific binding motifs relative to other sequences [362], however the overlap between experimentally determined binding sites and sequence matching the motif is not perfect. TF-binding motifs are small (6-12 bases) and flexible. This flexibility is represented by sequence logos in which the relative preference for the TF for each base in the binding site is represented through the size of the base relative to other bases at that position [363]. The identification of these motifs is critical for assigning function to a particular TF, as motifs can be identified at regulatory regions of DNA and the role of the TF at that region can then be assessed.

Systems-wide approaches have been undertaken to identify the transcription-factor-binding patterns in unicellular organisms, and these studies have been successful in identifying how limited cohorts of TFs are able to organize the large diversity of gene expression patterns [364, 365]. Similar studies in more complex organisms are challenging due to the size of the TF repertoire and genome, however TF regulatory networks have been successfully built in a few cell types, including embryonic stem cells (ESC) [355]. The challenge of these studies is further exacerbated by the observation that the same TF can regulate different genes in different cell types [366], and their regulatory patterns can change when mutated [367]. ChIP-seq has revolutionized the study of TF-binding sites by enabling the genome-wide identification of regions occupied by a TF of interest. Using ChIP-seq for transcription factor identification has several caveats. TF binding is a dynamic process, and the use of crosslinkers for ChIP does not enable the measurement of dynamic TF binding. ChIP data is also highly dependent on antibody quality, and the cross reaction of some antibodies eliminates the specificity of a particular TF binding site. Finally, ChIP-seq can detect indirect binding, which can lead to identification of binding sites for proteins other than the ChIPped protein [368]. This final caveat, while presenting a significant challenge for some experiments, also provides an opportunity for studying TF cooperativity. TFs are known to collaborate in a myriad of ways, including aiding each other in binding DNA, impacting chromatin state (synergistic regulation), binding cooperatively as homo- and hetero-dimers, or higher order structures [369]. Cooperative binding of TFs is often mediated

by protein-protein interactions, which confer additional stability when two or more interacting proteins bind DNA. This cooperation can impact the sequence preferences of TF complexes and constrain the DNA sequence in between two binding sites [370]. In some cases, TFs are able to enhance the binding of other TFs at a particular location through DNA-mediated cooperative binding. For example, the IFN-beta enhancer recruits eight DNA binding TFs which then allow for the recruitment of three non-DNA binding cofactors. Structural analysis reveals few contacts among the TFs, with stability instead being conferred by changes in DNA structure and interactions with cofactors [371].

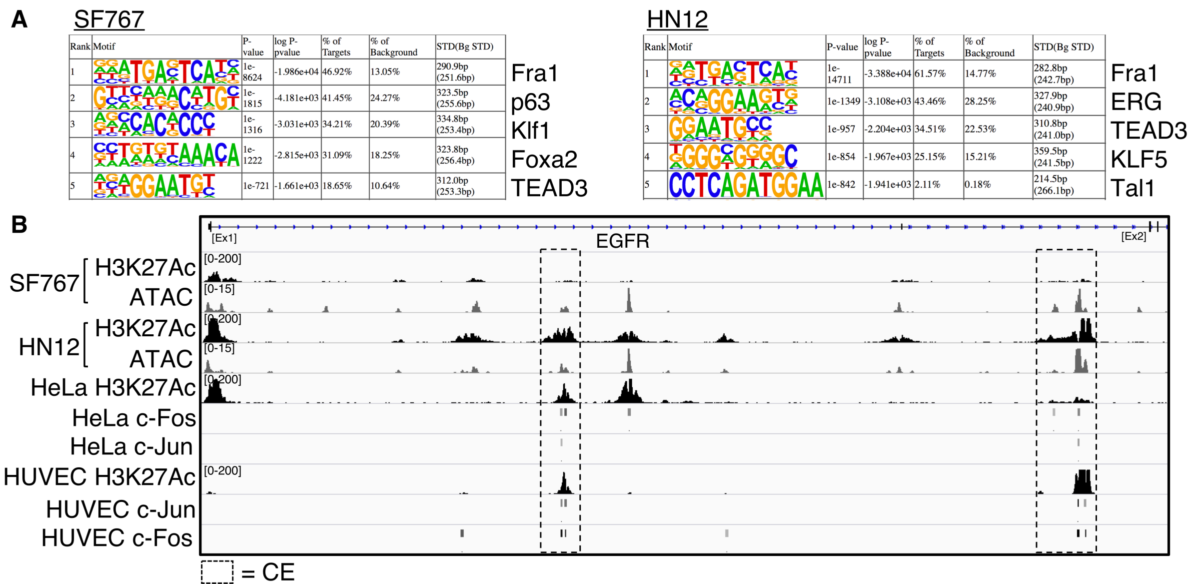
TFs can vary dramatically in how they impact transcription. Some TFs can directly recruit Pol II, provide steric hindrance by blocking binding of other proteins [372], or act by recruiting cofactors including coactivators and corepressors [373]. These cofactors are frequently large multi-subunit protein complexes or multi-domain proteins that regulate transcription through nucleosome remodeling, covalent modifications of histones or other proteins, or recruitment of Pol II [374]. Through their transcriptional activation activities, whether direct or indirect, TFs are critical for driving gene expression in a cell-type-specific and timing-dependent manner. Thus, identification of transcription factors critical for the activity of particular genes is an important step in understanding the regulation of that gene and recognizing how to restore transcriptional balance to aberrantly activated or repressed genes in diseased states.

5.2 Results

AP-1 Family Transcription Factors Bind to and Influence *EGFR* Intron 1 Enhancers.

The primary function of enhancers is to serve as a binding site for different TFs, which can recruit cofactors to mediate the recruitment of Pol II at core promoters. Every enhancer has a slightly different set of factors it can bind to, and this is influenced by many things including enhancer size, motifs present, and proximity to other TF motifs. We wanted to identify the critical motifs in CE1 and CE2 in order to find the crucial TFs mediating the interaction between each CE and the *EGFR* promoter. To begin identifying critical motifs we further analyzed our H3K27Ac ChIP-seq and ATAC-seq data in SF767 and HN12 cells. We utilized a recent paper

which identified critical TFs in macrophages as a guideline for this process [375]. In brief, Lavin et al. performed H3K27Ac/H3K4me1 ChIP-seq, ATAC-seq, and RNA-seq in purified macrophages from fresh mouse tissues. Enhancers were identified by overlapping H3K4me1 and H3K27Ac signals, and a strong correlation between ATAC-seq peaks and identified enhancers was identified, with the vast majority of enhancer regions containing at least one ATAC-seq peak. Because identification of small TF motifs would not be informative from broad enhancer peaks, they overlaid ATAC-seq peaks from corresponding cells with identified enhancers to narrow the search regions to the likely sites of TF binding. DNA lifted from overlaid ATAC-seq peaks could then be subjected to motif-finding algorithms to find highly enriched motifs. We utilized this strategy with some adjustment to find more enhancer-specific transcription factors. To eliminate non-enhancer regulatory regions (e.g. promoters), we intersected ATAC-seq peaks with enhancer peaks from H3K27Ac and kept only the TSS-distal (+/- 2.5kb) ATAC-seq peaks which mapped within an enhancer. Performing *de novo* motif analysis on these peaks in *EGFR* expressing cells (SF767 and HN12) identified an Activating Protein 1 (AP-1) transcription factor motif as the most significantly enriched motif (Figure 5.1A).



A

SF767

Rank	Motif	P-value	log P-pvalue	% of Targets	% of Background	STD(Bg STD)	
1		1e-8624	-1.986e+04	46.92%	13.05%	290.9bp (251.6bp)	Fra1
2		1e-1815	-4.181e+03	41.45%	24.27%	323.5bp (255.6bp)	p63
3		1e-1316	-3.031e+03	34.21%	20.39%	334.8bp (253.4bp)	Klf1
4		1e-1222	-2.815e+03	31.09%	18.25%	323.8bp (256.4bp)	Foxa2
5		1e-721	-1.661e+03	18.65%	10.64%	312.0bp (253.3bp)	TEAD3

HN12

Rank	Motif	P-value	log P-pvalue	% of Targets	% of Background	STD(Bg STD)	
1		1e-14711	-3.388e+04	61.57%	14.77%	282.8bp (242.7bp)	Fra1
2		1e-1349	-3.108e+03	43.46%	28.25%	327.9bp (240.9bp)	ERG
3		1e-957	-2.204e+03	34.51%	22.53%	310.8bp (241.0bp)	TEAD3
4		1e-854	-1.967e+03	25.15%	15.21%	359.5bp (241.5bp)	KLF5
5		1e-842	-1.941e+03	2.11%	0.18%	214.5bp (266.1bp)	Tal1

Figure 5.1: AP-1 family transcription factors are identified as possible modulators of *EGFR* intron 1 enhancers. **A:** Top 5 HOMER *de novo* motif search results in ATAC-seq peaks located within TSS-distal enhancers. % of Targets = Percentage of input peaks containing the indicated motif. % of Background = Percentage of randomly generated background sequences which contain the indicated motif. The number of background regions is 2x the total number of input peaks. STD(Bg STD) = average distance from the peak center where the motif was identified. **B:** Tracks of ENCODE ChIP-seq peaks from c-Jun and c-Fos ChIP-seq experiments in HeLa and HUVEC cells overlaid with tracks for H3K27Ac and ATAC-seq in glioma and HNSCC cell lines. Darker bars indicate stronger binding intensity.

AP-1 is a collective term referring to dimeric transcription factor composed of Jun, Fos, or ATF family subunits. A common feature of the proteins within these families is the conserved basic region leucine zipper (bZIP) DNA-binding domain, first identified in the initially discovered c-Jun protein [376]. The leucine zipper component of the bZIP domain is responsible for dimerization, which is a prerequisite for DNA binding mediated by the basic domain of bZIP. The primary AP-1 proteins in mammalian cells are c-Fos and c-Jun, however these proteins can form many combinations of both homo- and hetero-dimers depending on the cellular context, and these different dimerization patterns can have different effects on the genes that are regulated by AP-1 [377]. Different dimers are known to bind specific motifs, and this can drastically affect their transcriptional transactivation potential. The primary consensus motif, known as the TPA-responsive element (TRE), has the consensus sequence 5'-TGAG/CTCA-3'. Dimers of several AP-1 proteins, including c-Fos, c-Jun and FosB, can efficiently transform cells in culture and have potent transactivation domains [378]. Other AP-1 proteins, including JunB, JunD, Fra1 and Fra2, exhibit only weak transactivation potential and may even act as repressors of AP-1 activity by competing for binding sites or forming inactive dimers [379, 380]. Depending on the cellular context, the same AP-1 protein can have both pro- and anti-oncogenic activities [381, 382], thus it is critical to assess the phenotypic effects of perturbing AP-1 in our cell line models. Importantly, studies have shown the *EGFR* promoter to be a direct target of c-Jun transcriptional activity [383, 384]. Additionally, a previous study which identified an *EGFR* SE showed the binding of the AP-1 family member JunB to the identified SE in A549 NSCLC cells [287].

To validate the TF motifs identified in silico, we examined AP-1 family transcription factor ChIP-seq data deposited by the Encyclopedia of DNA Elements (ENCODE) consortium (Figure 5.1B). To compare ENCODE-generated ChIP-seq to data generated in-house, we overlaid tracks of H3K27Ac ChIP-seq and ATAC-seq from SF767 and HN12 with available H3K27Ac/c-Fos/c-Jun ChIP-seq data from HeLa and HUVEC cells. Importantly, H3K27Ac ChIP-seq in HeLa and HUVEC also shows high levels of enhancer marks in the AP-1 marked regions (Figure 5.1B). Using this approach multiple c-Jun and c-Fos peaks were identified within the CE1 and CE2 of

EGFR in HN12 and SF767 cells (Figure 5.2A). Of note, these c-Fos and c-Jun peaks appear to be enhancer specific within *EGFR* as no significant peaks were identified at the *EGFR* promoter in either HeLa or HUVEC cells (Figure 5.1B). For further analysis we chose c-Jun and c-Fos as the prototype AP-1 factors, as most AP-1 heterodimers contain at least one of c-Fos or c-Jun [385]. We validated and quantified c-Fos and c-Jun enrichment at the CE1 and CE2 regions in *EGFR* expressing (SF767 and HN12) and non-expressing (TS576) cells. We performed ChIP-qPCR at four regions within each constituent enhancer and identified significant fold enrichment of c-Fos and c-Jun within CE1 and CE2 in *EGFR* expressing cells over a negative control region in a gene desert on chromosome 12 (Figure 5.2B). Additionally, we identified significantly increased binding of c-Fos at the CE1-AP1-3 and c-Jun in CE1-AP1-3 and CE2-AP1-4 sub-regions in HN12 cells, indicating these regions may be important for the increased *EGFR* expression levels in these cells (Figure 5.2B).

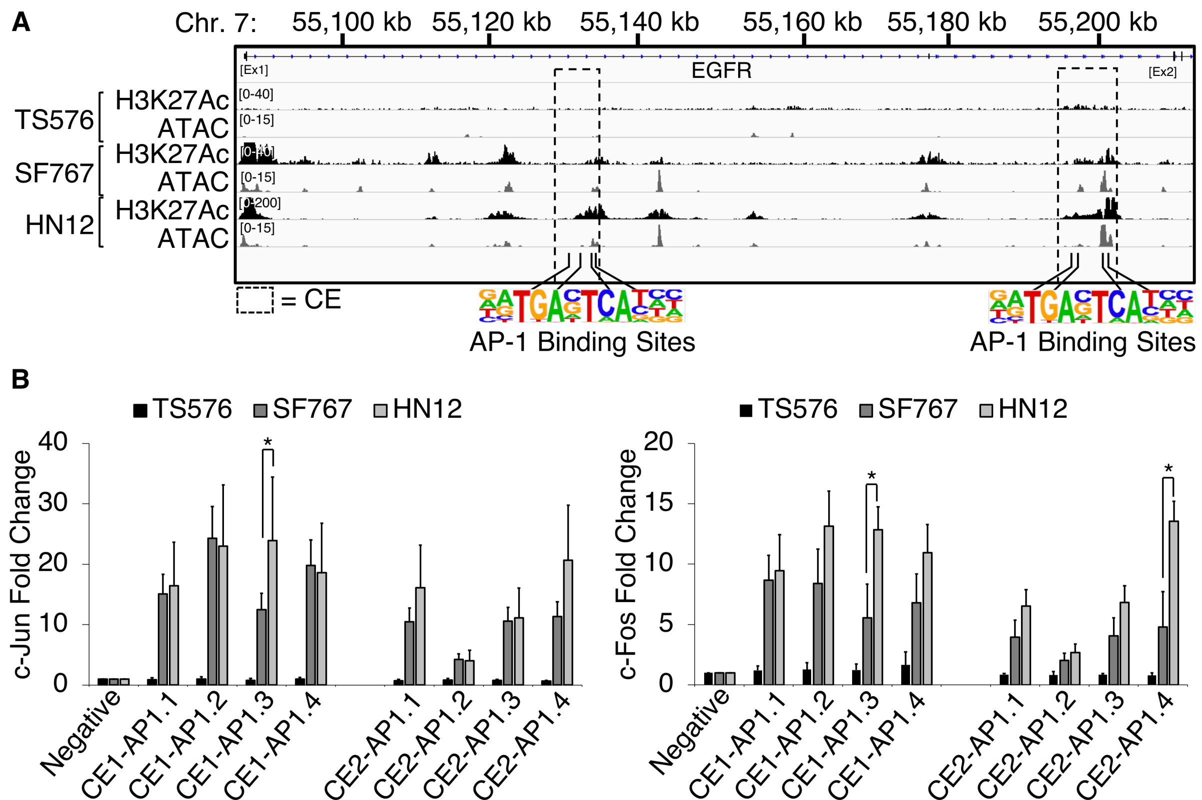


Figure 5.2: AP-1 family members bind to *EGFR* Intron 1. **A:** Schematic of positions of AP-1 binding positions based on ENCODE ChIP-seq data, shown relative to ChIP-seq and ATAC-seq peak density. CE1 and CE2 are highlighted. **B:** Analysis of (left) c-Jun and (right) c-Fos occupancy at the indicated sites. Transcription factor binding is represented as fold change over a negative control region located in Chr12. (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, $n = 3$ independent experiments, Students t test)

To validate the role of AP-1 transcription factors in *EGFR* transcription, we utilized a dominant-negative version of c-Jun (JunDN) [386]. The dominant negative was prepared as described in [386] and is missing the first 122 amino acids consisting of the N-terminal activation domain. JunDN can dimerize with other AP-1 family members and bind DNA, however the transcriptional activation capability is eliminated. A similar construct known as JunbZIP has been shown to downregulate *EGFR* expression through disruption of c-Jun in the fibrosarcoma cell line HT-1080 [387], however the effectiveness of a dominant negative c-Jun has not been shown in GBM or HNSCC. Constitutive expression of JunDN through transduction with a pMIEG3-JunDN retrovirus in HN12 cells showed decreased EGFR protein levels (Figure 5.3A), thus supporting a role for c-Jun heterodimers in the regulation of *EGFR* transcription. Additionally, we detected a decrease in c-Jun levels when JunDN was present, likely due to autoregulation of the *JUN* promoter by c-Jun heterodimers [388] (Figure 5.3A). To confirm the effect of JunDN was due to reduced c-Jun heterodimer activity, we utilized a luciferase reporter containing a trimerized AP-1 binding motif [389]. Transfection of the reporter construct in combination with a *Renilla* control plasmid in HN12 cells significantly decreases reporter activity when JunDN is present compared to the empty vector control (Figure 5.3B). These results confirm the specific transcriptional activation potential of JunDN is abrogated. Taken together, these data suggest that AP-1 family members specifically bind to *EGFR* enhancer regions and are critical for fine-tuned regulation of *EGFR* expression. Perturbation of this AP-1 transactivation effect by expression of a dominant negative results in a significant repression of c-Jun heterodimer targets including *EGFR* and *JUN*, confirming the role of this family of transcription factors in intron 1-mediated *EGFR* expression.

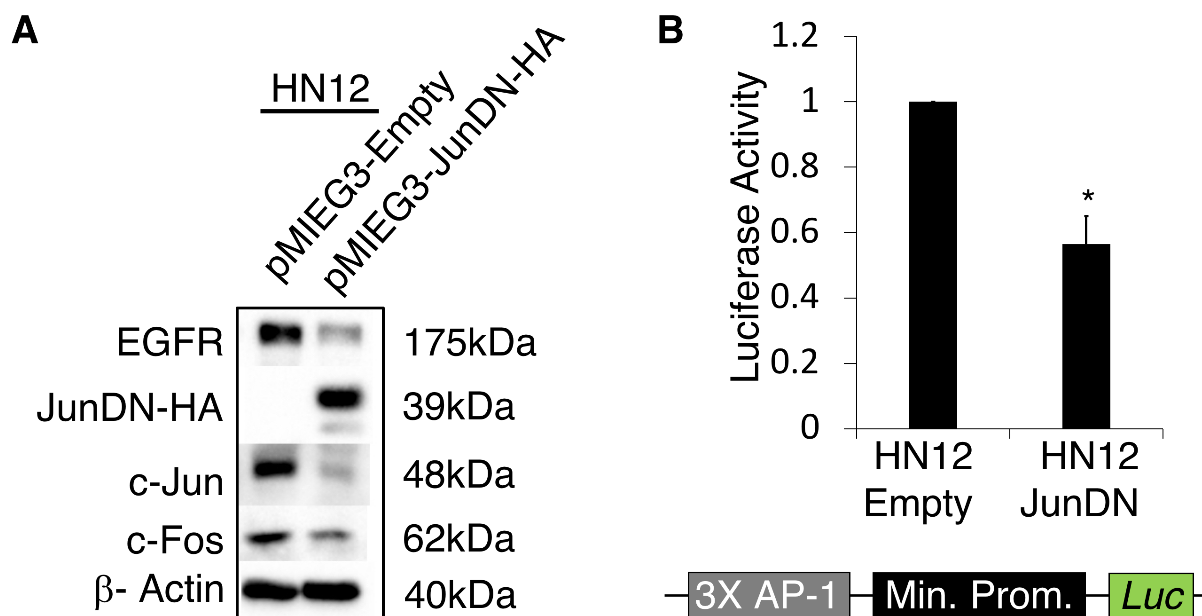


Figure 5.3: AP-1 family members modulate *EGFR* expression. **A:** Analysis of EGFR, JunDN-HA, c-Jun, and c-Fos protein expression in HN12 cells by western blotting after transduction with pMIEG3-JunDN-HA. β-Actin was used as a loading control. **B:** Analysis of JunDN efficacy on a luciferase reporter containing a trimerized AP-1 binding site. Relative luciferase activity after expression of JunDN is normalized to the empty vector. Relative luciferase expression is normalized against *Renilla* luciferase expression to control for transfection efficiency (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, $n = 3$ independent experiments, Student's t test)

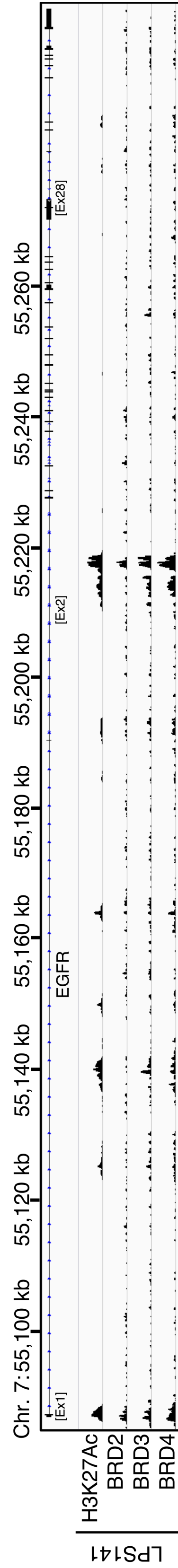
Treatment with JQ1 Reduces *EGFR* Expression by Modulation of TF Activity.

The number of proteins that have been found to interact with AP-1 components and regulate their transcriptional ability is both large and constantly increasing. One example of this interaction is the cooperative relationship between AP-1 and the glucocorticoid receptor (GR). GR can tether to AP-1 complexes without the need for DNA contact and was initially found to downregulate the activity of AP-1 [390], however subsequent genome-wide studies have indicated that the interaction is enriched at regulatory DNA and facilitates the binding and activity of GR through local relaxing of chromatin by AP-1 [391]. Another interaction exemplifying the crosstalk between AP-1 and other TFs is the relationship between AP-1 and NF-kB. NF-kB and AP-1 are activated by the same multitude of stimuli [392], and many genes are reported to require the combinatorial activation of AP-1 and NF-kB, suggesting they work cooperatively [393]. The bZIP domains of c-Fos and c-Jun have been shown to physically interact with the p65 subunit of NF-kB, and the transcriptional effect of AP-1 binding is robustly enhanced when p65 is already present at that region [394]. These results demonstrate that AP-1 has dual roles as both a DNA binding transcriptional activator through opening chromatin, as well as a cofactor with the ability to recruit additional DNA-binding or chromatin reader proteins to regulatory regions through protein-protein interaction.

Recently, AP-1 has been discovered to co-occupy enhancers with the bromodomain and extraterminal domain (BET) protein BRD4 [395]. Bromodomains occur in a variety of nuclear proteins that function as chromatin-binding proteins. The BET family of bromodomain proteins are distinguished by the presence of 2 bromodomains and at ET domain, and are known to bind to acetylated histones [168]. BET proteins including BRD2/3/4 have been shown to be important for enhancer function. BRD2/3 have been shown to bind to hyperacetylated regions and allow for the activity of RNA Pol II [396], and BRD4 in particular has been the subject of many studies. BRD4 is enriched at enhancers [269] and is a critical oncoprotein in AML [397], midline carcinomas [398], and GBM [399] and plays a role in autoimmunity and inflammatory diseases through its interactions with NF-kB [400]. The nature of the interaction between BET proteins and AP-1 is yet unknown, however previous research from our lab has shown treatment of mice

harboring GBM neurosphere PDX models with the pan-BET protein inhibitor JQ1 significantly prolongs survival [401], and combination of JQ1 with anti-EGFR therapy further increases this effect [399]. JQ1 functions by competing for the acetyl-lysine binding pocket of the bromodomain, displacing BET proteins from chromatin and altering the transcriptional activity of the target gene [401]. Additionally, recent data has shown that treatment of childhood sarcomas with JQ1 dramatically reduces AP-1 levels and activity through the suppression of the AP-1 family member FOSL1 [402], and reduced levels of both epidermal growth factor receptor (EGFR) and its ligand, heparin-binding EGF (HB-EGF), seem to be responsible for the severe proliferation defect in keratinocytes lacking c-Jun [317, 384].

Figure 5.4: BET bromodomain proteins bind in *EGFR* intron 1 acetylated regions. BET bromodomain protein and histone acetylation ChIP-seq tracks from the liposarcoma cell line LPS141.


Chr. 7:55,100 kb
55,120 kb
55,140 kb
55,160 kb
55,180 kb
55,200 kb
55,220 kb
55,240 kb
55,260 kb
[Ex1]
EGFR
[Ex2]
[Ex28]
H3K27Ac
BRD2
BRD3
BRD4
LPS141

To determine if the anti-tumorigenic effect of JQ1 as observed by [399] is partially attributable to downregulation of *EGFR* transcription by disruption of AP-1 and BET proteins, we treated HN12 and SF767 cells with JQ1 and measured the effects on *EGFR* expression. Interestingly, after 24 hours of 0.5µM JQ1 EGFR protein and transcript levels were decreased in both HN12 (Figure 5.5A, Figure 5.5B) and SF767 (Figure 5.6A, Figure 5.6B) cell lines. Additionally, BET family proteins BRD2 and BRD4 expression remained unchanged in both cell lines, however BRD3 expression increased in response to JQ1 in HN12 (Figure 5.5A).

To determine if JQ1 treatment was affecting EGFR levels through reduced activity of BRD family members, we first looked for evidence of binding of these factors to CE1 and CE2. Recent data in the liposarcoma cell line LPS141 [403] shows presence of H3K27Ac in CE1 and CE2, and has binding of BRD family members BRD2, BRD3 and BRD4 in those regions (Figure 5.4). To confirm binding of these factors in GBM and HNSCC and to interrogate their relationship with AP-1, we performed ChIP-qPCR for c-Fos, c-Jun, BRD2, BRD3, BRD4 and H3K27Ac at regions of open chromatin in CE1 and CE2. In HN12 cells, treatment with JQ1 significantly reduces occupancy of H3K27Ac at all measured regions, and significantly reduces binding of BET and AP-1 family transcription factors to CE1 and CE2 (Figure 5.5C). Interestingly, in contrast to steady state (Figure 5.2B) which suggests CE2-AP1-4 is a critical c-Fos and c-Jun binding site, treatment with JQ1 only affects binding of BRD4 at that region (Figure 5.5C). In SF767 cells, significant reductions in TF occupancy were observed primarily in CE2, with only BRD4 showing a significant reduction in binding to CE1 (Figure 5.6C).

To determine the specific BET protein critical for maintenance of *EGFR* expression we performed siRNA knockdowns of BRD2/3/4 individually as well as in combination. In HN12 cells single knockdown of BRD2 or BRD4, but not BRD3, resulted in downregulation of EGFR protein. Complete loss of BRD2/3/4 protein by combination siRNA treatment resulted in the strongest downregulation of EGFR protein (Figure 5.5D). In SF767 cells single knockdown of BRD2 produced the strongest downregulation of EGFR protein Figure 5.6D), indicating BRD2 and BRD4 activity at *EGFR* may be cell-type specific. In agreement with Figure 5.5A, knockdown of specific BRD proteins often resulted in upregulation of other BET family members.

These results are suggestive of some functional redundancy in these proteins, a result that is supported by previous data indicating partial redundancy of BRD2 and BRD3 in erythroid cells [404]. Despite this redundancy, increased expression of BRD3 after JQ1 treatment (Figure 5.5A) or RNAi (Figure 5.5D) fails to rescue the expression of EGFR, though triple siRNA treatment produced the strongest loss of EGFR protein. Thus, BRD3 alone is not sufficient for *EGFR* repression, but has some functional redundancy when other BET proteins are lost. These results argue against a role for BRD3 and implicate a role for BRD2 and BRD4 in cooperation with AP-1, in the maintenance of *EGFR* expression in GBM and HNSCC.

Figure 5.5: JQ1 treatment reduces *EGFR* transcription through inhibition of BRD2 and BRD4 activity in HN12 cells. **A:** Analysis of EGFR, BRD4, BRD3, BRD2 and β-Actin protein expression in HN12 cells by western blotting after treatment with 0.5μM JQ1 for 24 hours. β-Actin was used as a loading control. **B:** *EGFR* expression was analyzed by RT-qPCR in HN12 cells treated with 0.5μM JQ1 for 24 hours. *EGFR* transcript level was first normalized to *GAPDH* and subsequently calculated as fold change relative to DMSO control. **C:** Fold changes in enrichment of the indicated factors after 24 hours of 0.5μM JQ1 was measured at the indicated regions by ChIP-qPCR. ChIP enrichment is normalized to a negative control primer in chr12. **D:** Analysis of EGFR, BRD4, BRD3, BRD2 and β-Actin protein expression in HN12 cells by western blotting after treatment with indicated siRNA. A scrambled siRNA was used as treatment control and β-Actin was used as a loading control. **B-C:** (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, $n = 3$ independent experiments, Student's t test)

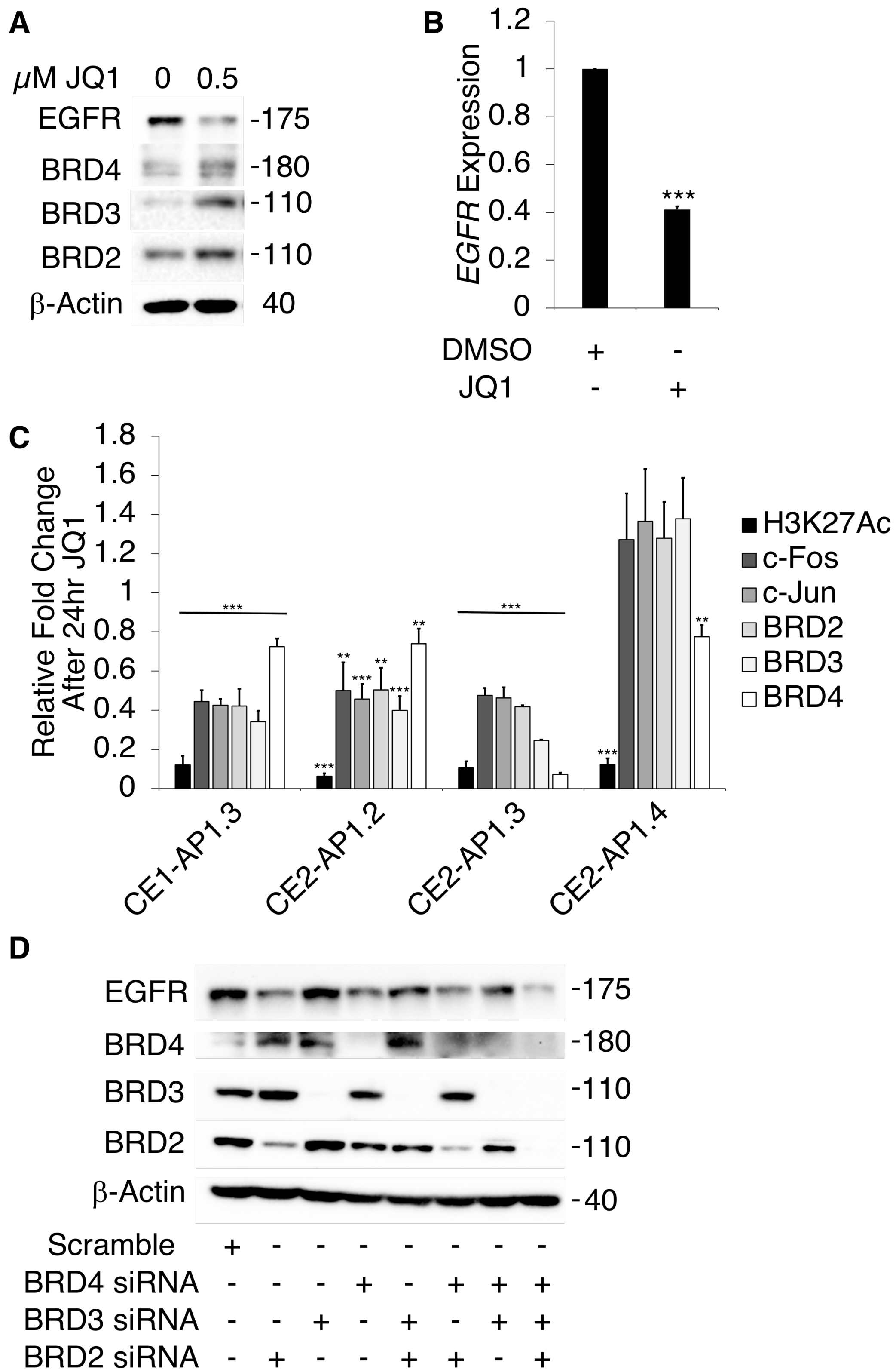
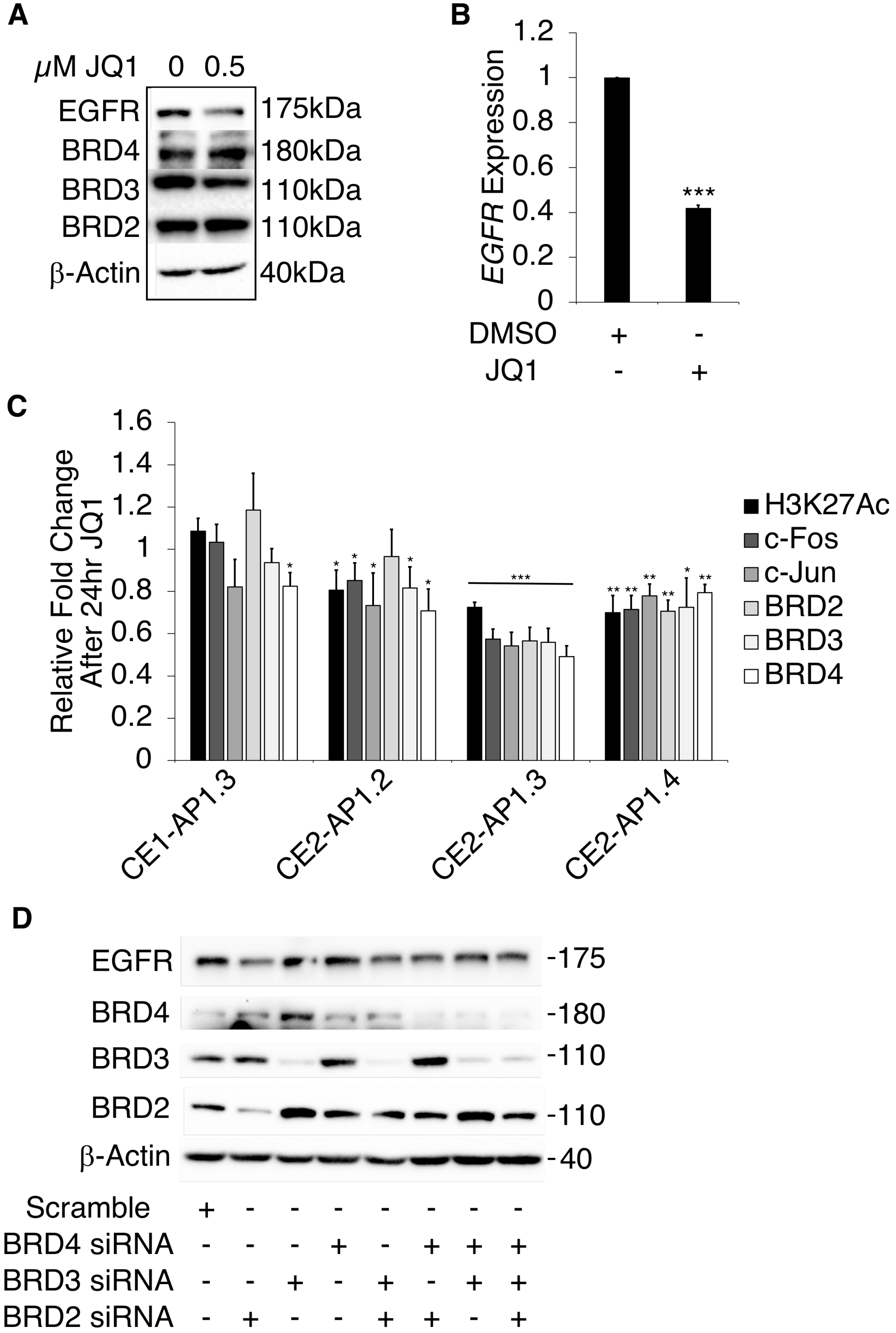

A
μM JQ1 0 0.5
EGFR -175
BRD4 -180
BRD3 -110
BRD2 -110
β-Actin 40
B
EGFR Expression
1.2
1
0.8
0.6
0.4
0.2
0
***
DMSO + -
JQ1 - +
C
Relative Fold Change After 24hr JQ1
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
H3K27Ac
c-Fos
c-Jun
BRD2
BRD3
BRD4
CE1-AP1.3
CE2-AP1.2
CE2-AP1.3
CE2-AP1.4
D
EGFR -175
BRD4 -180
BRD3 -110
BRD2 -110
β-Actin -40
Scramble + - - - - - - -
BRD4 siRNA - - - + - + + +
BRD3 siRNA - - + - + - + +
BRD2 siRNA - + - - + + - +

Figure 5.6: JQ1 treatment reduces *EGFR* transcription through inhibition of BRD2 and BRD4 activity in SF767 cells. **A:** Analysis of EGFR, BRD4, BRD3, BRD2 and β-Actin protein expression in SF767 cells by western blotting after treatment with 0.5μM JQ1 for 24 hours. β-Actin was used as a loading control. **B:** *EGFR* expression was analyzed by RT-qPCR in SF767 cells treated with 0.5μM JQ1 for 24 hours. *EGFR* transcript level was first normalized to *GAPDH* and subsequently calculated as fold change relative to DMSO control. **C:** Fold changes in enrichment of the indicated factors after 24 hours of 0.5μM JQ1 was measured at the indicated regions by ChIP-qPCR. ChIP enrichment is normalized to a negative control primer in chr12. **D:** Analysis of EGFR, BRD4, BRD3, BRD2 and β-Actin protein expression in SF767 cells by western blotting after treatment with indicated siRNA. A scrambled siRNA was used as treatment control and β-Actin was used as a loading control. **B-C:** (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, $n = 3$ independent experiments, Student's *t* test)


A
μM JQ1 0 0.5
EGFR 175kDa
BRD4 180kDa
BRD3 110kDa
BRD2 110kDa
β-Actin 40kDa
B
EGFR Expression
1.2
1
0.8
0.6
0.4
0.2
0
***
DMSO + -
JQ1 - +
C
Relative Fold Change After 24hr JQ1
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
H3K27Ac
c-Fos
c-Jun
BRD2
BRD3
BRD4
CE1-AP1.3
CE2-AP1.2
CE2-AP1.3
CE2-AP1.4
D
EGFR -175
BRD4 -180
BRD3 -110
BRD2 -110
β-Actin -40
Scramble + - - - - - - -
BRD4 siRNA - - - + - + + +
BRD3 siRNA - - + - + - + +
BRD2 siRNA - + - - + + - +

5.3 Summary

Many potent oncogenes are influenced by super enhancers, including *MYC* and *HER2* [316, 346]. These genes and others have been successfully targeted in cancers which express them by inhibiting the BET protein BRD4, a hallmark factor involved in super enhancer identity [269]. The BET-bromodomain inhibitor JQ1 has been shown to inhibit BRD4 at super enhancers [269] and additionally sensitizes *EGFR* amplified GBM [399] and HNSCC [405] cells to EGFR TKI. Our data suggests that this sensitization may also be due in part to JQ1-mediated inhibition of AP-1 and other BET proteins at *EGFR* intron 1 enhancers (Figure 5.5, Figure 5.6). Although site-specific inhibition of TF binding is cell-type dependent, whether targeting TF binding pharmacologically (Figure 5.5A-C, Figure 5.6A-C) or through RNAi (Figure 5.5D, Figure 5.6D), the effects on *EGFR* are consistently negative. These results support the global targeting of AP-1 and BET rather than site-specific repression (e.g. dCas9-KRAB). Additionally, our study shows significant reduction of EGFR protein and transcript after JQ1 treatment independent of gene amplification (Figure 5.5A-C, Figure 5.6A-C), indicating a broader effect of JQ1 on tumor models which express *EGFR* at varying levels. Indeed, *EGFR* transcript levels are reduced to similar levels in SF767 cells which express less *EGFR* and exhibit less significant TF occupancy differences in response to JQ1 treatment (Figure 5.6C). These data combined further support the combination of EGFR TKI and JQ1 as treatment for EGFR-positive malignancies.

It has been previously observed that Ras is a significant activator of AP-1 activity, and has been identified as one of the main cooperating partners of AP-1 [406, 407]. Oncogenic transformation of a cell with Ras or MEK significantly induces AP-1 protein expression [408, 409], specifically the Fra1 and c-Jun proteins [408]. This potent transformation appears to be through the N terminal phosphorylation of c-Jun by the Jun N-terminal Kinase (JNK) proteins [410, 411] and is supported by the observation that Ras-mediated transformation is suppressed in fibroblasts that lack c-Jun [412]. As introduced in chapter 1, Ras is potently activated upon EGFR phosphorylation [15] leading to the activation of many different pathways, including enhanced AP-1 transcription. This pathway indicates a potential positive feedback loop for the continued expression of EGFR. Activated EGFR (following ligand binding or amplification) activates

Ras and MAPK pathways, stimulating AP-1 production and phosphorylation, and leading to increased enhancer activation of *EGFR* transcription. There are many opportunities for pharmacological inhibition of this pathway as discussed earlier, however the wide range of kinases and transcription factors involved also introduces a high likelihood for bypass pathways. Further studies should evaluate the efficacy of combining various upstream and downstream pathway inhibitors, and determine the mechanism by which they are exacting their effects so that inevitable resistance mechanisms can then be targeted in a logical and systematic manner.

5.4 Materials and Methods

Cell Culture.

SF767 (RRID:CVCL_6950) was provided by Dr. Mitch Berger (UCSF Brain Tumor Center, San Francisco, CA). Head and neck cell line HN12 (RRID:CVCL_5518) was provided by Dr. Silvio Gutkind, UCSD Moores Cancer Center, San Diego, CA. Cells were maintained in DMEM (Hyclone, #SH30022.01) supplemented with 10% fetal bovine serum (Atlanta Biologicals, #S12450) and 1% penicillin-streptomycin (Gibco, #15140-122) and grown as adherent cultures.

TF Motif identification.

Tag directories were created from H3K27Ac alignment files using HOMER makeTagDirectory. Peaks from the H3K27Ac ChIP-seq were identified from the tag directories using HOMER findPeaks with parameters -style histone -size 250 -minDist 250. NarrowPeak files from individual ATAC-seq replicates and pooled peak files from MACS2 were sorted for high P-value peaks ($\geq$ 13 -log10 [$p < 0.05$]) with awk. In order to get a set of high P-value, replicated peaks, high P-value pooled peaks were intersected with high P-value peaks from each replicate using bedtools intersect with the -u parameter. In order to exclude promoter proximal peaks, replicated high P-value peaks were sorted for. The Gencode hg38 annotation was downloaded and TSS +/- 2kb were filtered out with awk. High p-value peaks were intersected with the Gencode TSS annotation with bedtools intersect with the -v parameter. H3K27Ac peaks were modified from hg19 to hg38 using the hgLiftOver tool for intersecting with ATAC-seq peaks.

Promoter distal high p-value ATAC peaks were intersected with the H3K27Ac ChIP-seq peaks to identify ATAC peaks located within regions of H3K27Ac using bedtools intersect with the -wa parameter. Motifs were identified from these peaks using HOMER findMotifsGenome with the parameter -size given. De novo motifs were identified from html files output by HOMER.

Data Access.

All raw and processed sequencing data generated in this study have been submitted to NCBI GEO under accession number GSE128275. Accession numbers for publicly available data accessed are as follows: HeLa c-Fos (GSM935317), HeLa c-Jun (GSM935341), HeLa H3K27Ac (GSM733684), HUVEC c-Fos (GSM935585), HUVEC c-Jun (GSM935278), HUVEC H3K27Ac (GSM733691), LPS141 H3K27Ac (GSM3027215), LPS141 BRD2 (GSM3027219), LPS141 BRD3 (GSM3027220), LPS141 BRD4 (GSM3027221).

Quantitative real-time PCR.

RNA was extracted with the RNeasy Plus kit (Qiagen, #74134) according to the manufacturers instructions. Reverse transcription of mRNA was performed using 1µg RNA with the iScript Reverse Transcription Supermix (BioRad, #1708841). For real-time PCR analysis, 5µl of cDNA (50ng of starting RNA) was amplified per reaction using the iTaq Universal SYBR Green Supermix (Bio-Rad, #1725124) and the Bio-Rad CFX96 qPCR system. Fold change analysis was performed using the $2^{-\Delta\Delta Ct}$ method and normalized as indicated.

Luciferase Reporter Assay.

pMIEG3-JunDN (RRID: Addgene_40350) and 3xAP1pGL3 (RRID:Addgene_40342) were gifts from Alexander Dent. pMIEG3-Empty was created by removing the JunDN sequence by EcoRI digestion. For each transfection reaction, 100ng control plasmid expressing Renilla luciferase (Promega, #E2241) and 1µg Firefly luciferase construct were co-transfected with Lipofectamine 2000 (ThermoFisher, #11668030) into 2 x 10^5 cells in a 12-well plate well. After 24 hours, cells were collected in 1X PLB. Luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega, #E1910) on a Tecan Spark 10M with injection

control. Transfection efficiency was controlled for by dividing firefly luminescence by *Renilla* luminescence, and final activity was normalized to a negative control.

Western Blotting.

Protein samples were collected in SDS sample buffer, separated using gel electrophoresis and transferred via wet transfer onto a PVDF membrane. The membrane was blocked with 5% milk in TBST and probed with primary antibodies at 1:1000 dilution overnight at 4°C and secondary horeseradish peroxidase (HRP) antibodies at 1:2000 for 1 hour at room temperature. Signal was assessed via chemiluminescence with the SuperSignal West Pico PLUS substrate (ThermoFisher, #34580) and visualized on a ChemiDoc MP system (Bio-Rad). anti-β-actin (Sigma, #A3854), anti-c-Jun (Cell Signal, #9165S), anti-c-Fos (Santa Cruz Biotechnology, #sc-52), anti-HA-HRP (Santa Cruz Biotechnology, #sc-805), anti-BRD4 (Active Motif, #39909), anti-BRD3 (Santa Cruz Biotechnology, #sc-515729), anti-BRD2 (Cell Signal, #5848S) and anti-EGFR (BD Biosciences, #610017) were used for analysis.

Chromatin Immunoprecipitation.

Chromatin immunoprecipitation was performed as described previously [317] with the following modifications. Chromatin was sheared in diluted lysis buffer to 200-500bp using a Covaris M220 Focused-Ultrasonicator with the following parameters: 10 minutes, peak incident power 75, duty factor 10%, 200 cycles/burst. Antibodies for ChIP were obtained from commercially available sources: anti-H3K27Ac (Active Motif, #39133), anti-BRD4 (Active Motif, #39909), anti-c-Jun (Cell Signaling, #9165T), and anti-c-Fos (Santa Cruz Biotechnology, #sc-166940). 5% of the chromatin was not exposed to antibody and was used as control (input). For ChIP-qPCR analysis DNA quantity for each ChIP sample was normalized against input DNA.

siRNA Transfection.

$1x10^5$ SF767 or $5x10^4$ HN12 cells were seeded in 12 well plates and grown overnight. siRNAs were transfected into each well with Lipofectamine 2000 in serum free and antibiotic free DMEM. Media was changed to complete media 6 hours later. Samples were collected in SDS sam-

ple buffer 48-72 hours later. siRNAs used for this study include BRD2 (Ambion, #s12070), BRD3 (Ambion, #s15544), BRD4 (Ambion, #s23902), and scramble control (Invitrogen, #12935-300).

JQ1 Treatment.

SF767 or HN12 cells were treated with 0.5µM JQ1 dissolved in DMSO (MedChemExpress, #HY-13030) for 24 hours. Vehicle control samples were treated with equal volume DMSO for 24 hours. Samples were collected in SDS sample buffer and analyzed.

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Chapter 6

Conclusion

In this study we identify regions of epigenetic regulation within the first intron of the *EGFR* gene, characterizing DNA regions which are cell type specific in their H3K27Ac deposition, but contain conserved regions of open chromatin and histone acetylation within EGFR-expressing cells. These regions pass the threshold to be considered super enhancers and contain individual constituents which demonstrate functional attributes of active enhancers, including transcriptional enhancement in reporter assays, 3D interactions with the *EGFR* promoter, and negative regulation of their target gene when removed or repressed. We identify the presence and activity of AP-1 and BET transcription factors in the CE1 and CE2, and when the activity of these transcription factors is eliminated significant effects are seen in expression of target genes including *EGFR* and *JUN*, indicating direct AP-1 and BET dependency of these genes. Pharmacologic disruption of the transcription factor complexes at these enhancers has significant effects on *EGFR* expression, providing a mechanism by which this transcriptional control mechanism may be targeted.

As mentioned throughout, few studies have attempted to elucidate the mechanisms of transcriptional control of *EGFR*, and the few early studies primarily focused on the *EGFR* promoter as the primary unit mediating any control. Though this chapter and this study focus exclusively on CE1 and CE2 for the control of *EGFR* transcription, it is important that the promoters role is not lost as part of this regulatory circuit. Although the dCas9-KRAB

transcriptional repression system has been extensively validated through the silencing of distal enhancer elements [413, 414], its initial utility was described at the promoter regions of genes targeted for silencing [341, 342]. Keeping in mind the ACH hypothesis introduced in Chapter 2 [195] and the fact that enhancers and promoters interact with each other directly through DNA bending in 3D chromosomal space, it could be hypothesized there are transcriptional-silencing mechanisms beyond KRAB-mediated deposition of H3K9me3 or de-acetylation of H3K27 at an enhancer. Targeting a KRAB domain to an enhancer region known to be interacting with a promoter could exact the same KRAB-mediated deposition of H3K9me3 or de-acetylation of H3K27 at the promoter of that gene at the same time. To my knowledge, this has not been experimentally validated, however evidence we have shown here indicates that this may be the case.

In chapter 4 Figure 4.4, in HN12 cells compared to the O-T control, the CE1.4 and CE2.4 gRNA targeted regions had significantly decreased enrichment of H3K27Ac at the opposing targeted region (e.g. H3K27Ac loss at CE1.4 when 2.4 was targeted). If these regions are cooperatively interacting with the *EGFR* promoter, as suggested by the strongest repression occurring when either both enhancers are deleted (Figure 4.3) or repressed (Figure 4.4, Figure 4.5), then the association with dCas9-KRAB at a specific region within CE1 or CE2 could be mediating the identified de-acetylation of H3K27 at the other CE. It stands to reason that this phenomenon could also be occurring at the *EGFR* promoter, and H3K27Ac ChIP-qPCR for regions around the promoter could help validate this hypothesis. Similarly, though significant repression of *EGFR* transcription was mediated by dCas9-KRAB targeting to CE1 and CE2, targeting additional KRAB domains specifically to the *EGFR* promoter could completely shut down the expression of the gene in combination with CE1 and CE2 repression. Further experiments are required to confirm this hypothesis in our cell line models.

Elevated EGFR is a well-established therapeutic target, however responses to EGFR tyrosine kinase inhibitors (TKI) are sporadic [66,70,72,93,133]. The mechanisms behind resistance to EGFR TKI are unique to each tumor type, and secondary resistance mutations are common, particularly in NSCLC [105, 109]. In HNSCC, high *EGFR* copy numbers are statistically associated

with cetuximab and gefitinib resistance [415], and although rare, kinase domain mutations may be associated with altered responses to EGFR inhibitors [416]. In GBM, resistance mechanisms are less well understood with prevailing theories including intratumoral heterogeneity [417], *EGFR* amplified extrachromosomal DNA (ecDNA) [48], and loss of PTEN [76]. With these challenges in mind, this study presents a kinase domain independent mechanism by which *EGFR* expression and activity can be prevented. Recent studies have shown targeted transcription factor blockade can overcome EGFR TKI resistance [418], thus this study presents an additional set of pathways which can be targeted alone or in combination with EGFR TKIs to treat EGFR-positive tumors.

Evidence for the effectiveness of this strategy has been published before by our lab and others. Treatment of mice harboring GBM neurosphere PDX models with the pan-BET protein inhibitor JQ1 significantly prolongs survival, and combination of JQ1 with anti-EGFR TKI further increases this effect [399]. This anti-EGFR effect of JQ1 is at least partially attributable to disruption of genes which associate with enhancers co-occupied by AP-1 and BRD4 [395]. Both BRD4 and AP-1 have been shown to interact with or co-occupy NF-kB targets [392,393,400], thus small molecule inhibitors targeting these molecules should prove effective in EGFR-overexpressing cells. For example, SP100030 is a molecule which shows potent inhibition of NF-kB and AP-1 transcription activation [419] and has been shown to be effective inhibiting AP-1 and NF-kB targets [420]. Other molecules targeting AP-1 binding to DNA [421] have even been utilized in clinical trials [422]. Further studies should investigate the effectiveness of these anti-AP-1 compounds in EGFR-positive tumors, both as single agents and in combination. Further, additional studies should interrogate the CE1 and CE2 in *EGFR*-mutated models to identify if the use of transcription factor blocking molecules can rationally be expected to have increased effects regardless of the status of the kinase domain of the protein.

Though the preclinical evidence for utilizing JQ1 is compelling, JQ1 has limited use in the clinic. JQ1 is a pan-BET inhibitor, and thus is not selective for any BRD proteins [401]. Initial hope for JQ1 was primarily based on its potent inhibition of BRD4, a protein which was known to cooperate with MYC to drive the transcription of oncogenic genes [423]. However, because JQ1 targets BRD2 and BRD3 in addition to BRD4, the off-target effects are significant.

Preclinical studies showed that knocking out BRD4 by RNAi produced widespread stem cell depletion [424], and BRD2 knockout mice are not viable [425]. JQ1 has a very short half-life and dose concentrations required to mediate single agent activity are above physiologic safety levels *in vivo* [426], therefore it has not even been tested in clinical trials. Other pan-BET inhibitors like, ABBV-075 [427], I-BET151 [428], CPI203 [429], and PFI-1 [430] function in a similar anti-MYC manner and have shown preclinical benefit, however clinical trials featuring these inhibitors have generally not passed phase I as single therapies. With the significant off-target effects of pan-BET inhibitors in mind, more selective compounds have begun to be developed. OTX015 is a BRD2/4 inhibitor [431], and RVX-208 is a BRD3 specific inhibitor [432]. OTX015 has progressed furthest and shown the most effectiveness overall of these inhibitors [433, 434], but still was withdrawn at Phase II. Toxicities for these drugs are often found in the stem cell populations, most often leading to thrombocytopenia (platelet loss) and anemia [435–438].

Additionally, it appears that BRD2/3/4 are not fully functionally redundant [425, 439] meaning pan-disruption is likely causing widespread toxicities. Because of their lack of selectivity, actual pan-BET bromodomain inhibitors constitute a major limiting issue for the elucidation of specific functions of BRD2, BRD3 or BRD4. Solving this problem by providing selective compounds of individual BET proteins is a crucial challenge to understand their biological roles, to unravel the molecular mechanisms of their signaling, and to develop relevant targeted therapeutic strategies. For the purposes of *EGFR* transcription, it will be important for future experiments to further specify which BRD protein is critical for driving enhancer activity and utilizing BRD protein-specific inhibitors, possibly in combination with EGFR TKIs, to identify truly effective combination therapies.

The mechanisms for *EGFR* transcriptional control which we have described here may have implications for *EGFR* mutations, specifically the EGFRvIII ED mutation. EGFRvIII is a critical oncogene in GBM, present in 30-60% of primary GBMs depending on the method of identification [43, 44]. As mentioned earlier, EGFRvIII results from in-frame deletion of 801 base pairs spanning exons 27 of the coding sequence [41, 42]. EGFRvIII shows constitutive tyrosine kinase activity [39] and is highly tumorigenic [40]. In GBM every incidence of *EGFRvIII*

has unique genomic breakpoints within intron 1 and intron 8, removing exons 2-7 including large fragments of intron 1 depending on breakpoint location, often including regions which we have identified as enhancers [440] (Figure 3.1). It is important to note the majority of identified *EGFRvIII* breakpoints occur nearer the 3 end of *EGFR* intron 1, often removing the region which we have identified as CE2 [440]. Interestingly, in some of our cell line models the data indicates a more significant role for CE2 in interaction with the *EGFR* promoter by 3C (Figure 3.6) and influence on the expression of EGFR by dCas9-KRAB (Figure 4.4, Figure 4.5). In other models the utilization of each CE is either equal (Figure 4.5) or demonstrates a CE1 bias Figure 4.2B, SF767/Cal27). But, targeting both CEs always produces a combinatorial effect (Figure 4.3B, Figure 4.4D, Figure 4.5D). These data demonstrate an intricate regulatory system that is cell type-specific and makes predicting the functional consequences of intron 1-loss on EGFRvIII expression difficult.

Further research should interrogate directly how extent of intron 1-loss affects EGFRvIII through analysis of patient derived and/or CRISPR/Cas9-generated EGFRvIII+ models. Engineered cells which express homozygous EGFRvIII in an isogenic background would provide a few advantages to directly study the effect of intron 1 loss in *EGFR*. First, progressively larger deletions could be made and compared against an EGFRvIII missing the minimal amount of intron 1. Second, each engineered cell line would have the same driver and passenger mutations, ensuring no compensatory pathways can activate the intron 1 enhancers. We would expect that loss of CE2 alone would either lead to increased activity of CE1, or overall decreased transcriptional activation of *EGFR* expression. This could be measured by ChIP-seq for H3K27Ac at the remaining enhancer regions paired with qPCR to assess *EGFR* expression levels. However, to make any clinically relevant conclusions, experiments would need to be further validated using neurospheres or clinical samples expressing EGFRvIII.

This study focuses exclusively on non-amplified *EGFR*, however significant fractions of both HNSCC and GBM tumors have high copy numbers of the gene [26, 118]. In GBM, *EGFR* can be amplified both as a homogeneously staining region (HSR) or on extrachromosomal DNA (ecDNA) [48], both of which include the entire *EGFR* gene and surrounding regions. Ampli-

fied enhancer regions maintain their enhancer signatures [348] and focal amplifications of super enhancers have been shown to drive transcription [353]. Previous data in our lab has shown this to be significant specifically in GBM neurospheres which contain ecDNA with EGFRvIII, as treatment with JQ1 significantly increases survival [399]. To most accurately establish a link between JQ1 and *EGFR* transcriptional inhibition in amplified *EGFR/EGFRvIII* cases, ChIP-seq for direct JQ1 targets (e.g. BRD4) should be done in treatment naïve and treated neurospheres. Because *EGFR* is often amplified on ecDNA and ecDNA is often stitched together from many non-adjacent genomic regions [441, 442], it is conceivable that other amplified genes on ecDNA are directly able to increase transcription through epigenetic interactions. Further analysis of ecDNA in a wider range of GBM clinical samples should be done to confirm whether chromatin-altering proteins are amplified on ecDNA at any meaningful rate. These data support a role for *EGFR* intron 1 enhancers in the control of amplified *EGFR* on ecDNA, and future studies should further explore the chromatin landscape of ecDNA and utilize treatment strategies which have already been discussed for disrupting the transcriptional activation of *EGFR* by intron 1 enhancers.

In conclusion, we found that *EGFR* expression is maintained in part through presence and activity of critical constituent enhancers present in intron 1 of the gene. Characterization of CE1 and CE2 in multiple cell line systems identified a novel role for BET transcriptional co-activators and AP-1 transcription factors in these enhancers, and provided the rationale for therapeutic targeting of *EGFR* through perturbation of BET and AP-1 in EGFR-positive malignancies. Therapeutic targeting strategies for mitigating the oncogenic effects of active EGFR, though numerous and wide in scope, have proven largely ineffective in many cancer types including HNSCC and GBM. Thus, we present a previously uncharacterized *EGFR* expression mechanism which we believe is an important modality which can be targeted in EGFR-positive malignancies (Figure 6.1).

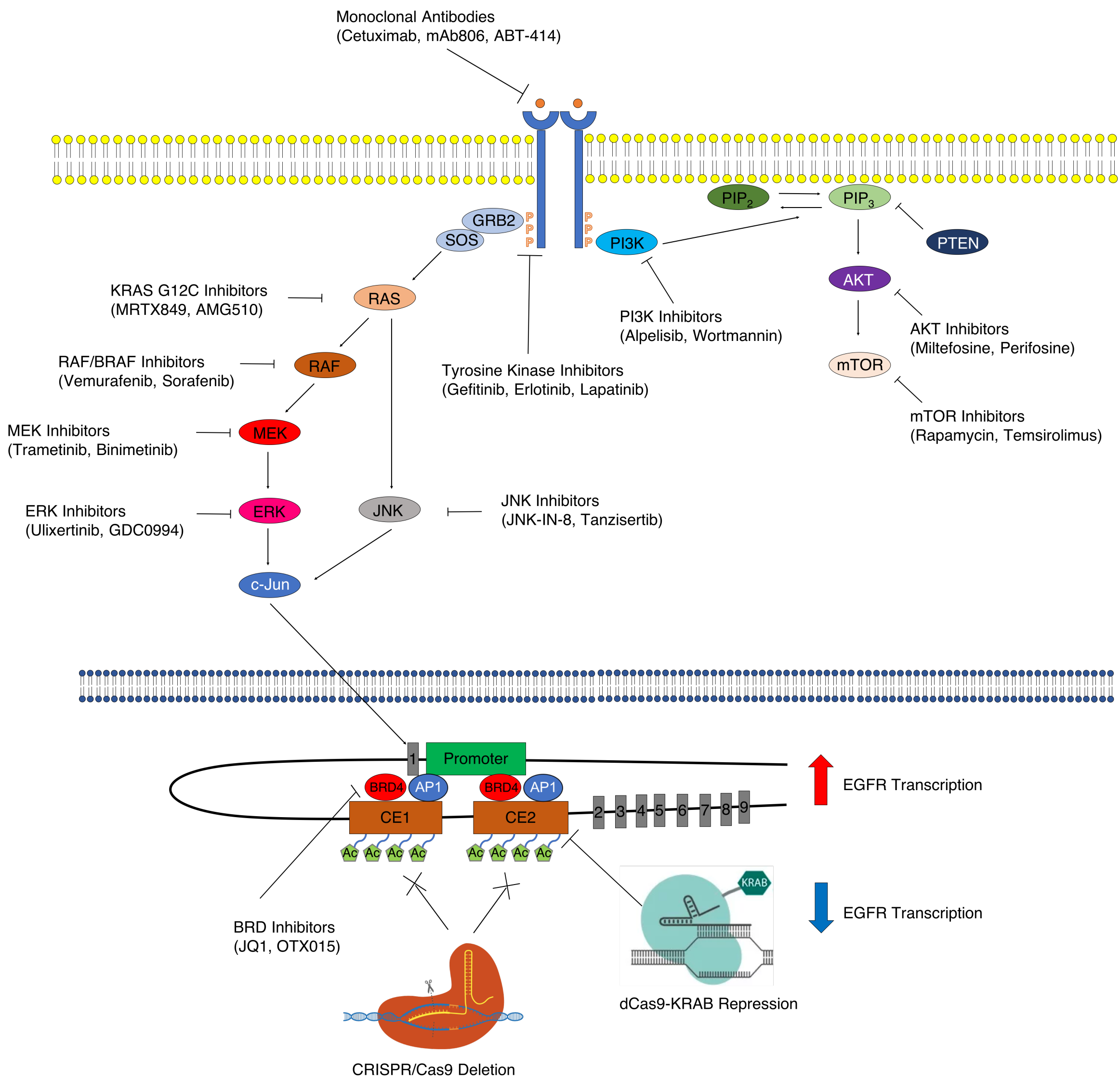

Figure 6.1: Summary of current anti-EGFR strategies and findings from this study.

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