Genetic, inflammatory, and epithelial cell differentiation factors control expression of human calpain-14

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RNAseq data: GSE113341

IRB approval: CCHMC 2008-0090

21 Running title: Regulation of *CAPN14* expression

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23 Keywords: CAPN14, Eosinophilic Esophagitis, IL-13, IL-4, STAT6

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28

ABSTRACT

Eosinophilic esophagitis (EoE) is a chronic, food-driven allergic disease resulting in eosinophilic esophageal inflammation. We recently found that EoE susceptibility is associated with genetic variants in the promoter of *CAPN14*, a gene with reported esophagus-specific expression. *CAPN14* is dynamically up-regulated as a function of EoE disease activity and after exposure of epithelial cells to interleukin-13 (IL-13). Herein, we aimed to explore molecular modulation of *CAPN14* expression. We identified three putative binding sites for the IL-13-activated transcription factor STAT6 in the promoter and first intron of *CAPN14*. Luciferase reporter assays revealed that the two most distal STAT6 elements were required for the ~10-fold increase in promoter activity subsequent to stimulation with IL-13 or IL-4, and also for the genotype-dependent reduction in IL-13-induced promoter activity. One of the STAT6 elements in the promoter was necessary for IL-13-mediated induction of *CAPN14* promoter activity while the other STAT6 promoter element was necessary for full induction. Chromatin immunoprecipitation in IL-13 stimulated esophageal epithelial cells was used to further support STAT6 binding to the promoter of *CAPN14* at these STAT6 binding sites. The highest *CAPN14* and calpain-14 expression occurred with IL-13 or IL-4 stimulation of esophageal epithelial cells under culture conditions that allow the cells to differentiate into a stratified epithelium. This work corroborates a candidate molecular mechanism for EoE disease etiology in which the risk variant at 2p23 dampens mediated *CAPN14* expression in differentiated esophageal epithelial cells following IL-13/STAT6 induction of *CAPN14* promoter activity.

49 **INTRODUCTION**

50 Eosinophilic esophagitis (EoE) is a chronic, allergic disease associated with marked mucosal
51 eosinophil accumulation (Liacouras et al. 2011). EoE remits after removal of specific food types, and
52 food re-introduction causes disease reoccurrence, including dysregulation of esophageal transcripts.
53 The etiology of EoE includes environmental, immunological, and genetic components (Alexander et
54 al. 2014; Kottyan et al. 2014; Kottyan et al. 2015). One of the central questions in the EoE field, and
55 allergy in general, is to understand why individuals develop certain manifestations of disease.

56

57 Until recently, studies have identified EoE-genetic risk loci that were broadly linked to other allergic
58 diseases (Sherrill and Rothenberg 2014). For example, genetic variants at 5q22, encoding *TSLP*
59 and *WDR36,* and 11q13, encoding *LRRC32* and *EMSY*, have been associated with allergic
60 sensitization, asthma, allergic rhinitis, atopic dermatitis, food allergy and/or EoE (Greisenegger et al.
61 2013; Tang et al. 2012; Ferreira et al. 2011; Li et al. 2015; Ferreira et al. 2014; Weidinger et al. 2013;
62 Ramasamy et al. 2011; Hinds et al. 2013; Sleiman et al. 2014). The shared association of risk
63 variants among these phenotypes suggests that these loci contain variants that participate in the
64 allele-dependent regulation of a molecular pathway that is central to the etiology of allergic disease.

65

66 We recently found that, in addition to genetic risk loci for allergic sensitization, EoE susceptibility is
67 linked to one or more genetic factors at 2p23, encoding the *CAPN14* gene (Kottyan et al. 2014). This
68 genetic linkage has been replicated at genome-wide significance in multiple cohorts, as well as in a
69 recent independent study (Sleiman et al. 2014), adding credence to the importance of the 2p23
70 genetic linkage. *CAPN14* (encoding calpain-14) belongs to the classical calpain sub-family, a set of
71 calcium activated intracellular regulatory proteases (Sorimachi et al. 2011). In our initial studies, we
72 identified *CAPN14* as dynamically up-regulated as a function of EoE disease activity as well as after

73 exposure of epithelial cells to IL-13 (Davis et al. 2016). Expression quantitative trait loci (eQTL)
74 analysis revealed that patients with active EoE expressed *CAPN14* in a genotype-dependent
75 manner (Kottyan et al. 2014). While *CAPN14* was expressed at a higher level in individuals with EoE
76 compared to those without EoE, patients with the risk genotype had decreased expression of
77 *CAPN14* compared to patients with the non-risk genotype . Consistent with these findings,
78 overexpression of the calpain-14 protein in esophageal epithelial cells leads to morphological
79 changes and barrier defects independently of IL-13-mediated inflammation, while *CAPN14* gene
80 silencing in these cells leads to defects in barrier repair after IL-13 stimulation (Davis et al. 2016),
81 suggesting that calpain-14 might contribute to EoE via a regulatory loop (Litosh et al. 2017).

82

83 In this study, we aimed to identify regulatory factors that control the expression of *CAPN14*. Using
84 luciferase reporter constructs, we determined that IL-13 is sufficient to induce *CAPN14* promoter
85 activity and identified one putative STAT6 binding sites as necessary for IL-13-induced *CAPN14*
86 promoter activity with a second putative STAT6 binding site in the promoter necessary for full
87 induction of *CAPN14* promoter activity. Using chromatin immunoprecipitation, we identified STAT6
88 binding to the promoter of *CAPN14* at the site of the two putative STAT6 binding sites in EPC2
89 esophageal epithelial cells. One single nucleotide polymorphism (SNP) that is highly associated with
90 EoE risk and located in the promoter of *CAPN14* was sufficient to decrease *CAPN14* promoter
91 activity in a manner that is consistent with the eQTL seen in individuals with and without EoE (Kottyan
92 et al. 2014). By measuring mRNA and protein expression of *CAPN14*/calpain-14 over a variety of *in*
93 *vitro* culture conditions, we further found that *CAPN14* expression is highest in differentiated
94 esophageal epithelial cells after IL-13 exposure. IL-4 also signals in esophageal epithelial cells in a
95 STAT6-dependent manner, and we obtained consistent results for IL-4 and IL-13 promoter activity
96 and gene expression. Altogether, this study identified immunological, genetic, and epithelial cell
97 differentiation mechanisms that regulate the expression of *CAPN14*.

98

99 **METHODS**

100 **Esophageal epithelial cell cultures.** EPC2 esophageal epithelial cells were grown in keratinocyte
101 serum-free media (K-SFM) (Life Technologies, Grand Island, NY) to various levels of confluence
102 (80% or 100%) with relatively low (0.09 mM) or high (1.8 mM) Ca^{2+} and with or without 100 ng/mL
103 IL-13 or IL-4 for 24 hours, as a monolayer submerged culture or in an air-liquid interface (ALI) culture
104 (Davis et al. 2016; Kottyan et al. 2014). For the ALI culture system, the esophageal epithelial EPC2
105 cell line was grown to confluence on 0.4 μm pore–size polyester permeable supports (Corning
106 Incorporated, Corning, NY) in K-SFM supplemented with 1.8 mM calcium. Epithelial differentiation
107 was then induced by removing culture media from the inner chamber of the permeable support and
108 maintaining the esophageal epithelial cells for 8 days in the ALI in the presence or absence of IL-13
109 or IL-4 (100 ng/mL). TE7 esophageal epithelial cells were grown in RPMI1640 supplemented with
110 10% FBS (HyClone, Pittsburgh, PA).

111 **Luciferase reporter assays.** The *CCL26* nanoluciferase reporter construct was engineered using
112 the firefly reporter construct reported previously (Lim et al. 2011). The first 964 bp before the *CCL26*
113 start codon were subcloned into the pNL1.1 nanoluciferase reporter vector (Promega, Madison, WI).
114 For the *CAPN14* promoter reporter construct, PCR primers (listed in **Table S1**) were used to amplify
115 the 1988 base pair region around rs76562819 and subsequently clone it into the pNL1.1
116 nanoluciferase reporter vector. Site-directed mutagenesis was performed to generate the construct
117 with scrambled STAT6 binding sites and the EoE non-risk allele of rs76562819 using the GeneArt
118 Site-Directed Mutagenesis System (Invitrogen, Darmstadt, Germany). Sequences were confirmed
119 by Sanger sequencing. All mutagenesis primers are provided in **Table S1**.

120 250 ng of nanoluciferase experimental constructs were transiently co-transfected into esophageal
121 epithelial cells with 250 ng of pGL3-control firefly luciferase reporter plasmid. After 24 hours, cells

122 were treated with 100 ng/mL of IL-13 or IL-4 (Promega, Madison, WI). 24 hours after cytokine
123 administration, cells were lysed and nano and firefly luciferase activity was assayed using the
124 Promega Nano-Glo Luciferase Assay System. The nanoluciferase measurement for each well was
125 divided by the firefly luciferase measurement in order to account for cytotoxicity, small differences in
126 cell number, and transfection efficiency. Each well was further normalized to the promoterless
127 pNL1.1 vector to control for baseline activity of the vector.

128 **Chromatin immunoprecipitation analysis.** EPC2 cells were treated with IL-13 at 100 ng/mL for 45
129 min and fixed with 1% formaldehyde for ChIP. ChIP was performed as reported previously (Rochman
130 et al. 2015) with the exception that the sonication was for 4 min and ChIP was performed manually
131 for the anti-STAT6 experiments. Anti-STAT6 antibody S-20 (D3H4 Rabbit mAb #5397, Cell
132 Signaling) anti-H3K4me3 antibody ab8580 (Abcam), and anti-H3K27Ac antibody ab4279 (Abcam).
133 We fixed 10–20×10^6 cells with 0.8% formaldehyde by adding 1 ml of 10X fixation buffer (50 mM
134 Hepes-KOH, pH 7.5; 100 mM NaCl; 1 mM EDTA; 0.5 mM EGTA; 8% formaldehyde) to 9 ml of growth
135 medium for 8 minutes at room temperature with shaking. The reaction was stopped by adding glycine
136 to a final concentration of 125 mM for an additional 5 minutes. After washing with PBS, pellets were
137 frozen at −80°C for at least overnight. Nuclei were prepared by re-suspending pellets in 1 ml of L1
138 buffer (50 mM Hepes-KOH, pH 7.5; 140 mM NaCl; 1 mM EDTA; 10% glycerol; 0.5% NP-40; 0.25%
139 Triton X-100) and incubated at 4°C for 10 min. Nuclei were pelleted and re-suspended in 1 ml of L2
140 buffer (10 mM Tris-HCl, pH 8.0; 200 mM NaCl; 1 mM EDTA, pH 8.0; 0.5 mM EGTA, pH 8.0) and
141 rotated for 10 min at room temperature. Nuclei were briefly washed with sonication buffer (Tris-EDTA
142 [TE] buffer + 0.1% SDS) and re-suspended in 1 ml of sonication buffer. All buffers were
143 supplemented with complete EDTA-free protease inhibitors (Roche Diagnostics Corporation
144 Indianapolis, IN). Sonication was performed by a Covaris S220 focused ultrasonicator (Covaris, Inc.
145 Woburn, MA) at 175W Peak Incident Power, 10% output, 200 bursts for 10 min in 12×12-mm, round-
146 bottom glass tubes. Efficient DNA fragmentation was verified by agarose gel electrophoresis. Anti-

147 histone mark ChIP was performed by SX-8G IP-Star® Automated System (Diagenode Inc., Denville,
148 NJ) in RIPA buffer (TE + 0.1% SDS, 1% Triton X-100, 150 mM NaCl, 0.1% sodium deoxycholate)
149 following the protocol of the manufacturer with 2–4 µg of indicated antibodies. Libraries were
150 sequenced on the Illumina HiSeq 2500, and ChIP-seq data were visualized and analyzed in
151 Biowardrobe (Kartashov and Barski 2015; Vallabh et al. 2018).

152 **RNA purification and expression analyses.** For the real-time PCR analysis of CAPN14
153 expression, total RNA was purified from esophageal epithelial cells using the MirVana miRNA
154 Isolation kit (ThermoFisher, Waltham, MA). RNA was reverse-transcribed with the High-Capacity
155 RNA-to-cDNA kit (Applied Biosystems, Grand Island, NY). Gene expression was determined by real-
156 time PCR using a 7500 Real-time PCR system (Applied Biosystems, Foster City, CA) using Taqman
157 Gene Expression Assays CAPN14- Hs00871882_m1, CCL26- Hs00171146_m1 and GAPDH-
158 Hs02786624_g1. Relative quantification was calculated by the comparative CT method (Livak and
159 Schmittgen 2001). Briefly, expression levels for *CAPN14* or *CCL26* were normalized to levels of
160 *GAPDH*. All samples were then normalized to cells grown at 80% confluence without high calcium
161 or IL-13 and the data were exponentially transformed.

162 For the RNA-sequencing study performed to identify the isoforms of CAPN14 expressed in the
163 esophageal biopsies of people with and without EoE (**Figure S2**), RNA was isolated from distal
164 esophageal biopsy RNA from EoE patients (5 male and 5 female) with active disease and from
165 unaffected controls (5 male and 5 female) as previously described (Kottyan et al. 2014; Blanchard
166 et al. 2006; Namjou et al. 2014). EoE biopsies showed active disease pathology at the time when
167 they were taken, and all patients reported no glucocorticoid treatment at the time of biopsy.
168 RNA sequencing acquiring 50 million mappable 125 base-pair reads from paired-end libraries was
169 performed at the Genetic Variation and Gene Discovery Core Facility at CCHMC. Data were aligned
170 to the GrCh37 build of the human genome using the Ensembl (Flicek et al. 2013) annotations as a

171 guide for TopHat (Trapnell et al. 2009). Expression analysis was performed using Kallisto's quant
172 function (Bray et al. 2016). Data has been deposited in NCBI Geo database as GSE113341.

173 **Protein isolation and analysis.** Whole cell lysate was extracted from EPC2 cells lysed with RIPA
174 Buffer (Millipore, Burlington, MA) and Halt™ phosphatase inhibitor cocktail. Samples were exposed
175 to electrophoresis in NuPAGE Novex 4-12% Bis-Tris Gels and transferred to nitrocellulose using the
176 iBlot system (Invitrogen, Carlsbad, CA). Primary antibodies: anti-CAPN14 (HPA035721, Sigma
177 Aldrich, St. Louis, MO) and anti-β-actin (4967L, Cell Signaling Technologies, Danvers, MA).
178 Secondary antibody: IRDye 800CW donkey anti-rabbit (926-32213, LI-COR, Lincoln, NE).
179 Nitrocellulose membranes were imaged using the Odyssey CLx system (LI-COR) and analyzed with
180 Image Studio (LI-COR). EPC2 cells that constitutively express *CAPN14* behind a CMV promoter
181 were used as a positive control.

182 Statistical analysis and data visualization were done with GraphPad Prism (GraphPad Software, La
183 Jolla, CA).

184 **Data and reagent availability**. Constructs are available upon request. For the *CAPN14* promoter
185 reporter construct, PCR primers (listed in **Table S1**) were used to amplify the 1988 base pair region
186 around rs76562819 and subsequently clone it into the pNL1.1 nanoluciferase reporter vector. All
187 mutagenesis primers are provided in **Table S1**. RNA sequencing data has been deposited in NCBI
188 Geo database as GSE113341.

189 **RESULTS**

190 IL-13 administration to esophageal epithelial cells is sufficient to induce *CAPN14* expression similar
191 to *CCL26*, a chemokine whose promoter activity is dependent on the STAT6 transcription factor (Lim
192 et al. 2011). Based upon these previous results, we hypothesized that IL-13 acted through the
193 transcription factor STAT6 to directly promote *CAPN14* expression. Thus, we first constructed
194 promoter reporters by placing the promoters of either *CAPN14* or *CCL26* in front of the

195 nanoluciferase gene (**Figure 1 A**). After treatment with IL-13 or IL-4, esophageal epithelial cells
196 transfected with the *CAPN14* reporter construct exhibited a similar upregulation of nanoluciferase
197 expression to the cells transfected with a *CCL26* reporter construct (**Figure 1 B and Figure S1 A**).
198 Based upon the canonical 5'-TTC...GAA-3' core motif of STAT6, we identified two putative STAT6
199 binding sequences in the promoter located within 90 base pairs of the transcription start site of
200 *CAPN14* and one putative STAT6 binding site in the first intron of *CAPN14*. We tested the necessity
201 of each individual predicted STAT6 binding site for IL-13-induced promoter activity by creating
202 luciferase reporter constructs with mutated putative STAT6 sites (**Figure 1 A**). Mutation of the first
203 STAT6 site attenuated IL-13-mediated induction of CAPN14 promoter activity, while mutation of the
204 second STAT6 site completely blocked CAPN14 promoter induction (**Figure 1 C**). Mutation of the
205 third putative STAT6 site in the first intron of CAPN14 did not affect the promoter activity of CAPN14
206 following treatment with IL-13 (**Figure 1 C**). From these experiments, we concluded that the two
207 sites in the promoter are necessary for full IL-13-induced promoter activity.
208

209 Chromatin immunoprecipitation of STAT6 in esophageal epithelial cells treated with IL-13 was used
210 to further establish STAT6 binding to the promoter of *CAPN14* at the two binding sites that were
211 necessary for IL-13-inducible *CAPN14* promoter activity. We identified ChIP-seq peaks in the
212 promoter of *CAPN14* spanning these two STAT6 sites from chromatin extracted from IL-13
213 stimulated esophageal epithelial cells (**Figure 2**). There was no evidence of STAT6 binding at the
214 third putative STAT6 site in the first intron of *CAPN14*. These STAT6 ChIP-seq peaks overlap ChIP-
215 seq peaks from active histone marks H3K4me3 and H3K27Ac that appear after induction by IL-13
216 (**Figure 2**). Altogether, these functional genomic data support our initial hypothesis that IL-13 and
217 IL-4 activate *CAPN14* expression through the binding of STAT6 to two sites in the promoter of
218 *CAPN14*.
219

220 Patients with EoE and the genetic-risk haplotype at 2p23, marked by single nucleotide polymorphism
221 (SNP) rs76562819**,** have 50% less *CAPN14* in their esophageal biopsies compared to patients with
222 the non-risk haplotype (Kottyan et al. 2014). An analysis of differential isoform usage of *CAPN14*
223 identified two major isoforms of CAPN14 that did not change with EoE disease status or sex, (**Figure**
224 **S2**). Of the two isoforms of *CAPN14* that are expressed, the major isoform (ENST00000444918)
225 includes exon 7, while the less highly expressed isoform of *CAPN14* (ENST00000398824) does not.
226 Furthermore, the less highly expressed isoform of *CAPN14* (ENST00000398824) is predicted to
227 undergo nonsense mediated decay based upon manual annotation from the VEGA/Havana projects
228 (Wilming et al. 2008). The EoE-risk haplotype at 2p23 includes 12 variants in linkage disequilibrium
229 (r^2>0.8), but functional genomic and biochemical evidence supported a specific role for rs76562819
230 (Davis et al. 2016; Kottyan et al. 2014). We tested the hypothesis that rs76562819 was sufficient to
231 result in genotype-dependent promoter activity of *CAPN14* using two identical luciferase constructs
232 containing the promoter and a portion of the first intron of *CAPN14* with either the risk or the non-
233 risk allele of rs76562819 (**Figure 3 A**). We found that the EoE risk allele at rs76562819 resulted in
234 a 40.0% reduction of IL-13 and IL-4-induced *CAPN14* promoter activity compared to the EoE non-
235 risk allele (**Figure 3 B**). Thus, the single base change from non-risk to risk at rs76562819 is sufficient
236 to explain most of the reduced *CAPN14* promoter activity in the luciferase reporter assays. These
237 results are consistent with the genotype at rs76562819 accounting for genotype-dependent
238 expression observed in EoE patient biopsies.

239

240 We next sought to determine the factors leading to expression of *CAPN14* in esophageal epithelium.
241 Unlike *CCL26*, *CAPN14* expression is highly dependent upon the cell culture conditions. Esophageal
242 epithelial differentiation is a physiological process that increases mechanical strength and barrier
243 function to squamous epithelia (Macara et al. 2014). Factors that are necessary for differentiation *in*
244 *vitro* are calcium concentration, cellular confluence, and the culture system (Macara et al. 2014; Kc

245 et al. 2015). Without esophageal epithelial differentiation by confluence, calcium, and growth in an
246 air-liquid interface (ALI), *CAPN14* mRNA expression is low (**Figure 4 A**). Following differentiation,
247 *CAPN14* is induced 10,000-fold in the ALI, $p<0.0001$ (**Figure 4 A**). At a protein level, calpain-14 was
248 detected only in the most differentiated system (ALI) following treatment with IL-13 (**Figure 4 B**). As
249 seen in the promoter reporter experiments, IL-4 and IL-13 are equally capable of induction of
250 *CAPN14* mRNA and protein (**Figure S3**).

251

252 **DISCUSSION**

253 Altogether, we identified immunological (IL-13/IL-4/STAT6), genetic (rs76562819), and epithelial
254 differentiation factors that regulate the expression of *CAPN14*. These results are important because
255 calpain-14 represents a candidate for treatment and prevention of EoE. Previous studies supported
256 a model in which IL-13 induction of *CAPN14* occurs in a STAT6-dependent manner. We identified
257 the STAT6 binding sites, the necessity of these sites for *CAPN14* promoter activity, and the binding
258 of STAT6 to the promoter of *CAPN14* in esophageal cells stimulated with IL-13. We demonstrated
259 genotype-dependent expression of *CAPN14* and identified rs76562819 as the genetic variant that is
260 sufficient to produce genotype-dependent promoter activity. Indeed, the 40.0% reduction of *CAPN14*
261 promoter activity in the genotype-dependent reporter assays mirrors the effect size of the eQTLs
262 measured in subjects with and without EoE (Kottyan et al. 2014). The reporter experiments did not
263 demonstrate statistically significant genotype-dependent promoter activity in cells that were not
264 stimulated with IL-13. This could be due to several factors, including the sensitivity of the reporter
265 system, the non-differentiated context of the reporter experiments, or the fact that IL-13 is needed
266 for genotype-dependent *CAPN14* promoter activity. The fact that relatively undifferentiated
267 esophageal epithelial cell culture systems are sufficient for luciferase reporter systems could be due
268 to how differentiation affects chromatin availability – a factor that is not assessed in the cytoplasmic
269 luciferase reporter assay. *CAPN14* mRNA and protein expression levels strongly support the

270 conclusion that both differentiation and stimulation with IL-13 or IL-4 is critical for the highest
271 expression of calpain-14 in esophageal epithelial cells. The requirement for esophageal epithelial
272 cell differentiation before *CAPN14* expression might be the primary mechanism through which
273 *CAPN14*'s expression is-induced in the esophageal mucosa.

274 IL-13 is a critical cytokine in the pathoetiology of EoE (Blanchard et al. 2011; O'Shea et al. 2017;
275 Davis et al. 2016; D'Mello et al. 2016; Cianferoni and Spergel 2016; Rothenberg et al. 2015; Sherrill
276 et al. 2014b; Sherrill et al. 2014a; Zuo et al. 2010; Blanchard et al. 2007; Mishra and Rothenberg
277 2003). Indeed, treating esophageal epithelial cells with IL-13 is an effective way to model
278 approximately 25% of the transcriptome and cytokine secretion profiles found in the esophageal
279 biopsies of patients with EoE (Sherrill et al. 2014b; Lu et al. 2012; Blanchard et al. 2007). IL-13
280 directly controls the expression of chemokines that recruit eosinophils to the esophagus (*CCL11*,
281 *CCL24*, *CCL26*) through STAT6-dependent mechanisms (Lim et al. 2011). IL-13 treatment can also
282 activate other cellular and transcriptional regulators including the protein kinase B (Akt) and mitogen-
283 activated protein kinase (MAPK) pathways (Demoulin and Renauld 1998; Harris et al. 2007), and
284 transcriptional regulation downstream of IL-13 signaling can also be indirect (e.g. genes transcribed
285 in response to IL-13 can regulate the expression of other genes). It was therefore important to
286 establish that two STAT6 sites in the *CAPN14* promoter control IL-13-stimulated expression *of*
287 *CAPN14*. IL-13 shares many functional properties with IL-4, stemming from the fact that they share
288 a common receptor subunit, the alpha subunit of the IL-4 receptor (Hershey 2003). Both IL-13 and
289 IL-4 signaling lead to STAT6 dimerization, phosphorylation, and nuclear translocation (Kuperman
290 and Schleimer 2008), so it is therefore not surprising that IL-4 might also regulate *CAPN14* mRNA
291 expression and promoter activity (**Figures S1 and S3**). Despite the evidence presented in this study,
292 future experiments in esophageal epithelial cells deficient in STAT6 signaling are needed to fully
293 conclude that STAT6 is necessary in the transcription of *CAPN14*.
294

295 Very little is known about the factors regulating the expression, function, and tissue specificity of
296 *CAPN14* (Litosh et al. 2017; Davis et al. 2016; Sleiman et al. 2014; Ueta et al. 2010; Kottyan et al.
297 2014; Dear and Boehm 2001). A growing body of literature suggests that there are factors specific
298 to the esophagus that affect gene regulation (Keane et al. 2015; Rochman et al. 2018; Rochman et
299 al. 2017). These and other studies are consistent with our findings that while some genes regulated
300 by IL-13 are expressed in various epithelial context (e.g. *CCL26*/eotain-3), other genes have more
301 nuainced regulatory factors affecting expression (e.g. *CAPN14*/calpain-14). In this study, we
302 demonstrated that epithelial differentiation was important; however, we were unable to establish
303 specific tissue-specific molecular pathways that lead to the differences in CCL26 and CAPN14
304 expression in response to IL-13.

305 This study focused on the regulation of *CAPN14* transcription. Future studies will assess the post-
306 translational regulation and activity of calpain-14. The structure of calpain-14 strongly suggests that
307 it might dimerize with calpain-S1 (Litosh et al. 2017). Other members of the calpain family are known
308 to undergo autolysis (Ono et al. 2016a; Ermolova et al. 2011; Taveau et al. 2003; Badugu et al.
309 2008), and proteomic analysis of calpain-14 expression found evidence consistent with cleavage of
310 calpain-14, especially in non-differentiated conditions (**Figure 4 B**). We used an overexpression
311 construct of calpain-14 to identify the specific band bound by the anti-calpain-14 antibody in the
312 Western blots (**Figure 4 B**). Calpains cleave substrates in a calcium-dependent manner based upon
313 the conformation, rather than the amino acid sequence, of their targets (Ono et al. 2016b; Sorimachi
314 et al. 2012). Full biochemical analyses of the substrates of calpain-14 will be critical as the functions
315 of calpain-14 in health and EoE are discovered.

316

317 The current study identifies critical factors that regulate *CAPN14* expression. Altogether, this study
318 makes an important advance towards identification of the factors that control the genotype-
319 dependent transcriptional regulation of *CAPN14* in esophageal epithelium.

320

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health [R37 AI045898, R01 AI124355, R01 DK107502, R01 NS099068, R21 HG008186, P30 AR070549, U01 HG008666]; the Campaign Urging Research for Eosinophilic Disease (CURED); the Buckeye Foundation; the Sunshine Charitable Foundation and its supporters, Denise A. Bunning and David G. Bunning; the American Partnership of Eosinophilic Disorders (APFED); Cincinnati Children's Hospital CpG Pilot Study and Endowed Scholar awards to M.T.W.

This project was supported in part by PHS Grant P30 DK078392 DNA sequencing core of the Digestive Disease Research Core Center in Cincinnati. N.W. and S.A.C. were supported by NIH Grant DA038017 and funds from CCRF, including an Arnold Strauss Fellowship to S.A.C.

CONFLICT OF INTEREST STATEMENT

M.E.R. is a consultant for Pulm One, Spoon Guru, ClostraBio, Celgene, Shire, Astra Zeneca, GlaxoSmithKline, Allakos, Adare, Regeneron and Novartis and has an equity interest in the first four listed and Immune Pharmaceuticals, and royalties from reslizumab (Teva Pharmaceuticals). M.E.R. is an inventor of patents, owned by Cincinnati Children's.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

342 **Figure 1. STAT6 binding sites in the promoter region of the *CAPN14* gene affect IL-13-induced**
343 ***CAPN14* promoter activity.** *A*, schematic representation of the nucleotide sequence of the promoter
344 of the human *CAPN14* gene. Putative binding sites for STAT6 are defined by grey boxes. *B*, Site-
345 directed mutagenesis elimination of putative STAT6-binding sites is denoted by crossed boxes. The
346 (*B*) CCL26 and CAPN14 constructs or the (*C*) mutagenized CAPN14 constructs were transfected
347 into esophageal epithelial cells followed by treatment with or without IL-13 (100 ng/mL) for 24 h. For
348 each sample, nanoluciferase activity was normalized to firefly luciferase activity. Data are shown as
349 mean ± S.E.M. (***, $p < 0.001$; $n = 3$ per group; data representative of three independent
350 experiments).

351

352 **Figure 2. Epigenetic signature and STAT6 binding to the promoter region of *CAPN14* in**
353 **response to IL-13 stimulation.** An image capture from the UC Santa Cruz genome browser
354 shows the tag density profile of aligned ChIP-seq reads in untreated and IL-13-treated EPC2 cells.
355 The read depths of the aligned sequences are of the same scale. Arrow represents direction of
356 transcription; black boxes represent exons. In the IL-13-treated cells, elevated levels of H3K4me3
357 and H3K27Ac are detected in the promoter region. The scale is the same for all track pairs
358 (untreated and IL-13).Three putative STAT6 binding sites around the TSS are indicated by blue
359 lines. TSS – transcription start site.

360

361 **Figure 3. The risk variant of rs76562819 results in a genotype-dependent decrease in IL-13**
362 **induced promoter activity of *CAPN14*.** *A,* Diagrams of constructs containing the *CAPN14* promoter
363 are shown. *B,* Reporter constructs were transfected into esophageal epithelial cells followed by
364 treatment with or without IL-13 (100 ng/mL) for 24 h. For each sample, nanoluciferase activity was
365 normalized to firefly luciferase activity. Data are shown as mean ± S.E.M. (***, t-test p-value<0.001;

366 2-way ANOVA: $p<0.05$, genotype $p<0.0001$ accounting for 12.6% of total variation; $n = 3$ per group;
367 data representative of three independent experiments).
368
369 **Figure 4. The expression of *CAPN14* in esophageal epithelial cells is dependent upon**
370 **calcium, confluency, and IL-13.** EPC2 esophageal epithelial cells were grown to various levels of
371 confluence (80%- indicated by -, or 100%-indicated by +) with relatively low (0.09 mM) or high (1.8
372 mM) Ca^{2+} and with or without 100 ng/mL IL-13 for 24 hours. Cultures were grown either as a
373 monolayer submerged culture or in an air-liquid interface (ALI) setup. *A,* RNA was isolated and
374 *CAPN14* was measured relative to the expression of the house keeping gene *GAPDH*. All RNA
375 expression values are normalized to cells at 80% confluence without high calcium or IL-13. *B,*
376 Calpain-14 protein expression levels. EPC2 cells that constitutively express *CAPN14* behind a CMV
377 promoter were used as a positive control for calpain-14 protein expression. RNA and protein level
378 expression: ***, t-test p-value<0.001, n=3-5 per group, data representative of 4 independent
379 experiments.
380
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382

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518

Figure 1. Three STAT6 binding sites in promoter region of the *CAPN14* gene affects IL-13-induced *CAPN14* promoter activity.

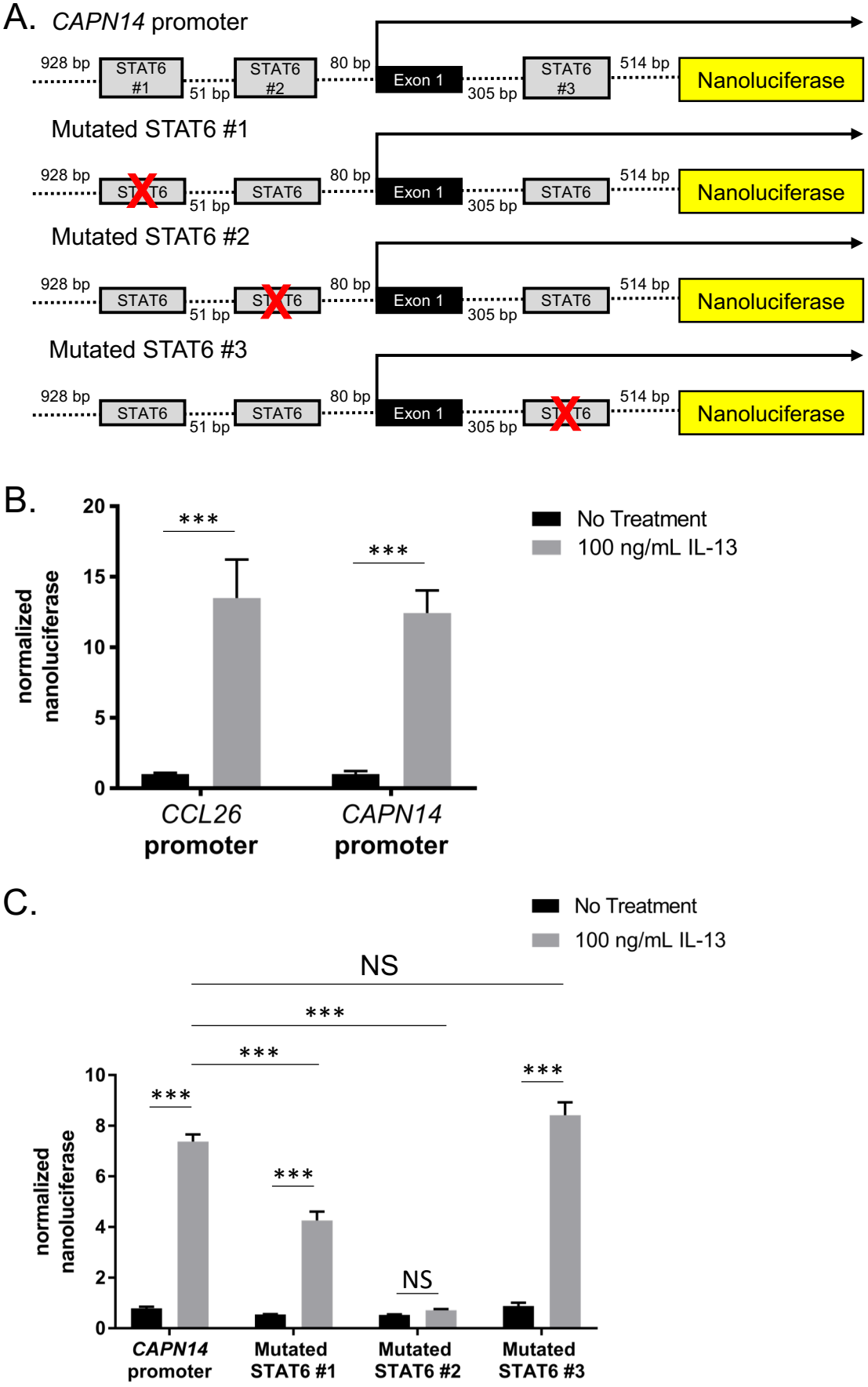


Figure 2. Chromatin immunoprecipitation of STAT6 confirms STAT6 binding at the the promoter of *CAPN14*.

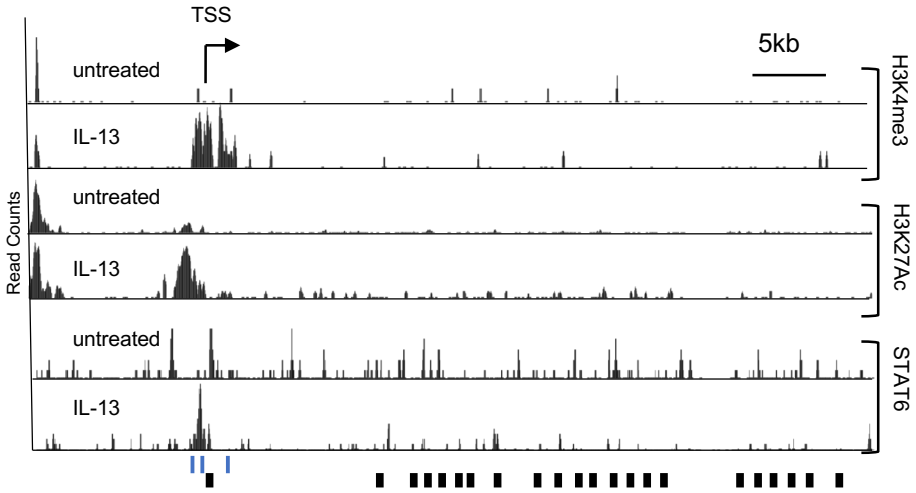


Figure 3. The risk variant of rs76562819 results in genotype-dependent decrease in IL-13 induced promoter activity of *CAPN14*

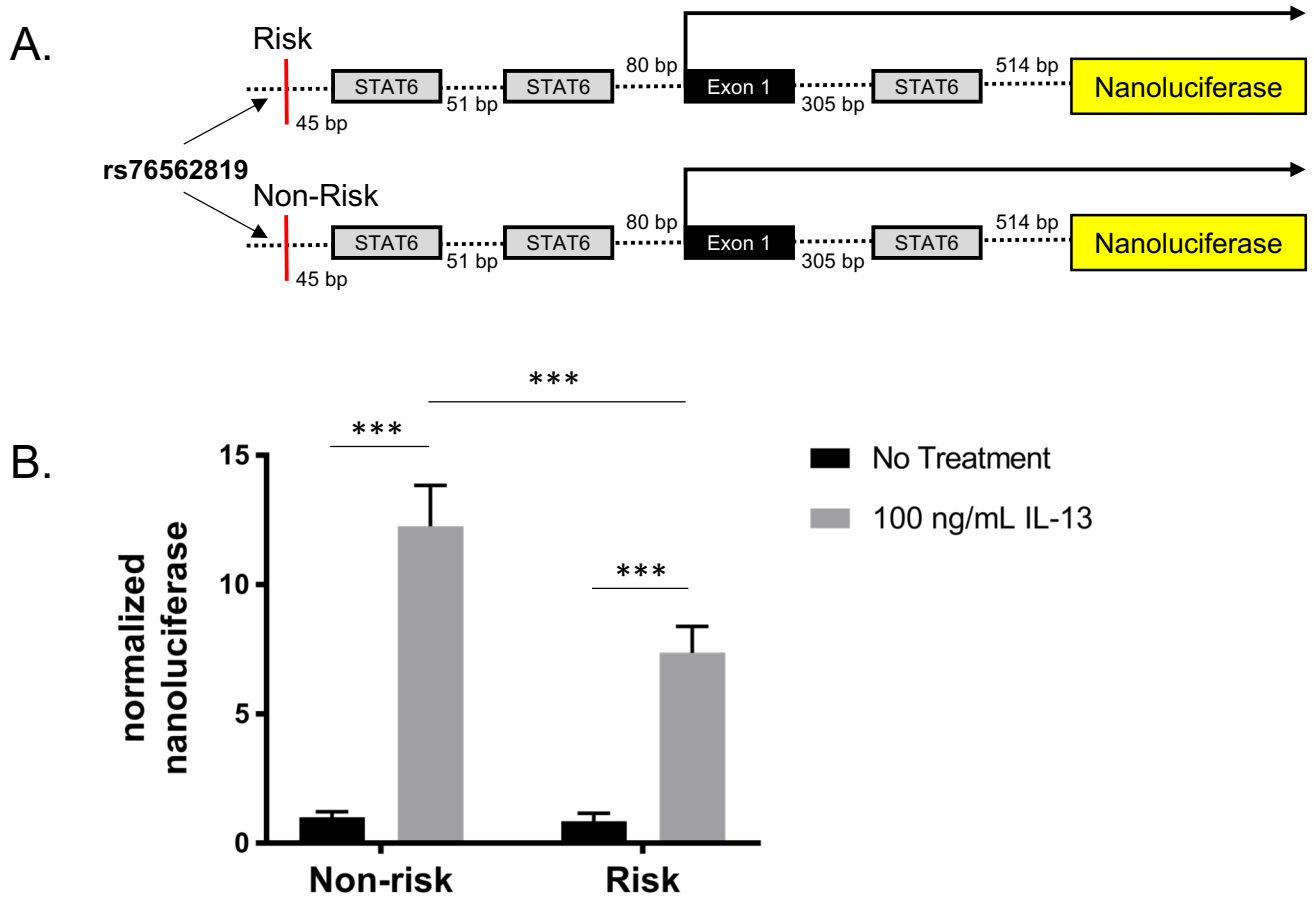
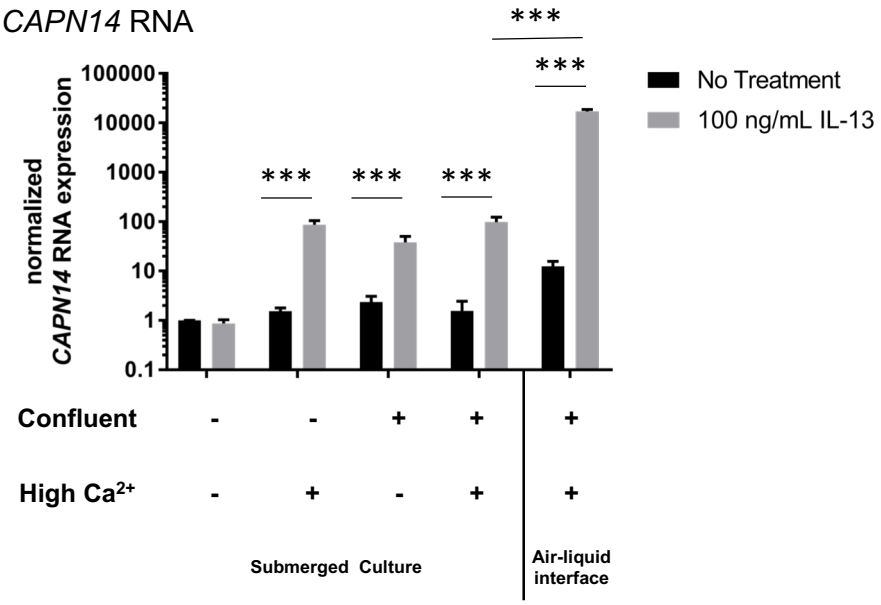
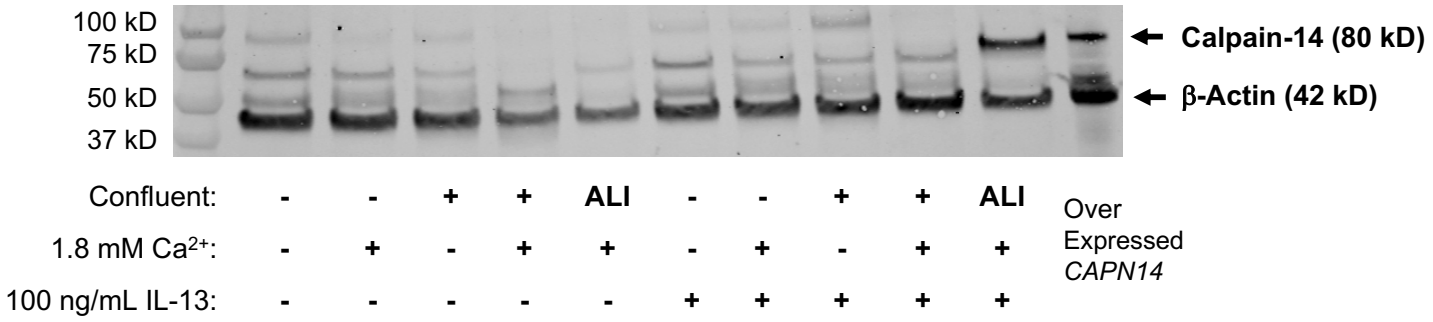


Figure 4. The expression of *CAPN14* in esophageal epithelial cells is dependent upon calcium, confluency, and IL-13.

A. *CAPN14* RNA



B. Calpain-14 Protein