# K811 acetylation regulates ZEB1 dimerization, protein stability, and NuRD complex interactions to promote lung adenocarcinoma progression and metastasis

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## Introduction

Lung cancer is the leading cause of cancer-related death worldwide due to the ability of cancer cells to metastasize. Therefore, it is essential to expand our current knowledge of the biological processes that contribute to metastasis to guide the discovery of novel therapeutic modalities. The Epithelial-to-mesenchymal transition (EMT) is a mechanism for metastasis, which changes polarized epithelial cells into invasive mesenchymal cells. High expression of the Zinc finger E-box binding homeobox 1 (ZEB1) transcription factor is correlated to poor outcomes in cancer, including therapeutic resistance and EMT-mediated metastasis. ZEB1 has a predicted molecular weight of 125kDa; however, multiple groups have reported discrepancies in the observed molecular weight (~ 190-250kDa). This has been attributed to dimerization mediated by post-translational modifications (PTMs). Therefore, we performed mass spectrometry and identified a novel PTM - K811 acetylation - that may regulate ZEB1 dimerization and function. To define the role of ZEB1 acetylation, we generated ZEB1 acetyl mimetic (K811Q) and deficient (K811R) mutants in a panel of lung adenocarcinoma cell lines. We aim to characterize a *novel regulatory mechanism of the transcriptional repressor ZEB1* with the goal of identifying its functional and pathological relevance to the metastatic process.

### Lung Cancer is the leading cause of cancer-related deaths

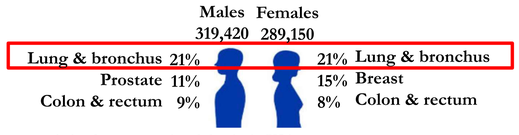
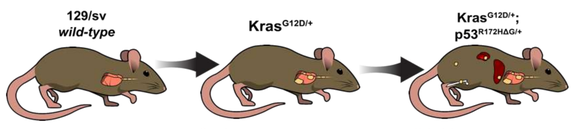



Figure 1. 2022 cancer statistics for cancer related deaths in USA. Siegel et al., 2022

### Mouse model of metastatic lung adenocarcinoma




| NAME | ROUTE OF INJECTION | LOCAL | METASTASIS | METASTATIC SITES | EMT STATE |
|---|---|---|---|---|---|
| 393P | SQ | 7/10 | 0/10 | None | Epithelial Cell |
| 344SQ | SQ | 13/13 | 13/13 | Lung, pancreas, adrenal, muscles, diaphragm, bone | Mesenchymal Cell |

Gibbons et al., 2009

Figure 2. The genetically engineered mouse model (GEMM) acquires the somatic activation of the Kras$^{G12D}$ allele to develop spontaneous lung adenocarcinoma, without metastatic formation. Introduction of a mutant p53$^{R172HΔG}$ allele was found to recapitulate features of metastasis-prone lung adenocarcinoma cancer patients. The derived cell lines from this GEMM were subcutaneously injected into syngeneic mice to evaluate the propensity to metastasize. The cell line 393P was defined as a metastasis incompetent cell line, and 344SQ was a metastasis prone cell line.

### ZEB1 transcriptional repressor regulates Epithelial-to-mesenchymal transition (EMT)-contributed to lung cancer metastasis

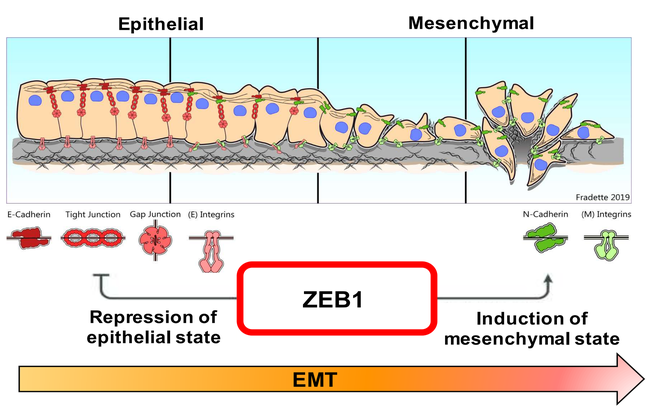



Figure 3. Epithelial-to-mesenchymal transition (EMT) is a mechanism for metastasis that results in a loss of apical-basal polarity and epithelial cell-cell contacts to acquire mesenchymal front-back polarity that confers migration and invasion. The Zinc finger E-box binding homeobox 1 (ZEB1) transcriptional repressor is a master regulator of EMT.

### 250 kDa ZEB1 is an SDS-PAGE-resistant homodimer

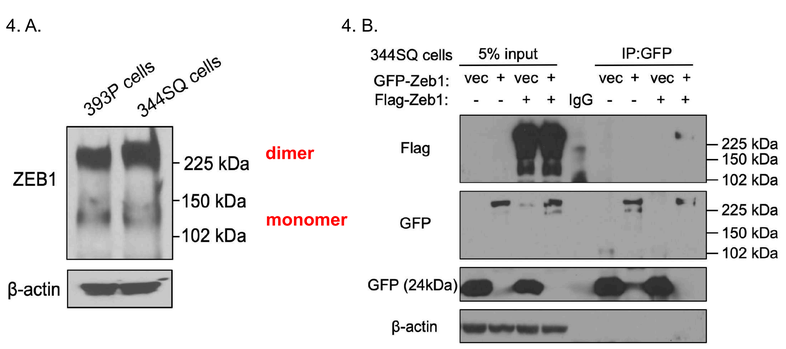



Figure 4. (A) Immunoblotting of ZEB1 and β-actin in non-metastatic 393P and metastatic 344SQ murine cell lines. Blot confirms the presence of both 125 kDa and 225 kDa ZEB1 bands. (B) GFP-vector alone, GFP-vector plus FLAG-ZEB1, GFP-ZEB1 alone, or GFP-ZEB1 plus FLAG-ZEB1 were co-transfected into 344SQ cells and induced with doxycycline for 24 h. The whole-cell lysate was co-immunoprecipitated with antibodies against mouse IgG and GFP, and immunoblotted with antibodies against Flag, GFP, and β-actin. Blot for Flag antibody confirms that GFP-ZEB1 co-immunoprecipitates with FLAG-ZEB1.

### K811 acetylation site is a novel PTM in ZEB1 identified by mass spectrometry

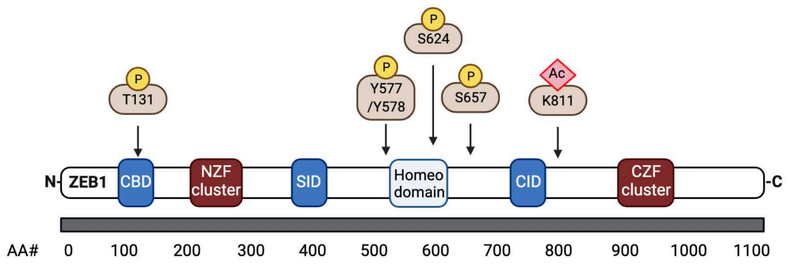



Figure 5. Schematic diagram of the PTMs in ZEB1 identified by tandem-mass spectrometry (MS/MS) analysis: T131, Y577/Y578, S624, S657, K811. The circles indicate phosphorylation (P), and the diamond indicates acetylation (Ac).

## Hypothesis

ZEB1 acetylation regulates dimerization and protein stability to promote lung adenocarcinoma progression and metastasis

## Results

### K811 acetylation regulates ZEB1 dimerization and protein stability

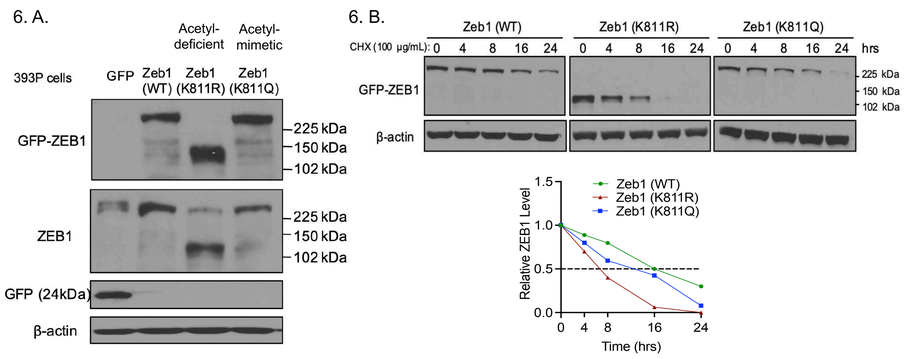



Figure 6. (A) GFP-vector, GFP-ZEB1, GFP-ZEB1_K811R (acetyl-deficient), and GFP-ZEB1_K811Q (acetyl-mimetic) were co-transfected into 393P cells and induced with doxycycline for 24 h. Blots for GFP, ZEB1 and β-actin confirm that ZEB1 WT and acetyl-mimetic mutant are a 250 kDa dimer while the acetyl-deficient mutant is a 125 kDa monomer. (B) GFP-ZEB1 acetylation mutant and control 393P cells were treated with 100 μg/ml cycloheximide (CHX), collected for the indicated time point, and immunoblotted with antibodies against GFP and β-actin. Densities of ZEB1 bands were measured using ImageJ software and normalized to the expression of actin (loading control). The value of normalized ZEB1 at time 0 was set at 1.0. Blots and graphs confirm that 250 kDa ZEB1 band prolongs half-life.

### K811 acetylation protects ZEB1 from proteasomal degradation by the action of the E3 Ubiquitin Ligase (UBL) SIAH1

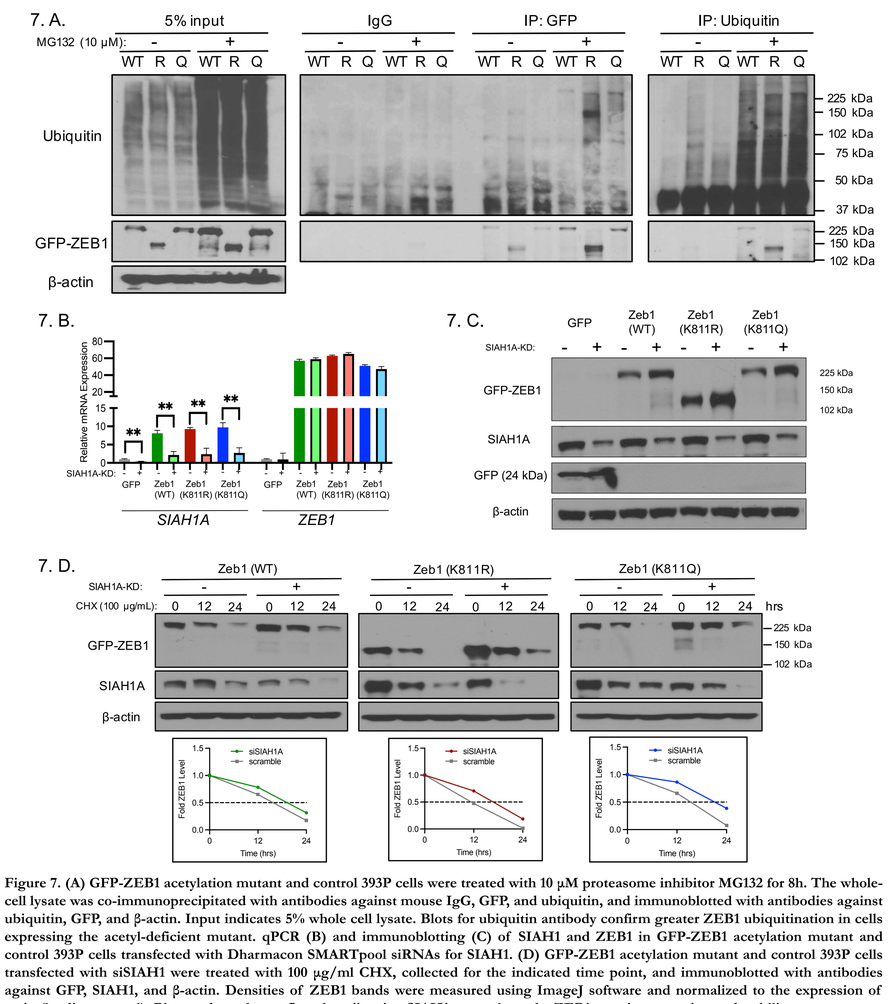



Figure 7. (A) GFP-ZEB1 acetylation mutant and control 393P cells were treated with 10 μM proteasome inhibitor MG132 for 8h. The whole-cell lysate was co-immunoprecipitated with antibodies against mouse IgG, GFP, and ubiquitin, and immunoblotted with antibodies against ubiquitin, GFP, and β-actin. Input indicates 5% whole cell lysate. Blots for ubiquitin antibody confirm greater ZEB1 ubiquitination in cells expressing the acetyl-deficient mutant. qPCR (B) and immunoblotting (C) of SIAH1 and ZEB1 in GFP-ZEB1 acetylation mutant and control 393P cells transfected with Dharmacon SMARTpool siRNAs for SIAH1. (D) GFP-ZEB1 acetylation mutant and control 393P cells transfected with siSIAH1 were treated with 100 μg/ml CHX, collected for the indicated time point, and immunoblotted with antibodies against GFP, SIAH1, and β-actin. Densities of ZEB1 bands were measured using ImageJ software and normalized to the expression of actin (loading control). Blots and graphs confirm that silencing SIAH1 upregulates the ZEB1 protein expression and stability.

### Dimerization facilitates ZEB1/NuRD complex interaction and binding at the promoter of its target genes

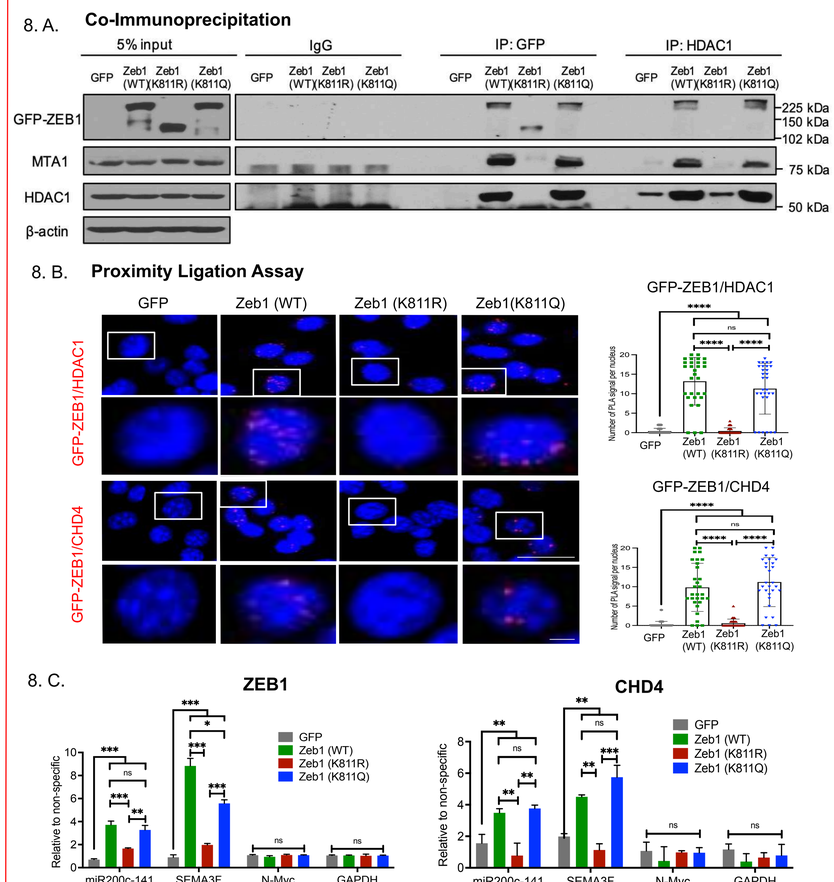



Figure 8. (A) GFP-ZEB1 acetylation mutant and control 393P cells were co-immunoprecipitated with antibodies against mouse IgG, GFP, and HDAC1, and immunoblotted with antibodies against GFP, MTA1, HDAC1, and β-actin. Blots confirm that ZEB1 dimers interact with the NuRD complex. (B) GFP-ZEB1 acetylation mutant and control 393P cells were probed with ZEB1 and NuRD complex members antibodies using the Duolink proximity ligation assay (PLA). Images and graphs confirm the ZEB1 dimer/NuRD interaction in situ. (C) ChIP with antibodies against ZEB1, CHD4 and control IgG in GFP-ZEB1 acetylation mutant and control 393P cells. ****P < 0.0001; ***P < 0.005; **P < 0.001; *P < 0.05.

### K811 acetylation promotes NSCLC invasion *in vitro* and metastasis *in vivo*

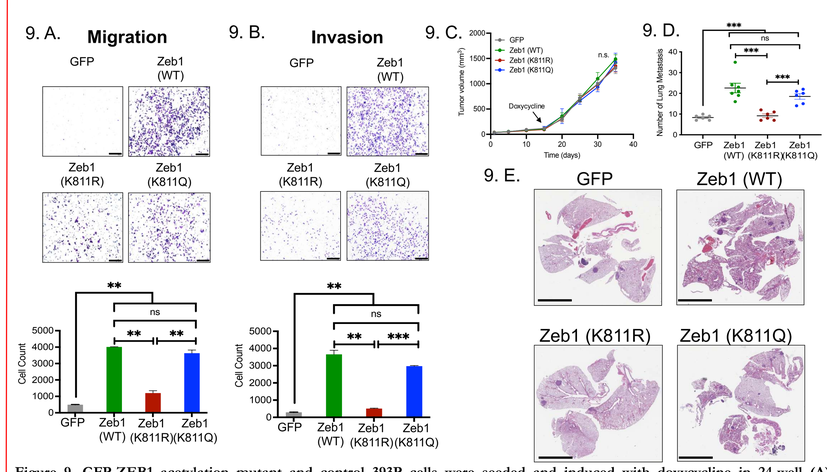
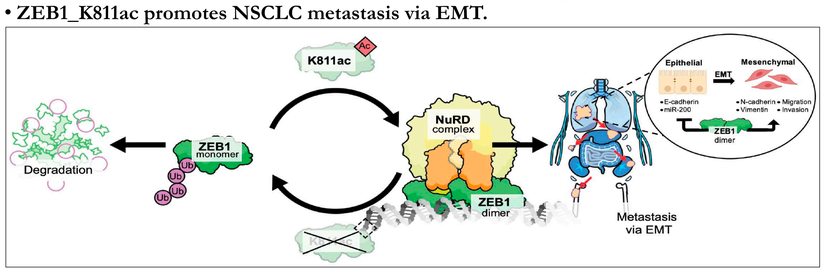



Figure 9. GFP-ZEB1 acetylation mutant and control 393P cells were seeded and induced with doxycycline in 24-well (A) chemotaxis or (B) haptotaxis chambers. (C) GFP-ZEB1 acetylation mutant and control 344SQ cells were subcutaneously implanted into the flank of syngeneic WT 129Sv mice. The growth of the primary tumor was measured. Once tumors reached a volume of 100 mm$^3$, we gave the mice doxycycline, and when the tumors reached 1500 mm$^3$, we killed the mice via $CO_2$. (D) Metastatic lung nodules were quantified and (E) validated by hematoxylin and eosin staining of paraffin-embedded lung tissue.

## Summary and working model

- K811 acetylation regulates ZEB1 dimerization and protein stability.
- Acetylation protects ZEB1 from proteasomal degradation by the action of the UBL SIAH1.
- Dimerization facilitates recruitment of the NuRD complex to genomic promoters.
- ZEB1_K811ac promotes NSCLC metastasis via EMT.




## Acknowledgements

This research is funded by the Ruth L. Kirschstein National Research Service Award Individual Pre-doctoral Fellowship to Promote Diversity in Health-Related Research (NCI 1F31CA268343-01). M. Perez-Oquendo acknowledges Drs. Gibbons and Barton, as well as her advisory committee for their continued mentorship and support throughout this project.