Transactivation of Lung Lysozyme Expression by Ets Family Member ESE-1

Infectious Diseases Program, Lovelace Respiratory Research Institute, Albuquerque, NM

Wanli Lei, Richard J. Jaramillo and Kevin S. Harrod*

Running title: ESE-1 transactivates human lysozyme gene

Correspondent:

*Kevin S. Harrod, PhD

Infectious Disease Program

Lovelace Respiratory Research Institute

2425 Ridgecrest Drive, SE

Albuquerque, NM 87108

Ph: 505-348-9488

Fax: 505-348-8567

Email: kharrod@lrri.org

ABSTRACT

Epithelial-specific Ets (ESE) transcription factors, consisting of ESE-1, ESE-2, and ESE-3, are constitutively expressed in distinct epithelia of mucosal tissues, including the lung. Each ESE member exhibits alternative splicing and yields at least two isoforms (a and b) with transcriptional targets largely unidentified. The studies described herein define a novel role for ESE transcription factors in transactivation of the human lysozyme gene (*LYZ*), an essential component of innate defense in lung epithelia. Of the six ESE isoforms, ESE-1a and ESE-1b transactivated *LYZ* promoter in reporter gene assays, while only ESE-1b dramatically upregulated transcription of endogenous *LYZ* in both nonpulmonary and pulmonary epithelial cells. Importantly, ESE-1a and ESE-1b could transactivate the *LYZ* promoter in cultured primary airway epithelial cells. ESE-2 and ESE-3 isoforms were unable to substantially transactivate the lysozyme promoter or up-regulate transcription of endogenous *LYZ*. Two functional consensus Ets sites located in the proximal 130bp *LYZ* promoter were responsive to ESE-1b as identified by site-directed mutagenesis and DNA binding assays. Short hairpin RNA (shRNA) attenuation of endogenous ESE-1b mRNA levels in lung epithelia resulted in decreased *LYZ* transcription. Furthermore, ESE-1 antibody specifically enriched the 130bp proximal *LYZ* promoter in chromatin immunoprecipitation analyses. These findings define a novel role for ESE transcription factors in regulating lung innate defense and suggest distinct regulatory functions for ESE family members.

Keywords: airway epithelia; Ets proteins; transcriptional regulation; lysozyme; mucosal innate defense.

INTRODUCTION

Gene expression is tightly controlled, in part, through transcription factors binding to DNA motifs(21). In different tissue subsets, the transcriptomes are imparted by expression or functional control of distinct transcriptional regulators. Ets (E26 transforming specific) proteins are a superfamily of transcription factors containing a highly conserved "Ets domain" that recognizes the core motif GGA, with specificities further defined by flanking nucleotide sequences(19, 27). Based on amino acid identities of the Ets domain and the presence of other conserved domains, such as the pointed domain, Ets proteins can be classified into several subfamilies(27), including the newly emerging subfamily epithelial-specific Ets (ESE) proteins. Consisting of three distinct genes, *ESE-1*(1, 8, 10, 23, 30), *ESE-2*(24, 33), and *ESE-3*(4, 15, 29), each ESE member yields at least two isoforms (**a** and **b**) through alternative splicing(15, 23, 24).

ESE proteins are constitutively expressed in many subtypes of mucosal epithelia with highest expression found in the airway epithelia(14). Their transcriptional targets in these uniquely differentiated cells have not been well defined. Mucosal epithelia are essential components of innate immunity, not only providing an anatomic barrier, but also contributing actively to host defense by secreting a plethora of proteins such as lysozyme and lactoferrin(3, 12, 28). Transcriptional mechanisms coordinating the expression of these host-defense proteins in mucosal epithelia are poorly understood. Various reports of Ets family members regulating expression of host defense genes are suggestive, but definitive elucidation of mechanisms in the lung epithelium are not defined yet.

Herein, *LYZ* is identified as a transactivation target of ESE-1, but not ESE-2 and ESE-3 isoforms. Specifically, ESE-1b transactivates the *LYZ* promoter through two consensus Ets binding sites in the proximal promoter region. We also provide evidence that ESE isoforms have differential

regulating potentials on the *LYZ* promoter. These studies provide a foundation for elucidating the transcriptional networks in mucosal tissues, such as regulating coordinated host defense gene expression.

MATERIALS AND METHODS

Cell Culture, Transfection, and Reporter Gene Assays —Normal human bronchial epithelial cells (NHBE) were purchased from Lonza and kept in BEBM (Cambrex # CC-3171) without retinoic acid (RA); air-liquid interface (ALI) culture of NHBE cells were carried out with protocols provided in mixture of BEBM and DMEM-H (Sigma # D6429) at the ration of 1:1. The epithelial cell lines A549, H292 and H441, immortalized or transformed cells of human lung epithelial origin, were maintained in RPMI 1640 (Invitrogen) medium containing 10% fetal bovine serum (FBS); Human cervical epithelial cells (HeLa) were maintained in Advanced MEM (Invitrogen) containing 10% FBS. Cells were seeded into 48-well plates (2×10^4 cells per well) 24 hours before the experiment and transfected with FuGENE 6 (Roche) as recommended by the manufacturer. Typically, 100 ng of reporter plasmid, 25 ng of ESE expression vector, and various amount of empty expression vector was added to total 200 ng per well. For competition assays, 25 ng each of ESE-1b and ESE-2 or ESE-3 isoform expression vector were co-transfected. Twenty-four hours after transfection, cells were washed once with cold phosphate buffered saline (PBS), followed by adding 100 μl 1X lysis buffer (Promega) per well and kept at -70˚C for 1 hour. After 30 minutes of rotation at room temperature, 5 μl of lysates were mixed with 25 μl of substrate (Promega) to detect luciferase activity. Because ESE factors affect transcriptional activity of CMV or TK promoters, we omitted internal control plasmid based on these promoters and carried out the transfections in duplicate; each experiment was repeated at least three times.

Construction of ESE Protein Expression Vectors — ESE-1 and ESE-3 cDNAs were isolated from H441 cells; ESE-2a and ESE-2b cDNAs were isolated from human lung RNA samples. All acquired cDNAs were FLAG-tagged through PCR. RNAs were extracted from H441 cells and

human lung tissue with RNeasy mini kits (Qiagen) according to the protocol provided. First-strand cDNA was synthesized with Superscript First Strand Synthesis System (Invitrogen) and amplified with AccuPrime Taq polymerase (Invitrogen). The primers used are as follows (restriction enzyme sites are shown in italics, and the antisense of FLAG-tag coding sequences are underlined): ESE-1 Upstream:5′-a*aagctt*atggctgcaacctgtgag-3′ (HindIII); Down-stream: 5′-a*ctaga*tcacttatcgtcgtcatccttgtaatcgttccgactctggagaacc-3′ (XbaI); ESE-2a Upstream: 5′-a*ggatcc*atgccatctctgcctcactc-3′ (BamHI); ESE-2b Upstream: 5′-a*ggatcc*Atgttggactcggtgacacac-3′ (BamHI); ESE-2 Downstream: 5′-a*gaattc*tcacttatcgtcgtcatccttgtaatctagcttgtcttcctgccacc-3′ (EcoRI); ESE-3 Upstream: 5′-a*ggatcc*atgattctggaaggaggtgg-3′ (BamHI); ESE-3 Down-stream: 5′-a*ctcgag*tcacttatcgtcgtcatccttgtaatcgttttcattttctctccatcctc-3′ (XhoI). The cDNAs of alternative spliced isoform ESE-1a and ESE-3a were acquired through overlapping/splicing by PCR. PCR products were cloned into pGEM-T vector (Promega) and verified through sequencing. Corresponding cDNAs were subsequently transferred into pcDNA3 (Invitrogen) to achieve an expression vector for each ESE protein. Over-expressed FLAG-tagged ESE-1 proteins were detected with immunoblotting as described in reference(18).

Construction of LYZ Reporter Plasmids and Site-directed Mutagenesis — DNA was extracted from H441 cells with TRIZOL (Invitrogen) according to the protocol provided by the manufacturer and used as the template in the PCR with primers hLysoS (5′-aggtaccactggcctaacaccctatct-3′) and hLysoAS (5′-aaagctttgctaggctgaccagggtg-3′) to isolate the 3-kb promoter sequence of *LYZ* (KpnI and HindIII sites are underlined). After cloning into the pGEM-T vector the sequence was verified through sequencing, the co-rresponding DNA fragment was inserted into pGL3 Basic (Promega) between the KpnI and HindIII sites to get the reporter construct $^{3.0}$hLyso. Deletions within the truncated *LYZ* promoter conferring expression of the *luc* gene were acquired through enzyme digestion and treatment ($^{1.6}$hLyso at PvuII, $^{0.7}$hLyso at

SmaI, $^{0.38}$hLyso at NsiI and $^{0.13}$hLyso was constructed through PCR). Transcription factor motifs were analyzed using software MATCH10.0 provided within the TRANSFAC database, and altered through PCR-based site-directed mutagenesis. In brief, 200 ng of purified $^{0.38}$hLyso plasmid was amplified using Pfx DNA polymerase (Invitrogen) with corresponding mutation primer pairs as listed in Table 1 for 14 cycles. Purified PCR products were digested with DpnI for 4 hours and the precipitated DNAs were used to transform XL-2 Blue competent cells (Stratagene). The mutated sites were verified through sequencing.

Electrophoretic Mobility Shift Assay (EMSA) — Due to dozens of Ets proteins expressed in all kinds of epithelial cells, HeLa cells were transfected with pcDNA3 or ESE-1a and ESE-1b expression vector using DreamFect (OZ Biosciences) with the ratio of plasmid to DreamFect at 1:5. Nuclear proteins were extracted with NE-Per Nuclear and Cytoplasmic Extraction Reagents (Pierce) and quantified with Bradford reagent purchased from Bio-Rad. Oligos were tagged with a Biotin 3' End DNA Labeling kit (Pierce) and annealed. EMSA was performed by pre-incubating each DNA binding reaction (5 mM Tris, 0.5 mM DTT, 0.05 μg/μl polydI:dC, 5 mM $MgCl_2$, 80 mM KCl, 0.05% NP-40, 10 mM EDTA, 2.5% glycerol and 10 μg extracted nuclear proteins) at room temperature for 20 minutes followed by adding 2 μl of biotin-labeled oligos and incubating at room temperature for an additional 20 minutes. For supershift assay and competition assays, 1 μl of anti-FLAG monoclonal antibody (Sigma) or 100X unlabeled oligos were added into the corresponding binding reaction during pre-incubation. Twenty microliters of binding mixture containing 1X-loading buffer was separated on 5% polyacrylamide gel in 1X Tris-Borate-EDTA (TBE) at 100 V and oligos were electro-transferred to a HyBond nylon membrane (GE Healthcare) in 0.5X TBE. After crosslinking with UV Stratalinker (Stratagene), the biotin-labeled oligos were detected with a LightShift^{TM} Chemiluminescent EMSA Kit (Pierce).

RT-PCR, semi-quantitative RT-PCR and quantitative PCR Analysis — To analyze the transcription of the endogenous *LYZ*, RNAs were extracted directly from NHBE, HeLa, A549, H292 and H441 cells with the RNeasy mini kit (Qiagen) and used for RT-PCR analysis. One well of ALI cells cultured two weeks were used for RNA extraction with TRIZOL (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA was used to synthesize the first-strand cDNA with QuantiTect Reverse Transcription Kit (Qiagen). FastStart Taq DNA Polymerase (Roche) was used to amplify full *LYZ* coding sequence (CDS) with primers FF (ctgacctagcagtcaacatg) and FR (ctggagttacactccacaac). In parallel, full *GAPDH* CDS was amplified as an internal control with primer GS (atcactgccacccagaagac) and primer GR (ttactccttggaggccatgtg). To assess the effects of ESE proteins on the endogenous *LYZ*, $2x10^5$ per well of cells were seeded into 6-well plates 1 day prior to transfection with 500 ng of empty pcDNA3 or corresponding ESE expression vectors. RNAs were extracted 24 hours after transfection with TRIZOL and reverse transcribed with QuantiTect Reverse Transcription Kit (Qiagen). Due to multiple transcripts containing partial *LYZ* coding sequence detected from the epithelial lineage cells, a semi-quantitative RT-PCR procedure was employed as reported (20) to co-amplify full *LYZ* CDS and *GAPDH* with optimized primers at 300nM of each FF and FR *vs.* 50nM each of QGF (cgtggaaggactcatgacca) and QGR (gccatcacgccacagtttc). Densities of ethidium stained PCR bands were analyzed with software provided by Bio-Rad and normalized to corresponding internal controls. QuantiTect SYBR Green PCR Kit (Qiagen) was used to detect mRNA levels of ESE-1 and used in chromatin immunoprecipitation assay with ABI 7300 real time PCR system (Applied Biosystems). The quantitative PCR results were analyzed with software provided. All assays were repeated at least three times.

Lentivirus-mediated delivery of ESE-1 shRNA. The 293TN packaging cells (System Biosciences) were co-transfected with lentiviral vector pGIPZ (empty) or V2LHS_17990 (encoding ESE-1

shRNA) (Open Biosystems) and packaging plasmids psPAX2/pMD2.G (Addgene) according to reference(34). The packaged lentiviral vectors were transduced into A549 cells in the presence of polybrene (6ug/ml, Millipore) for 6 hours, followed by selecting with puromycin (3ug/ml). Total RNAs were extracted from the survived A549 cells and used for quantitative RT-PCR to detect ESE-1 mRNA levels with primer EQF (gccagatacctcagcgctac) and EQR (ggacacctacgctcttgct c) or used for semi-quantitative RT-PCR to detect lysozyme mRNAs as described above.

Chromatin immunoprecipitation analyses (ChIP) . ChIP analyses were carried out with ChIP-IT™ Express Enzymatic kit (Active Motif) according to the protocol provided by manufacturer. In brief, three 15cm dishes of A549 cells (90% confluence) were fixed with 1% formaldehyde for 10 minutes at room temperature and neutralized with 0.125M glycine. Released nuclei from collected cells were enzymatically treated at 37°C for 10 minutes in 1ml volume and 50µl of sheared chromatins were used for immunoprecipitation with normal rabbit serum or polyclonal antibody against ESE-1 (Orbigen). The 110bp *LYZ* proximal promoter was amplified with primers LPF (gagtcagtggatcaatagacagttcctg) and LPR (taggctgaccagggtgagctgg). The 130bp secretoglobin 3A2 promoter contains no consensus Ets sites and was amplified with primers SPF (accctccaaattgtttggtgagaa) and SPR (gcacacacatatttatatgcccattgagg) as negative control to verify the specific enrichment of *LYZ* proximal promoter fragments by ESE-1 antibody. All ChIP experiments were repeated in triplicate. Specific enrichment of LYZ promoter by ESE-1 antibody was quantified as described above and normalized to the non-specific precipitation of SCGB3A2 promoter, which does not contain Ets sites.

RESULTS

3.0kb LYZ promoter is active in various epithelial cells — Lysozyme is constitutively expressed in some subtypes of mucosa epithelia(3, 11, 28). To determine if the human lysozyme gene (*LYZ*, GeneBank accession number: NM_000239) is expressed in NHBE and cell lines derived from nonpulmonary (HeLa) as well as pulmonary epithelia (A549, H292, and H441), RT-PCR was carried out with RNAs extracted from these cells as templates. The full *LYZ* CDS could not be detected in undifferentiated NHBE and HeLa cells, but was readily detected in A549, H441 and H292 cells (Fig. 1A). Interestingly, the differentiated NHBE cells express higher levels of *LYZ* mRNAs as detected after two weeks of ALI culture (Fig. 1B). To assess *LYZ* promoter function across different epithelial cells, a reporter construct containing 3.0 kb of the *LYZ* promoter ($^{3.0}$hLyso) was constructed to confer the expression of firefly luciferase gene (*luc*). This reporter construct exhibited a strong basic transcriptional activity in all cells studied (Fig. 1C), indicating there are positive regulators for *LYZ* promoter activity in pulmonary and nonpulmonary epithelial cells. These findings suggest that other mechanisms beyond transcriptional activation may regulate *LYZ* expression across various epithelial cells. Furthermore, these findings provide a basis for studying transcription factor mediated regulation of *LYZ*.

Transactivation of the LYZ Promoter by ESE-1 — ESE proteins are potential candidates for regulating transcription of *LYZ* due to the co-expression pattern in many mucosal epithelia and numerous putative Ets binding sites located upstream of the *LYZ* transcription start site. Expression vectors for the six ESE family isoforms were constructed as detailed in Materials and Methods and used for co-transfection studies with $^{3.0}$hLyso to assess their effects on the *LYZ* promoter. As shown in Fig. 2A, ESE-1a and ESE-1b dramatically transactivated the *LYZ* promoter in NHBE, A549 and HeLa cells with ESE-1a exhibiting weaker transactivating

capacities, whereas ESE-2 and ESE-3 isoforms differentially transactivated this reporter construct in different cells, yet never reaching the levels of transactivation induced by ESE-1.

To test if the ESE proteins could transactivate endogenous *LYZ*, an individual ESE expression vector was transfected into A549 and HeLa cells. We optimized a semi-quantitative RT-PCR protocol based on the primer FF and FR to co-amplify the full length *LYZ* CDS and internal control *GAPDH* with QGF and QGR. As shown in Fig. 2B, ESE-1b markedly upregulated transcription of endogenous *LYZ* in both A549 and HeLa cells. ESE-2 and ESE-3 isoforms could not up-regulate the basal transcription of endogenous *LYZ* in either cell line. The differential transactivating capacities of ESE-1a and ESE-1b are not related to their protein levels as detected by immunoblotting (Fig. 2C). These findings using both promoter-driven reporter assays and endogenous gene expression indicate that ESE-1 isoforms, particular ESE-1b, can induce *LYZ* expression in mucosal epithelial cells.

Attenuation of ESE-1b Transactivation by Other ESE Members — Ets transcription factors are known to bind overlapping consensus DNA motifs(27), suggesting that competition between co-expressed ESE members may have biological significance, such as the ablation of *LYZ* transcription in A549 cells. To test this in *LYZ* promoter regulation, ESE-2 and ESE-3 isoform expression vectors were co-transfected in conjunction with the ESE-1b expression vector and $^{3.0}$hLyso. Co-expression of ESE-2a, ESE-3a, or ESE-3b reduced the transactivation of the *LYZ* promoter by ESE-1b in both A549 and HeLa cells (Fig. 3). Interestingly, co-expression of ESE-2b with ESE-1b did not alter transactivation of the *LYZ* promoter in A549 cells but was able to attenuate ESE-1b transactivation in HeLa cells, suggesting ESE-2b may have unique cell-specific mechanisms for transactivation of the *LYZ* promoter. The overall findings suggest that

competition by multiple ESE family members for distinct Ets consensus sites may not be a critical mechanism in ESE-mediated transcriptional regulation.

Identification of the Proximal Promoter Sequences Responsive to ESE-1b — To map the corresponding ESE-1b responsive *cis*-element(s) in the *LYZ* promoter, reporter plasmids containing truncated *LYZ* promoters conferring the *luc* reporter gene were constructed (Fig. 4A). Each of the truncated promoter constructs was functional in HeLa, A549, H292, and H441 cells (data not shown). The 0.13 kb truncated promoter was sufficient for transactivation by ESE-1b in both A549 and HeLa cells (Fig. 4B). Interestingly, the 3.0 kb and 1.7 kb promoters exhibited diminished transactivation by ESE-1b in HeLa cells but not in A549 cells, suggesting that there are potential inhibitory elements functional in the 1.7-3.0kb upstream region in HeLa but not A549 cells. Regardless, the findings from these experiments indicate that the proximal 130 bp *LYZ* promoter contains *cis*-element(s) responsive to ESE-1b in both pulmonary and nonpulmonary cell lineages.

Two putative Ets sites within the 130 bp proximal *LYZ* promoter were identified computationally using Match10.0 of the TRANSFAC database, and these core motifs were designated as motifs I and II with motif I being the most proximal to the transcription start site (Fig. 4C). To assess whether these sites are responsive to ESE-1b, site-directed mutagenesis was used to alter the core-binding sequence in each putative site with oligos shown in Table 1. Mutation of either motif I or II abolished the transactivation by ESE-1b in A549 cells (Fig. 4D). Similar results were obtained in HeLa cells, with the mutation of motif II showing somewhat less functionality in ESE-1b transactivation than that for motif I. Mutation of either Ets sites resulted to decreased basic transcriptional activities and loss of ESE-1b transactivation in NHBE cells (Fig. 4E), strongly supporting a role for ESE-1b transactivation of the *LYZ* promoter in primary lung

epithelial cells. These findings indicate that both Ets sites located at 39 bp (site I) and 84 bp (site II) upstream of the transcription start site are important for ESE-1b to transactivate *LYZ* promoter in pulmonary or nonpulmonary epithelial cells.

ESE-1b Binding of Functional Ets Sites — Electrophoretic mobility shift assays were carried out to assess whether ESE-1b could directly bind the two putative Ets sites identified by site-directed mutagenesis. Nuclear proteins from HeLa cells transfected with ESE-1b expression plasmid formed a higher shifted complex with site I or site II oligos (Fig. 5, arrowhead; sequence of oligos are shown in Table 1). Competition with 100X extra unlabeled *wt* site I and site II oligos abolished all the bands shifted as expected, while competition with the *mut* site I or site II oligos had little effect on the larger shifted complex. In the absence of available antibodies for supershift analyses, the ESE-1b expression vector was constructed as detailed in Materials and Methods with a FLAG peptide tagged on the C-terminus. Addition of anti-FLAG antibody specifically abolished binding of the *wt* oligo due to the localization of the FLAG epitope adjacent to the DNA binding domain on the C-terminus of ESE-1b. ESE-1a showed similar binding patterns on site I and site II to that of ESE-1b (data not shown), suggesting that the reduced transactivation of the *LYZ* promoter by ESE-1a is likely not related to its DNA binding activities.

Endogenous ESE-1b transactivates LYZ. High levels of ESE-1b but not ESE-1a mRNAs could be detected in A549 cells (data not shown). To assess if endogenous ESE-1b could regulate endogenous *LYZ* expression, siRNA mediated knock-down studies were performed. A549 cells were transduced with empty or ESE-1 shRNA encoded lentiviral vectors as detailed in Materials and Methods. The shRNA recognizes both ESE-1a and ESE-1b mRNA targets; however, ESE-1b has much greater expression than ESE-1a in A549 cells (data not shown). As shown in Fig. 6A, the *LYZ* mRNA levels decreased concomitantly with knockdown of ESE-1 mRNA, indicating

endogenous *LYZ* is a transcriptional target of ESE-1b. To further assess ESE-1b binding to the endogenous *LYZ* promoter, chromatin immunoprecipitation was employed as detailed in Materials and Methods. The rabbit polyclonal antibody against ESE-1 (α ESE-1), but not the normal rabbit sera (Normal), specifically enriched the 110bp *LYZ* promoter containing the ESE-1b responsive elements. The 110bp proximal secretoglobin 3A2 promoter contains no consensus Ets sites and was used as a negative control and no differences could be detected, indicating the specific enrichment of *LYZ* proximal promoter by ESE-1 antibody. These findings together support that endogenous *LYZ* is a target for endogenous ESE-1b transactivation in lung epithelial cells.

DISCUSSION

The findings presented here indicate the *LYZ* promoter as a target for the highly tissue-restricted Ets transcription factor ESE-1 in lung epithelia. Interestingly, isoforms encoded by ESE-1 exhibited differential transactivating capacities, with ESE-1b showing greater transactivation activity than ESE-1a in lung epithelial cells. Transactivation of the *LYZ* promoter by ESE-1b was confined to the proximal 130 bp promoter region containing two functional Ets sites as identified by site-directed mutagenesis analysis and DNA binding assays. Isoforms encoded by ESE-2 or ESE-3 activated the *LYZ* promoter only marginally. Studies utilizing experimental approaches of both over-expression and endogenous ESE-1b proteins on regulating *LYZ* confirmed these findings. These studies provide strong evidence for the role of the ESE-1 in conferring *LYZ* expression in epithelial lineage cell types and extend our knowledge of transcriptional mechanisms regulating lung epithelial-specific gene expression.

The ESE transcription factors constitute an emerging subgroup of the Ets family of winged helix-turn-helix DNA-binding proteins(27). Of the three ESE members, the first identified protein ESE-1 has been well studied and shown to have multiple functions. ESE-1 can transactivate numerous genes including squamous differentiation marker *SPRR1B*(25), *angiopoietin-1*(5), *transforming growth factor-β type II receptor*(9, 10, 16), *inducible nitric-oxide synthase*(26), *cyclooxgenase 2* (13)and *MIP-3 alpha*(17). Studies relating ESE-1 to tissue functions have primarily focused on regulating differentiation of kerotinocytes(6) and corneal epithelia(32), as well as involution of mammary glands during development(22). The findings presented here indicate a role for ESE-1 in regulation of lung epithelial gene expression. Despite the nomenclature of ESE, recent reports indicate ESE family members are expressed in non-epithelial cell types(2, 13, 31), suggesting that their function may extend beyond tissues of epithelial origin. In light of this and the notion that

lysozyme can be expressed by non-epithelial cells under certain activated conditions, other transcriptional mechanisms may exist for *LYZ* gene expression.

Although ESE members demonstrate different target-gene specificity(15), the studies presented here also indicate that ESE isoforms probably have unique roles as well. For example, ESE-1b markedly upregulated transcription of endogenous *LYZ* and *LYZ* promoter activity in both pulmonary and nonpulmonary epithelial subsets, whereas ESE-1a only marginally upregulated transcription of endogenous *LYZ* in nonpulmonary epithelial cells. Compared with ESE-1a, ESE-1b has 23 additional amino acids (23)following the acidic transactivation domain encoded by exon 4(7). This additional stretch of peptide sequence appears to confer stronger transactivating capacities to ESE-1b on *LYZ* in multiple epithelial cell types. Compared with ESE-2 or ESE-3 isoforms, ESE-1 isoforms have an additional DNA binding domain designated as the "A/T hook" located between the N-terminal "pointed domain" and the C-terminal "Ets domain"(1, 23). The role of this A/T hook domain in the *LYZ* promoter requires further investigation; however, the findings presented here would suggest this region to be critically important in *LYZ* transactivation.

One critical element in defining the role of novel transcription factors is the delineation of their *cis*-elements for future comparisons across multiple promoters. Ets proteins have a highly conserved DNA binding domain, suggesting these transcription factors may bind similar consensus *cis*-element(s)(27). However, a recent report indicates that different Ets proteins can bind and transactivate unique sets of promoters(14). Our findings reiterate the importance of functional analysis specific to each gene target. In addition, our results are consistent with the notion that ESE transactivation is specific to certain family members with regards to *LYZ* transactivation, and such specificity may be defined by the binding of other transcription factors

to flanking sequences adjacent to the Ets motifs. Indeed, DNA binding studies of Ets consensus motifs identified nuclear proteins that bound to elements aside from Ets sites. Future identification of ESE-1 responsive gene promoters will assist in the verification of specific consensus sites and interactions with other transcription factors.

The data presented here provide new insights into the role of ESE transcription factors in regulation of host-defense genes in mucosal tissues. Further studies will be necessary to assess whether ESE-mediated regulation extends to other epithelial-specific host defense components such as defensins, chitinases and other antimicrobial proteins. Additionally, further studies will be needed to assess the roles of ESE proteins in conjunction with other transcription factors in epithelial-specific regulatory networks.

FOOTNOTES

[*]**Abbreviations:** Ets, E26-transforming specific; ESE, epithelial specific Ets, *LYZ*: human lysozyme gene; *GAPDH*, human Glyseraldehyde-3-phosphate dehydrogenase gene; EMSA, electrophoretic mobility shift assay; *luc*, firefly luciferase gene; *mut,* mutant; PBS, phosphate buffered saline; TBE ,Tris-Borate-EDTA; *wt*, wild type; shRNA, short hairpin RNA; ChIP, chromatin immunoprecipitation.

ACKNOWLEDGEMENTS

We thank Yong Lin, Juanita M. Martinez, and Albert P. Senft for helpful discussions and Kimberly Ortega for assistance with manuscript preparation. KSH was supported by NIH grants HL071547 and HL067790.

Reference:

1.**Andreoli JM, Jang SI, Chung E, Coticchia CM, Steinert PM, and Markova NG**. The expression of a novel, epithelium-specific ets transcription factor is restricted to the most differentiated layers in the epidermis. *Nucleic Acids Res* 25: 4287-4295, 1997.
2.**Appel S, Bringmann A, Grunebach F, Weck MM, Bauer J, and Brossart P**. Epithelial-specific transcription factor ESE-3 is involved in the development of monocyte-derived DCs. *Blood* 107: 3265-3270, 2006.
3.**Bals R, and Hiemstra PS**. Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. *Eur Respir J* 23: 327-333, 2004.
4.**Bochert MA, Kleinbaum LA, Sun LY, and Burton FH**. Molecular cloning and expression of Ehf, a new member of the ets transcription factor/oncoprotein gene family. *Biochem Biophys Res Commun* 246: 176-181, 1998.
5.**Brown C, Gaspar J, Pettit A, Lee R, Gu X, Wang H, Manning C, Voland C, Goldring SR, Goldring MB, Libermann TA, Gravallese EM, and Oettgen P**. ESE-1 is a novel transcriptional mediator of angiopoietin-1 expression in the setting of inflammation. *J Biol Chem* 279: 12794-12803, 2004.
6.**Cabral A, Fischer DF, Vermeij WP, and Backendorf C**. Distinct functional interactions of human Skn-1 isoforms with Ese-1 during keratinocyte terminal differentiation. *J Biol Chem* 278: 17792-17799, 2003.
7.**Chang CH, Scott GK, Baldwin MA, and Benz CC**. Exon 4-encoded acidic domain in the epithelium-restricted Ets factor, ESX, confers potent transactivating capacity and binds to TATA-binding protein (TBP). *Oncogene* 18: 3682-3695, 1999.
8.**Chang CH, Scott GK, Kuo WL, Xiong X, Suzdaltseva Y, Park JW, Sayre P, Erny K, Collins C, Gray JW, and Benz CC**. ESX: a structurally unique Ets overexpressed early during human breast tumorigenesis. *Oncogene* 14: 1617-1622, 1997.
9.**Chang J, Lee C, Hahm KB, Yi Y, Choi SG, and Kim SJ**. Over-expression of ERT(ESX/ESE-1/ELF3), an ets-related transcription factor, induces endogenous TGF-beta type II receptor expression and restores the TGF-beta signaling pathway in Hs578t human breast cancer cells. *Oncogene* 19: 151-154, 2000.
10.**Choi SG, Yi Y, Kim YS, Kato M, Chang J, Chung HW, Hahm KB, Yang HK, Rhee HH, Bang YJ, and Kim SJ**. A novel ets-related transcription factor, ERT/ESX/ESE-1, regulates expression of the transforming growth factor-beta type II receptor. *J Biol Chem* 273: 110-117, 1998.
11.**Fahlgren A, Hammarstrom S, Danielsson A, and Hammarstrom ML**. Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. *Clin Exp Immunol* 131: 90-101, 2003.
12.**Ganz T**. Antimicrobial polypeptides. *J Leukoc Biol* 75: 34-38, 2004.
13.**Grall FT, Prall WC, Wei W, Gu X, Cho JY, Choy BK, Zerbini LF, Inan MS, Goldring SR, Gravallese EM, Goldring MB, Oettgen P, and Libermann TA**. The Ets

transcription factor ESE-1 mediates induction of the COX-2 gene by LPS in monocytes. *Febs J* 272: 1676-1687, 2005.
14.**Hollenhorst PC, Jones DA, and Graves BJ**. Expression profiles frame the promoter specificity dilemma of the ETS family of transcription factors. *Nucleic Acids Res* 32: 5693-5702, 2004.
15.**Kas K, Finger E, Grall F, Gu X, Akbarali Y, Boltax J, Weiss A, Oettgen P, Kapeller R, and Libermann TA**. ESE-3, a novel member of an epithelium-specific ets transcription factor subfamily, demonstrates different target gene specificity from ESE-1. *J Biol Chem* 275: 2986-2998, 2000.
16.**Kim JH, Wilder PJ, Hou J, Nowling T, and Rizzino A**. Activation of the murine type II transforming growth factor-beta receptor gene: up-regulation and function of the transcription factor Elf-3/Ert/Esx/Ese-1. *J Biol Chem* 277: 17520-17530, 2002.
17.**Kwon JH, Keates S, Simeonidis S, Grall F, Libermann TA, and Keates AC**. ESE-1, an enterocyte-specific Ets transcription factor, regulates MIP-3alpha gene expression in Caco-2 human colonic epithelial cells. *J Biol Chem* 278: 875-884, 2003.
18.**Lei W, Liu F, and Ness SA**. Positive and negative regulation of c-Myb by cyclin D1, cyclin-dependent kinases, and p27 Kip1. *Blood* 105: 3855-3861, 2005.
19.**Li R, Pei H, and Watson DK**. Regulation of Ets function by protein - protein interactions. *Oncogene* 19: 6514-6523, 2000.
20.**Marone M, Mozzetti S, De Ritis D, Pierelli L, and Scambia G**. Semiquantitative RT-PCR analysis to assess the expression levels of multiple transcripts from the same sample. *Biol Proced Online* 3: 19-25, 2001.
21.**Mitchell PJ, and Tjian R**. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* 245: 371-378, 1989.
22.**Neve R, Chang CH, Scott GK, Wong A, Friis RR, Hynes NE, and Benz CC**. The epithelium-specific ets transcription factor ESX is associated with mammary gland development and involution. *Faseb J* 12: 1541-1550, 1998.
23.**Oettgen P, Alani RM, Barcinski MA, Brown L, Akbarali Y, Boltax J, Kunsch C, Munger K, and Libermann TA**. Isolation and characterization of a novel epithelium-specific transcription factor, ESE-1, a member of the ets family. *Mol Cell Biol* 17: 4419-4433, 1997.
24.**Oettgen P, Kas K, Dube A, Gu X, Grall F, Thamrongsak U, Akbarali Y, Finger E, Boltax J, Endress G, Munger K, Kunsch C, and Libermann TA**. Characterization of ESE-2, a novel ESE-1-related Ets transcription factor that is restricted to glandular epithelium and differentiated keratinocytes. *J Biol Chem* 274: 29439-29452, 1999.
25.**Reddy SP, Vuong H, and Adiseshaiah P**. Interplay between proximal and distal promoter elements is required for squamous differentiation marker induction in the bronchial epithelium: role for ESE-1, Sp1, and AP-1 proteins. *J Biol Chem* 278: 21378-21387, 2003.
26.**Rudders S, Gaspar J, Madore R, Voland C, Grall F, Patel A, Pellacani A, Perrella MA, Libermann TA, and Oettgen P**. ESE-1 is a novel transcriptional mediator of inflammation that interacts with NF-kappa B to regulate the inducible nitric-oxide synthase gene. *J Biol Chem* 276: 3302-3309, 2001.
27.**Sharrocks AD**. The ETS-domain transcription factor family. *Nat Rev Mol Cell Biol* 2: 827-837, 2001.

28.**Thompson AB, Robbins RA, Romberger DJ, Sisson JH, Spurzem JR, Teschler H, and Rennard SI**. Immunological functions of the pulmonary epithelium. *Eur Respir J* 8: 127-149, 1995.
29.**Tugores A, Le J, Sorokina I, Snijders AJ, Duyao M, Reddy PS, Carlee L, Ronshaugen M, Mushegian A, Watanaskul T, Chu S, Buckler A, Emtage S, and McCormick MK**. The epithelium-specific ETS protein EHF/ESE-3 is a context-dependent transcriptional repressor downstream of MAPK signaling cascades. *J Biol Chem* 276: 20397-20406, 2001.
30.**Tymms MJ, Ng AY, Thomas RS, Schutte BC, Zhou J, Eyre HJ, Sutherland GR, Seth A, Rosenberg M, Papas T, Debouck C, and Kola I**. A novel epithelial-expressed ETS gene, ELF3: human and murine cDNA sequences, murine genomic organization, human mapping to 1q32.2 and expression in tissues and cancer. *Oncogene* 15: 2449-2462, 1997.
31.**Wang H, Fang R, Cho JY, Libermann TA, and Oettgen P**. Positive and negative modulation of the transcriptional activity of the ETS factor ESE-1 through interaction with p300, CREB-binding protein, and Ku 70/86. *J Biol Chem* 279: 25241-25250, 2004.
32.**Yoshida N, Yoshida S, Araie M, Handa H, and Nabeshima Y**. Ets family transcription factor ESE-1 is expressed in corneal epithelial cells and is involved in their differentiation. *Mech Dev* 97: 27-34, 2000.
33.**Zhou J, Ng AY, Tymms MJ, Jermiin LS, Seth AK, Thomas RS, and Kola I**. A novel transcription factor, ELF5, belongs to the ELF subfamily of ETS genes and maps to human chromosome 11p13-15, a region subject to LOH and rearrangement in human carcinoma cell lines. *Oncogene* 17: 2719-2732, 1998.
34.**Zufferey R, Nagy D, Mandel RJ, Naldini L, and Trono D**. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotechnol* 15: 871-875, 1997.

FIGURE LEGENDS

FIGURE 1. **Transcription of the endogenous *LYZ* and *LYZ* promoter function in pulmonary and nonpulmonary epithelial cells.** (A) Transcription of *LYZ* in different epithelial cells was detected through RT-PCR (35 cycles). Abundance of *GAPDH* was used as an internal control. (B) Full length *LYZ* CDS could be detected in differentiated ALI cultures, but not in the undifferentiated NHBE cells. (C) Basic transcriptional activity of $^{3.0}$hLyso in epithelial cells (hatched bar) was compared to empty reporter vector pGL3 (set to 1.0, black bar). All the reporter assays in this report were carried out in duplicate and repeated at least three times. The data displayed here is a representative sample. Values represent means ± SE.

FIGURE 2. **Transactivation of the *LYZ* promoter by ESE-1.** (A) ESE-1 isoforms transactivate the *LYZ* promoter in A549, HeLa and NHBE cells. Basic transcriptional activity of $^{3.0}$hLyso is set as 1.0. None of the ESE proteins showed obvious transactivation on pGL3 (not shown). (B) The effects of ESE isoforms on transcription of endogenous *LYZ* were analyzed by semi-quantitative RT-PCR (35 cycles), shown in the order of CMV3, ESE-1a, ESE-1b, ESE-2a, ESE-2b, ESE-3a, and ESE-3b. Densitometry was carried out with software Quantity One (Bio-Rad). The fold changes after normalized with corresponding internal control were showed in the bottom. Density of the pcDNA3 treated sample was set as 1 in A549 cells. In HeLa cells, only ESE-1b induced the expression of endogenous *LYZ* and could not be normalized; therefore, its density was set as 1 and others set as 0 indicating a lack of expression. (C) Over expressed ESE-1a and ESE-1b in A549 as well as HeLa cells were detected by immunoblotting with Anti-FLAG monoclonal antibody, β-actin was used as loading control.

FIGURE 3. **Competition between ESE-2 or ESE-3 isoforms and ESE-1b**. Co-transfection of ESE-2 or ESE-3 isoforms with ESE-1b reduced transactivation of the *LYZ* promoter by ESE-1b. ESE-2b showed potentiation in pulmonary cell A549 and ablation in nonpulmonary HeLa cells. Values represent fold changes (means ± SE.).

FIGURE 4. ***Cis*-elements responsive to ESE-1 transactivation are located in the proximal *LYZ* promoter.** (A) Schematic structure of the truncated *LYZ* promoter constructs conferring expression of the *luc* reporter gene. (B) The proximal *LYZ* promoter (130 bp) contains the *cis*-element(s) responsive to ESE-1b transactivation in both A549 and HeLa cells. (C) Two putative Ets sites in the *LYZ* proximal promoter were identified by TRANSFAC software analysis and each core motif was labeled as I and II (see Materials and Methods). (D) Both site I and site II are required for transactivation by ESE-1b in A549 and HeLa cells as assessed by site-directed mutagenesis. (E) Core-motif mutation of either site I or site II resulted in decreased basic transcriptional activities of *LYZ* promoter and lost or decreased response to ESE-1b transactivation in NHBE cells. Values represent fold (means ± SE.).

FIGURE 5. **Binding of ESE-1b proteins with site I and site II.** The FLAG-tagged ESE-1b expression vector was transfected into HeLa cells and nuclear proteins were extracted for gel shift assay. The additional band specifically shifted by ESE-1b is shown next to the arrowhead. Binding of *wt* oligos was wrestled away by excess unlabeled site I or site II oligos, but not by mutated excess unlabelled oligos. Sequence of oligos is shown in Table 1. The anti-FLAG antibody specifically abolished the binding of ESE-1b.

FIGURE 6. **Endogenous ESE-1b regulates *LYZ*.** (A) A549 cells were transduced with lentiviral vectors pGIPZ (empty) or pGIPZ encoding ESE-1 shRNA and total RNAs were used to detect

ESE-1b mRNA through real time quantitative RT-PCR (left panel). *LYZ* mRNA was quantified by densitometry analyses of ethidium bromide stained PCR bands and normalized to that of *GAPDH* (right panel). (B) ChIP analysis of ESE-1b binding to the Ets motifs in the endogenous *LYZ* promoter. Quantitative PCR was carried out to verify the specific enrichment of *LYZ* promoter fragments by antibody against ESE-1 (left Panel). Representative ethidium bromide staining of amplified products after ChIP with normal rabbit serum (Normal) or ESE-1 antibody (α ESE-1). The enzymatically sheared DNAs prior to immunoprecipitation were used as positive control, and 110bp SCGB3A2 promoter was amplified as the negative control (Right Panel).

TABLES

TABLE 1. **Sequences of oligos used for EMSA and mutating putative Ets sites in *LYZ* proximal promoters.**

Site	Wild-type Oglios[a]	Mutant Oglios
I		
Sense	aggggaagaa**gg**aagttaaaagatg	aggggaagaa**tt**aagttaaaagatg
Antisense	catcttttaactt**cc**ttcttcccct	catcttttaactt**aa**ttcttcccct
II		
Sense	caatagacagtt**cc**tgttttccacac	caatagacagtt**tt**tgttttccacac
Antisense	gtgtggaaaaca**gg**aactgtctattg	gtgtggaaaaca**aa**aactgtctattg

[a]The changed nucleotides are shown in boldface type.

Fig. 1

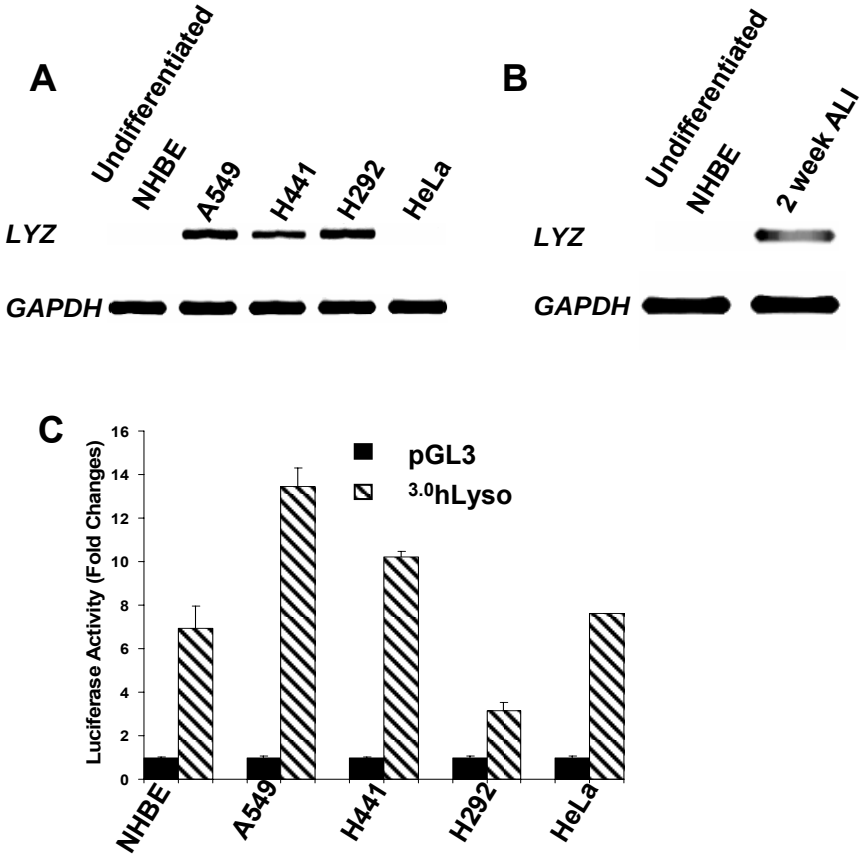
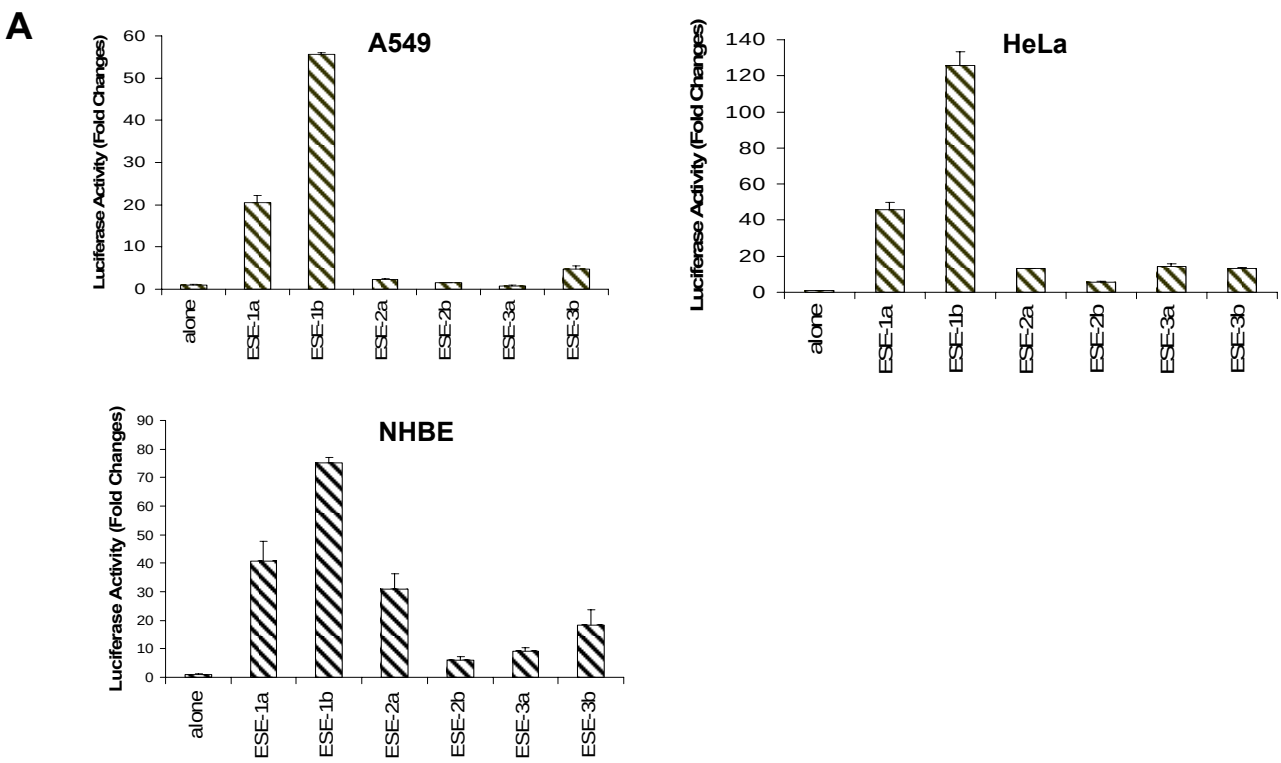

A
Undifferentiated
NHBE
A549
H441
H292
HeLa
LYZ
GAPDH
B
Undifferentiated
NHBE
2 week ALI
LYZ
GAPDH
C
Luciferase Activity (Fold Changes)
pGL3
3.0hLyso
NHBE
A549
H441
H292
HeLa

Fig. 2


A
A549
HeLa
NHBE
Luciferase Activity (Fold Changes)
alone
ESE-1a
ESE-1b
ESE-2a
ESE-2b
ESE-3a
ESE-3b

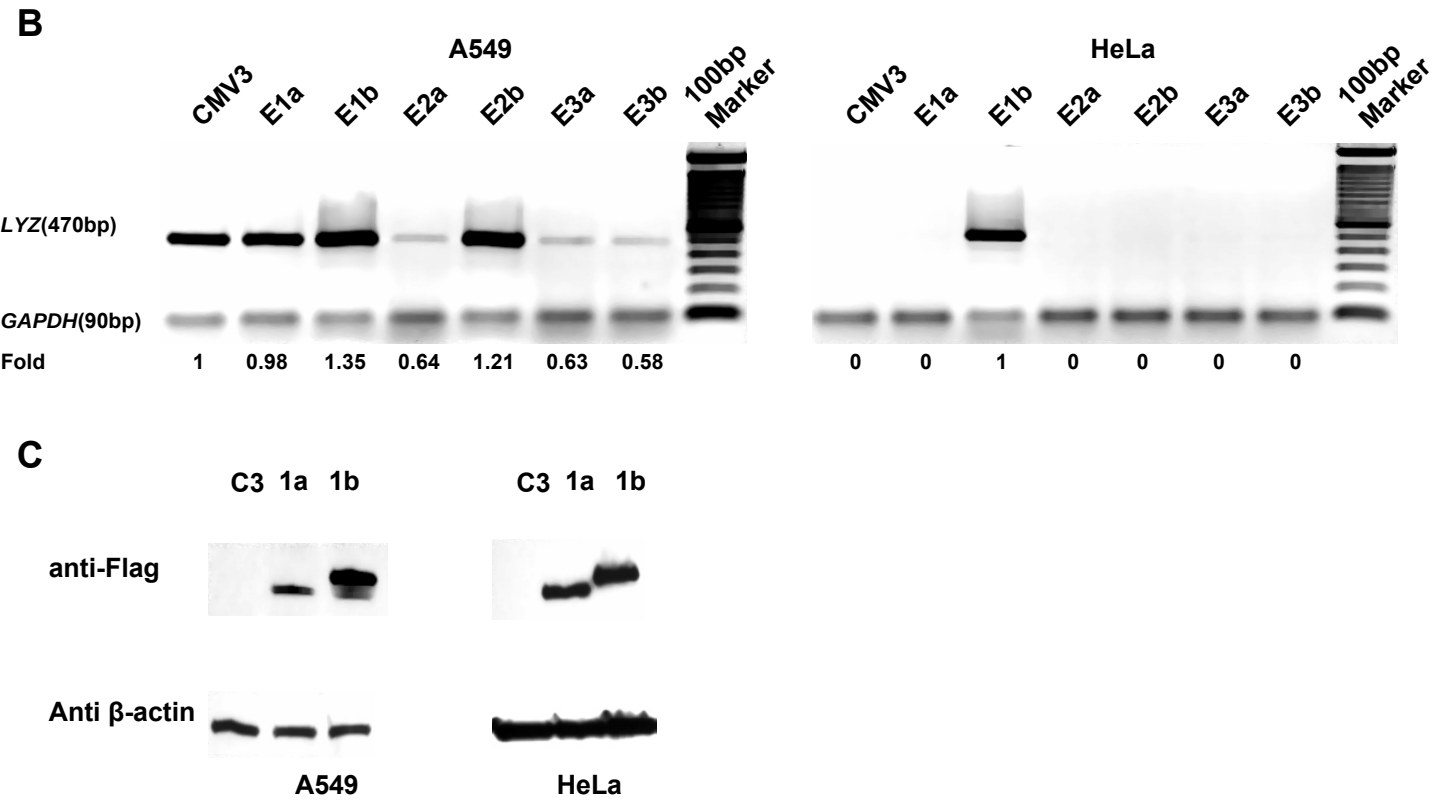

B
A549
HeLa
CMV3
E1a
E1b
E2a
E2b
E3a
E3b
100bp Marker
LYZ(470bp)
GAPDH(90bp)
Fold
1 0.98 1.35 0.64 1.21 0.63 0.58
0 0 1 0 0 0 0
C
C3 1a 1b
C3 1a 1b
anti-Flag
Anti β-actin
A549
HeLa

Fig. 3

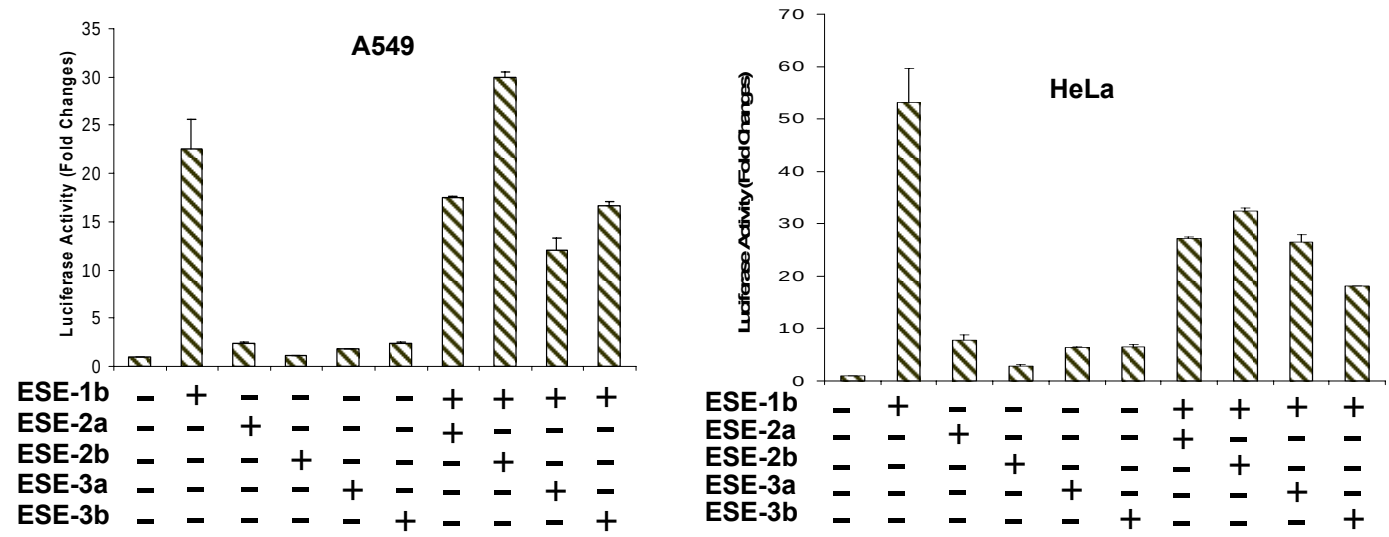
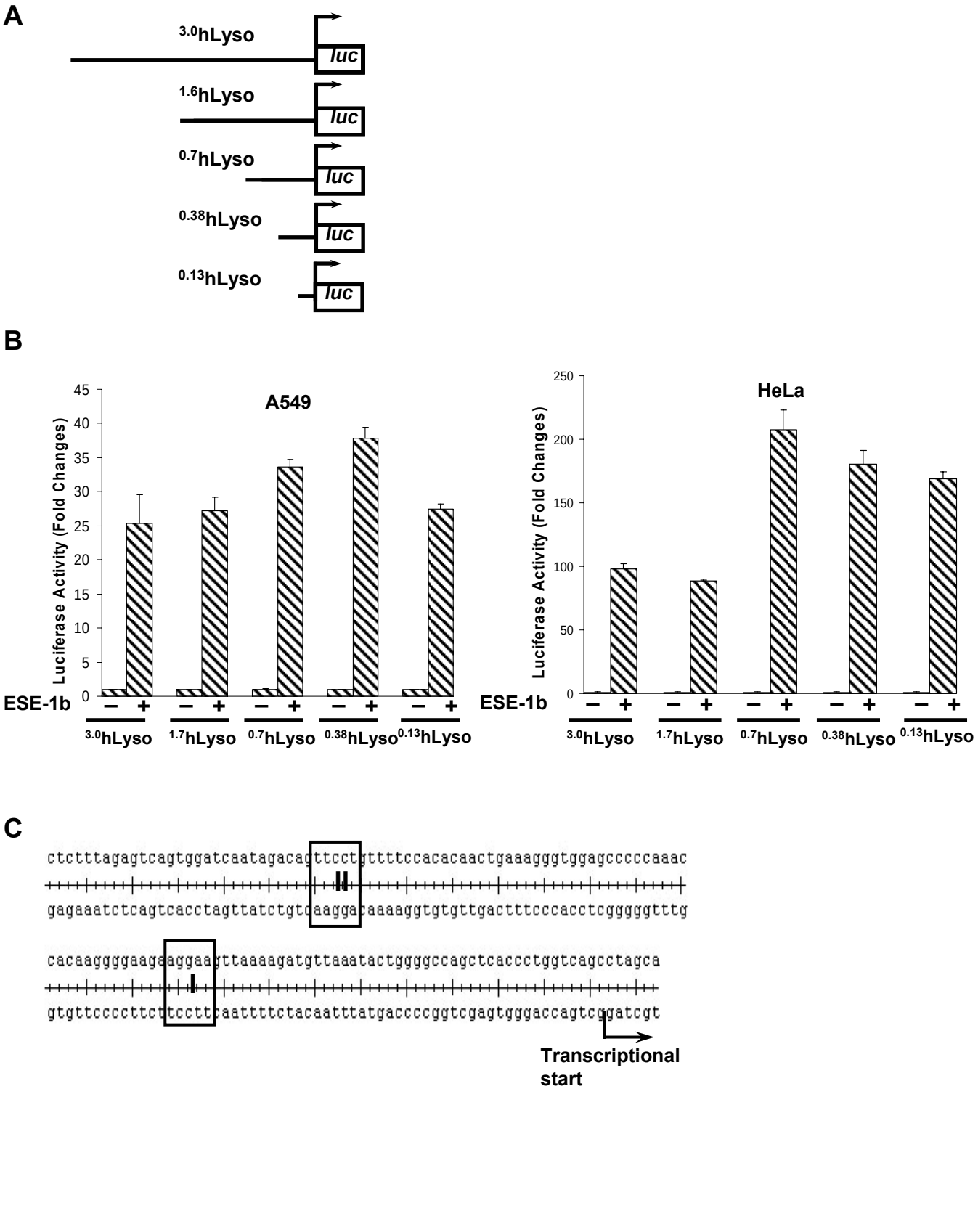

A549
Luciferase Activity (Fold Changes)
0 5 10 15 20 25 30 35
ESE-1b
ESE-2a
ESE-2b
ESE-3a
ESE-3b
HeLa
Luciferase Activity (Fold Changes)
0 10 20 30 40 50 60 70
ESE-1b
ESE-2a
ESE-2b
ESE-3a
ESE-3b

Fig. 4


A
3.0hLyso
luc
1.6hLyso
luc
0.7hLyso
luc
0.38hLyso
luc
0.13hLyso
luc
B
A549
Luciferase Activity (Fold Changes)
45
40
35
30
25
20
15
10
5
0
ESE-1b
– + – + – + – + – +
3.0hLyso
1.7hLyso
0.7hLyso
0.38hLyso
0.13hLyso
HeLa
Luciferase Activity (Fold Changes)
250
200
150
100
50
0
ESE-1b
– + – + – + – + – +
3.0hLyso
1.7hLyso
0.7hLyso
0.38hLyso
0.13hLyso
C
ctctttagagtcagtggatcaatagacagttcctgttttccacacaactgaaagggtggagcccccaaac
gagaaatctcagtcacctagttatctgtcaaggacaaaaggtgtgttgactttcccacctcgggggtttg
cacaaggggaagaaggaagttaaaagatgttaaatactggggccagctcaccctggtcagcctagca
gtgttcccccttcttccttcaattttctacaatttatgaccccggtcgagtgggaccagtcggatcgt
Transcriptional
start

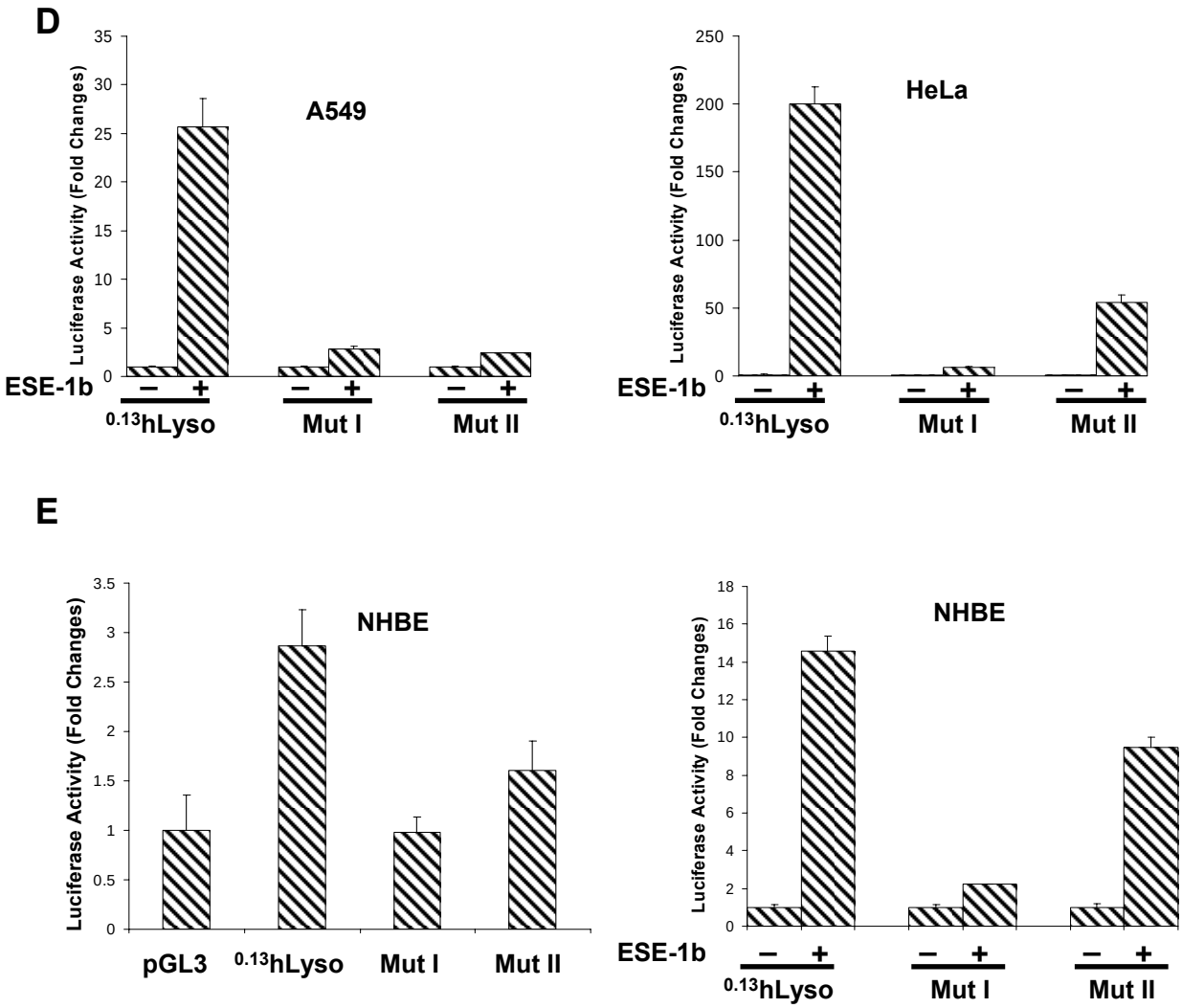

D
A549
HeLa
Luciferase Activity (Fold Changes)
ESE-1b
0.13hLyso
Mut I
Mut II
E
NHBE
pGL3

Fig. 5

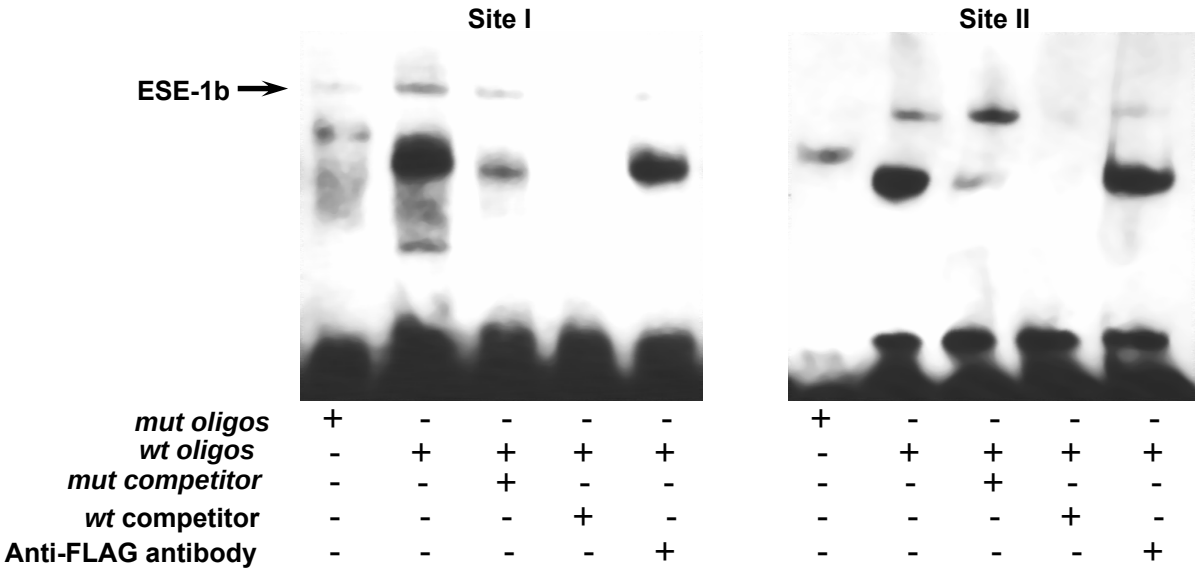
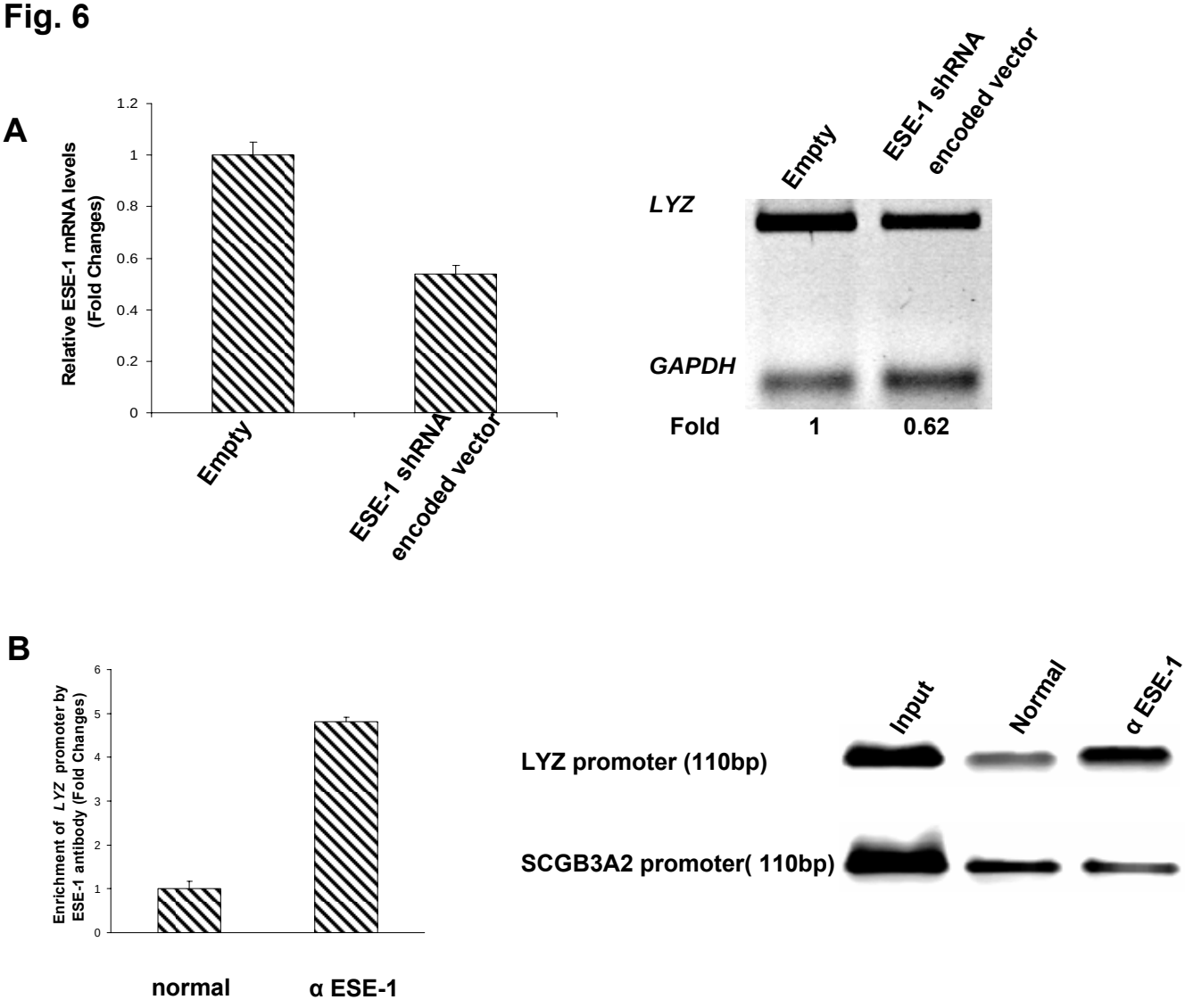

Site I
Site II
ESE-1b
mut oligos
wt oligos
mut competitor
wt competitor
Anti-FLAG antibody

Fig. 6


A
Relative ESE-1 mRNA levels (Fold Changes)
1.2
1
0.8
0.6
0.4
0.2
0
Empty
ESE-1 shRNA encoded vector
Empty
ESE-1 shRNA encoded vector
LYZ
GAPDH
Fold
1
0.62
B
Enrichment of LYZ promoter by ESE-1 antibody (Fold Changes)
6
5
4
3
2
1
0
normal
α ESE-1
Input
Normal
α ESE-1
LYZ promoter (110bp)
SCGB3A2 promoter(110bp)