HHS Public Access

Author manuscript

Mucosal Immunol. Author manuscript; available in PMC 2016 January 01.

Published in final edited form as:

Mucosal Immunol. 2015 July ; 8(4): 785–798. doi:10.1038/mi.2014.109.

Neurotrophic tyrosine kinase receptor 1 is a direct transcriptional and epigenetic target of IL-13 involved in allergic inflammation

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Abstract

Although IL-13 and neurotrophins are functionally important for the pathogenesis of immune responses, the interaction of these pathways has not been explored. Herein, by interrogating IL-13–induced responses in human epithelial cells we show that neurotrophic tyrosine kinase receptor, type 1 (NTRK1), a cognate, high-affinity receptor for nerve growth factor (NGF), is an early transcriptional IL-13 target. Induction of *NTRK1* was accompanied by accumulation of activating epigenetic marks in the promoter; transcriptional and epigenetic changes were STAT6-dependent. Using eosinophilic esophagitis (EoE) as a model for human allergic inflammation, we found that NTRK1 was increased in inflamed tissue, dynamically expressed as a function of disease activity, and the downstream mediator of NTRK1 signaling early growth response 1 (EGR1) protein was elevated in allergic inflammatory tissue compared with control tissue. Unlike NTRK1, its ligand NGF was constitutively expressed in control and disease states, indicating that IL-13–stimulated NTRK1 induction is a limiting factor in pathway activation. In epithelial cells, NGF and IL-13 synergistically induced several target genes, including CCL26 (eotaxin-3). In summary, we have demonstrated that IL-13 confers epithelial cell responsiveness to NGF by

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Conflict of interest: M.H.C. is a consultant for Meritage Pharma, Novartis, Receptos, Regeneron, and Aptalis. M.E.R. is a consultant for Immune Pharmaceuticals, Celsus Therapeutics and Receptos and has an equity interest in each of these; MER has royalties from reslizumab, a drug being developed by Teva Pharmaceuticals. MER is an inventor of several patents, owned by Cincinnati Children's, and set of these patents, related to molecular diagnostics, has been licensed to Diagnovus, LLC. All of the other authors have no potential conflicts to disclose.

Supplemental material

Supplemental material includes 1 table and 2 supplemental figures.

Disclosure

M.H.C. is a consultant for Meritage Pharma, Novartis, Receptos, Regeneron, and Aptalis. M.E.R. is a consultant for Immune Pharmaceuticals and Receptos and has an equity interest in Immune Pharmaceuticals and royalties from reslizumab, a drug being developed by Teva Pharmaceuticals. MER is an inventor of several patents, owned by Cincinnati Children's, and set of these patents, related to molecular diagnostics, has been licensed to Diagnovus, LLC. All of the other authors have no potential conflicts to disclose.

regulating NTRK1 levels by a transcriptional and epigenetic mechanism and that this process likely contributes to allergic inflammation.

Introduction

Interleukin 13 (IL-13)–mediated allergic inflammation is a hallmark of a number of diseases including asthma, atopic dermatitis, and eosinophilic esophagitis (EoE)[1–3]. IL-13 induces robust, cell-specific changes in gene expression and a majority of IL-13–mediated transcriptional and pathological changes are signal transducer and activator of transcription 6 (STAT6) dependent [4–6]. For example, in a murine model of EoE induced by IL-13 delivery into the lungs, eosinophilic infiltration and epithelial hyperplasia in the esophagus occur in a STAT6-dependent manner [7]. Likewise, in human intestinal, airway, and esophageal epithelial cells, induction of the eosinophil-specific chemokine (C-C Motif) ligand 26 (*CCL26* [eotaxin-3]) by IL-13 requires STAT6 expression [8–10]. To date, nearly all targets of IL-13 have been signaling molecules and/or soluble mediators of inflammation. Herein, we focus on a novel induction pathway in which IL-13 confers epithelial cell responsiveness to nerve growth factor (NGF) by inducing the NGF cognate, high-affinity receptor neurotrophin tyrosine kinase receptor 1 (NTRK1).

NGF was originally described as a critical factor for the survival and maintenance of sympathetic and sensory neurons [11], yet NGF is also considered a biomarker of asthmatic inflammation, with increased levels correlating with the severity of the disease [12]. Accordingly, NTRK1 is expressed on various structural and hematopoietic cells including basophils and eosinophils [13, 14]. Moreover, early growth response protein 1 (*EGR1*), a central transcriptional target of NGF [15], has a key role in IL-13–induced inflammation, fibrosis, alveolar remodeling and cytokine response [16], however, a connection between NTRK1 and these EGR1-associated inflammatory and/or remodeling pathways has not been established.

We herein employed RNA-sequencing analysis of the IL-13–mediated transcriptional response in the human esophageal epithelial cell line TE-7. We identified a set of 24 early IL-13 target genes, which included *NTRK1*. In response to IL-13, *NTRK1* was dramatically induced in epithelial cells and the *NTRK1* promoter showed rapid accumulation of multiple activating epigenetic marks; both transcriptional and epigenetic changes occurred in a STAT6-dependent manner. Notably, NTRK1 was the only receptor tyrosine kinase (RTK) with these characteristics. Functional analysis showed that IL-13–induced NTRK1 responded to NGF by activating EGR1 signaling and synergistically inducing a number of IL-13 target genes including *CCL26*, Serpin Peptidase Inhibitor, Clade B, Member 4 (*SERPINB4*) and KIT Ligand (*KITLG*). EGR1 induction was significantly diminished by gene silencing or by pharmacological inhibition of NTRK1. Translational studies showed elevated expression of NTRK1 in allergic tissue from patients with eosinophilic esophagitis (EoE). In summary, we have identified *NTRK1* as a direct transcriptional and epigenetic target of IL-13 with a contributory role in allergic inflammation.

Results

Transcriptional signature of IL-13 response in TE-7 esophageal epithelial cells

To gain insight into the transcriptional signature of IL-13–mediated allergic inflammation, we analyzed the kinetics of the transcriptional response to IL-13 in the human esophageal epithelial cell line TE-7 [17]. Cells were stimulated with IL-13 for 2, 6, and 24 hr and subjected to RNA-sequencing analysis. By applying differential expression analysis for sequence count data (DESeq) [18], we identified 767 unique genes significantly affected by IL-13 during the course of stimulation ($p < 0.05$, Figure 1A and Suppl. Table 1); 24, 328, and 573 genes were affected after 2, 6, and 24 hr of stimulation, respectively. Comparing the transcriptional response in TE-7 cells with the transcriptome of diseased tissue (esophageal biopsies from patients with active EoE) and of IL-13–induced primary esophageal epithelial cells revealed a remarkable similarity in the regulation of overlapping genes, including induction of the EoE hallmark genes *CCL26* and cadherin 26 (*CDH26*) [10, 19] (Figure 1B and Suppl. Figure 1). Kinetic analysis of the IL-13–mediated transcriptome of TE-7 cells revealed dynamic changes in gene expression, with early response genes mostly affected after 2 hr of stimulation and late response genes significantly changed only after 24 hr of IL-13 stimulation.

Of the 24 early target genes that responded to IL-13 within 2 hr of stimulation, 20 genes were significantly altered at 6 hr and 24 hr. Of these 20 genes, only inhibitor of DNA binding 3 (*ID3*) was decreased after 24 hr, whereas other genes were significantly up regulated at all assessed time points (Figure 1C). In agreement with previous findings, *CCL26* was one of the early transcriptional targets of IL-13 [10]. Among early target genes, we found known inhibitors of cytokine signaling, suppressor of cytokine signaling 1 (*SOCS1*) and cytokine inducible SH2-containing protein (*CISH*), which can provide a negative feedback loop for cytokine signaling [20]. We also detected genes such as hyaluronan synthase 3 (*HAS3*), heparan sulfate (glucosamine) 3-O-sulfotransferase 1 (*HS3ST1*), and glucosaminyl (N-acetyl) transferase 3, mucin type (*GCNT3*), which are involved in the synthesis of unbranched glycosaminoglycan hyaluronan, heparan sulfate, and mucin, major constituents of the epithelial extracellular environment [21–23]. These genes have been previously identified in EoE biopsies and in primary human esophageal epithelial cells induced with IL-13 [10, 19]. Notably, among early responsive genes, there was a member of the AT-rich interaction domain family of DNA-binding proteins, AT-rich interactive domain 5B (*ARID5B*), which functions as a cofactor in a histone demethylation process [24], and mitogen-activated protein kinase kinase kinase 14 (*MAP3K14*), a NF-κB–inducing kinase that participates in regulating tumor necrosis factor (TNF) and interleukin 1 (IL-1) receptor signaling [25], suggesting that initial stimulation by IL-13 can affect subsequent stimuli by modulating chromatin structure and signaling events. A member of the epithelial-specific ETS transcription factors ETS homologous factor (*EHF*) [26], a RUNX family protein runt-related transcription factor 2 (*RUNX2*), and the zinc finger protein B-cell CLL/lymphoma 11B (*BCL11B*) were also identified as early targets of IL-13 in TE-7 cells. These transcription factors have been previously implicated in regulating epithelial cell proliferation and differentiation [27–29]. Interleukin 31 (*IL-31*), which is normally produced by activated T cells [30], was highly induced in TE-7 epithelial cells. Interestingly, epithelial cells

have been shown to respond synergistically to IL-13 and IL-31 by secreting inflammatory cytokines, such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and chemokine (C-C motif) ligand 2 (CCL2) [31], suggesting a positive feedback loop in inducing allergic inflammation.

We identified *NTRK1*, as one of most highly IL-13–induced genes in TE-7 cells. NTRK1 is highly expressed in the nervous system and has a role in the survival of neurons, as well as in sensitivity to pain [32]. A growing body of evidence links the NGF/NTRK1 pathway to the pathogenesis of allergic inflammation, such as asthma and allergic rhinitis [33–35], yet a direct link between IL-13– and NGF-mediated pathways has not been shown.

NTRK1 is a unique RTK transcriptionally induced by IL-13

In light of the emerging role of NGF/NTRK1 signaling in the pathogenesis of allergic inflammation, we were intrigued by the robust induction of *NTRK1* by IL-13 in TE-7 cells. We hypothesized that the NGF/NTRK1 and IL-13/STAT6 pathways cooperate in propagating allergic inflammation. We therefore focused on exploring the mechanism of IL-13–mediated *NTRK1* induction in epithelial cells and the functional outcome of this induction. First, we validated our RNA-sequencing finding by testing the kinetics of *NTRK1* expression in TE-7 cells by real-time polymerase chain reaction (RT-PCR). In agreement with RNA-sequencing data, this analysis revealed that the *NTRK1* transcript was detectable as early as 2 hr after stimulation and progressively increased at the assessed time points over a 24-hr period mirroring induction of *CCL26* (Figure 2A). We further assessed expression of *NTRK1* in the esophageal epithelial cell line EPC2 grown at air-liquid interface (ALI), primary epithelial cells obtained from esophageal biopsies, and human bronchial epithelial cells after stimulation with IL-13. The results demonstrated that *NTRK1* was highly induced by IL-13 in epithelial cells of different origin; the kinetics of *NTRK1* induction paralleled those of *CCL26* (Figure 2B–D). Finally, we tested whether induction of *NTRK1* was dependent on IL-13 signaling. By stably integrating shRNA against STAT6 in TE-7 cells, we decreased levels of STAT6 mRNA and protein to 20–30% of that of control cells (Suppl. Figure 2). By stimulating these cells with IL-13, we found that transcriptional induction of both *CCL26* and *NTRK1* was significantly decreased, indicating STAT6 dependency (Figure 2E).

As approximately 90 RTKs have been identified in the human genome [36], we investigated how many of these molecules besides *NTRK1* were altered in the course of IL-13 induction. From RNA-sequencing data, we identified 29 RTKs that were expressed in either untreated or IL-13–stimulated TE-7 cells (reads per kilobase per million mapped reads [RPKM]>1) and analyzed the effect of IL-13 on their transcription. We found that transcription of 6 members of the RTK family was significantly affected by IL-13 in at least one time point of stimulation ($p < 0.05$); however, *NTRK1* was unique in that it was the only RTK strongly and consistently up regulated throughout IL-13 stimulation (Figure 3A, B).

IL-13 epigenetically regulates *NTRK1* in a STAT6-dependent manner

We analyzed the levels of the activating epigenetic marks H3K9Ac, H3K27Ac, and H3K4me3, which reside on actively transcribed genes [37], in the promoter of *NTRK1*.

Quantitative kinetic analysis revealed the presence of activating chromatin marks prior to IL-13 stimulation and the progressive increase in the levels of H3 acetylation and methylation with IL-13 stimulation, starting from the 2-hr time point (Figure 4A, *NTRK1*). Similarly, the promoter of *CCL26* (positive control) was marked by histone acetylation, especially H3K27Ac, prior to IL-13 stimulation, and the level of acetylation was significantly increased after IL-13 stimulation; the level of H3K4me3 was not significantly affected by IL-13 (Figure 4A, *CCL26*). As a negative control, activating epigenetic marks were absent in the promoter of the myogenic differentiation 1 (*MYOD*) gene, which was neither expressed nor induced in TE-7 cells (Figure 4A, *MYOD*). We next examined the requirement for STAT6 in epigenetic regulation of NTRK1. We utilized TE-7 STAT6 knockdown cells to assess the level of acetylation and methylation chromatin marks in the *NTRK1* promoter following IL-13 stimulation. We found that the IL-13—mediated increase in activating chromatin modifications was dependent on STAT6 (Figure 4B). Collectively, these data identified *NTRK1* as a direct early transcriptional and epigenetic target of IL-13 in human epithelial cells.

IL-13–induced NTRK1 confers epithelial cell responsiveness to NGF

NTRK1 belongs to the family of mitogen-activated protein (MAP) kinase receptors, which undergo dimerization and phosphorylation on tyrosine residues in response to NGF stimulation. Subsequently, downstream signaling events are induced including stimulation of the RAS/RAF pathway, PLCγ recruitment, and PI3 kinase activation [34]. To assess the functionality of IL-13–induced NTRK1 in epithelial cells, we tested the ability of NGF to induce signal transduction in primary epithelial and TE-7 cells pre-treated with IL-13. Western blot analysis showed that NTRK1 was not detected in resting cells but was induced after IL-13 stimulation in primary esophageal epithelial cells and TE-7 cells (Figure 5A, B, NTRK1 panel). Subsequent stimulation with NGF caused rapid phosphorylation of tyrosine residues Tyr674/675 in the catalytic domain of NTRK1 (Figure 5A, B, pNTRK1 panel), as well as increased phosphorylation of ERK1/2 (Figure 5B, pERK1/2 panel) in IL-13–treated cells. In agreement with previous reports, NTRK1 showed rapid kinetics of Tyr674/675 phosphorylation with peak expression at 5 min and a progressive decrease over 30 min after NGF stimulation [38] (Figure 5C). To demonstrate that NGF stimulation caused a transcriptional response, we measured the levels of *EGR1* and *EGR3* mRNA, which are known to be transcriptional targets of NGF in PC12 pheochromocytoma cells [15, 39]. In IL-13–pre-treated cells, but not in untreated cells, we observed a rapid and transient increase in *EGR1* and *EGR3* transcripts, which peaked at 1 hr after NGF addition (Figure 5D). Accordingly, increased EGR1 and EGR3 protein levels were detected after NGF stimulation of IL-13-pre-treated cells (Figure 5E). Taken together, these data show that IL-13 induces functional NTRK1 and thereby confers NGF sensitivity to human epithelial cells.

NTRK1 and EGR1, but not NGF, are increased in esophageal biopsies from EoE patients

In vitro experiments with epithelial cells prompted us to investigate whether NTRK1 is increased in epithelial cells *in vivo* during allergic responses. First, we assessed expression of *NTRK1* mRNA in esophageal biopsies of control and EoE patients. RT-PCR analysis showed a highly significant increase of *NTRK1* expression in the biopsies of patients with active EoE compared to unaffected control individuals (Figure 6A, $p<0.001$). In addition,

NTRK1 was dynamically expressed as a function of disease activity, as the esophagus of patients who responded to swallowed steroid treatment (fluticasone propionate responders [FPR]) had normalized levels (Figure 6A, FPR). Levels of *NTRK1* mRNA highly correlated with *IL-13* mRNA (Figure 6B, r = 0.75, p = 0.0001) and with eosinophil counts in biopsies of EoE patients (Figure 6C, r = 0.46, p = 0.006). Accordingly, NTRK1 protein was significantly increased in biopsies from patients with active EoE compared to unaffected individuals (Figure 6D, E). Notably, neither mRNA nor protein levels of NGF were significantly altered in the biopsies from EoE patients; as a positive control, *CCL26* mRNA and protein were highly increased (Figure 6F, G). Immunohistochemistry of control and EoE biopsies substantiated our findings by showing positive NTRK1 staining in the cytoplasm and membrane of epithelial cells in the basal layer in active EoE samples but not in control unaffected samples (Figure 6H). Notably, NGF showed diffuse staining throughout the esophageal epithelium independent of the disease activity. Therefore, whereas the ligand NGF is constitutively expressed, NTRK1 is induced by IL-13 and during allergic inflammation. Collectively, these data establish a mechanism wherein the receptor, but not the ligand, is the limiting checkpoint in the pathway. Furthermore, we tested the expression level of EGR1, the main transcriptional target of NGF/NTRK1 signaling [15] in the biopsies of patients with active EoE and found that both the mRNA and protein levels of EGR1 were significantly increased in the biopsies from patients with active EoE compared to control samples (Figure 6I–K).

Characterization of TE-7 cells stably expressing NTRK1

In order to further investigate the functional link between IL-13 and NGF signaling we generated two pools of TE-7 cells stably expressing NTRK1 that allowed us to simultaneously induce IL-13 and NGF/NTRK1 responses. The majority of the cells expressed NTRK1 in the cytoplasm and membrane at comparable levels between cell lines (Figure 7A, B, NTRK1 panel), efficiently responded to NGF by phosphorylating NTRK1, and transduced intracellular signaling leading to ERK1/2 phosphorylation (Figure 7B, pNTRK1 and pERK 1/2 panels). Subsequently, rapid and transient induction of *EGR1* was observed, indicating that NTRK1 efficiently elicited a transcriptional response (Figure 7C). Notably, while IL-13 efficiently induced *CCL26* expression in both control and NTRK1-expressing cells, it failed to induce *EGR1* and *EGR3* transcription in control clones. As expected, NGF stimulation had no effect on control TE-7 cells, suggesting that NTRK1 is necessary for the activation of EGR genes. Indeed, a decrease in the level of expression of NTRK1 in stable pools and prevention of NTRK1 induction by IL-13 in control pools significantly diminished *EGR1* and *EGR3* activation by NGF (Figure 7D, E).

We further hypothesized that inhibition of NTRK1 kinase activity will prevent induction of EGR genes following NGF stimulation. To test this hypothesis we utilized two tyrosine kinase inhibitors lestaurtinib (CEP-701) and crizotinib that were previously reported as efficient blockers of NTRK1 auto-phosphorylation and downstream signaling [40, 41]. As expected both drugs dramatically reduced the NGF-mediated increase in *EGR1* and *EGR3* mRNA (Figure 7F). These data suggest that functional NTRK1 is required for efficient EGRs stimulation in epithelial cells.

NTRK1 and IL-13 synergize in propagating allergic responses

As readout of functional interaction between NGF/NTRK1 and IL-13, we measured transcription of several known IL-13 target genes, including *CCL26*, the molecular driver of eosinophilic infiltration in EoE. We stimulated TE-7 cells with IL-13 and NGF either alone or in combination for the period of 6 hr and quantified mRNA levels of the genes. Exposure to NGF alone had no effect on the level of *CCL26*, *SERPINB4*, *KITLG*, or *ID3* mRNA, whereas induction with IL-13 caused increased expression of these genes. In NTRK1-expressing cells, the transcription of *CCL26*, *SERPINB4*, *KITLG* but not *ID3* was synergistically increased by NGF and IL-13 (Figure 8A–D). In agreement with these data, secretion of CCL26 from NTRK1-expressing cells after co-stimulation with IL-13 and NGF was significantly higher compared with either signal alone (Figure 8E). Collectively, these findings demonstrated the potential of synergistic interaction between NGF/NTRK1 and IL-13 in propagating allergic inflammation.

EGR1 is required for efficient induction of IL-13 target genes

EGR1 has been previously implicated in IL-13—mediated allergic inflammation and tissue remodeling in mice [16]. Although we could not detect increased *EGR1* expression after IL-13 stimulation (Figure 7C), synergistic induction of several IL-13 target genes by IL-13 and NGF prompted us to test whether EGR1 is required for efficient IL-13 response in esophageal epithelial cells. As TE-7 cells express detectable levels of EGR1 at baseline (Figure 5E), we utilized an siRNA approach to decrease EGR1 expression in TE-7 cells prior to IL-13 stimulation. We efficiently downregulated *EGR1*, but not *EGR3* expression in cells by about 3 to 5-fold (Figure 8F, *EGR1*, *EGR3*). In TE-7 cells with decreased *EGR1* level, the ability of IL-13 to induce expression of some target genes (*CCL26*, *CDH26* and *SERPINB4*), but not others (*KITLG*, *NTRK1*) was significantly diminished (Figure 8F). These findings implicate EGR1 in the propagation of IL-13—mediated allergic inflammation in human epithelial cells and provide mechanistic insight into synergistic interaction between NGF and IL-13 pathways.

Discussion

By analyzing the kinetics of gene expression in IL-13–stimulated human epithelial cells, we have identified NTRK1, a high-affinity receptor for NGF, as a direct transcriptional and epigenetic target of IL-13. In support of this finding, we showed that NTRK1 was highly induced by IL-13 in epithelial cells, including those derived from several sources (e.g. bronchi and esophagus). This induction was STAT6 dependent and readily detectable as early as 2 hr after IL-13 addition, and the level of NTRK1 decreased as IL-13 was withdrawn (data not shown). In addition, in response to IL-13, the *NTRK1* promoter acquired a set of activating epigenetic marks including histone H3 acetylation and trimethylation as early as 2 hr after IL-13 addition and continued accumulating these marks throughout the stimulation in a STAT6-dependent manner, mirroring the expression pattern of the gene.

Translational studies showed elevated *NTRK1* expression in the biopsies of patients with active EoE and that this increased *NTRK1* expression normalized after steroid treatment.

Moreover, *NTRK1* mRNA levels strongly correlated with the markers of disease activity, such as *IL-13* mRNA level and eosinophil counts. This pattern of expression resembles that of CCL26, a critical eosinophil chemo attractant in the pathogenesis of EoE [19]. Our *in vitro* finding that NGF and IL-13 synergistically induce a number of hallmark genes including *CCL26* in conjunction with *in vivo* data showing dynamic expression of NTRK1 as a function of disease severity collectively suggest that the NGF/NTRK1 and IL-13/STAT6 pathways functionally cooperate in propagating allergic inflammation. We indeed observed increased levels of EGR1, a central transcriptional target of NGF [15], in biopsies of patients with active EoE. EGR1 has been previously shown to be induced in lung epithelial cells exposed to dust mite allergen [42] and to play an important role in the induction of Th2-related chemokines [16]. *EGR1* is rapidly and transiently induced in response to various stimuli, including growth factors or cytokines [43, 44], mechanical stress [45], and hypoxia [46]. Therefore, we cannot definitively state that EGR1 elevation is solely due to NGF/NTRK1 signaling. Regardless, these findings suggest potential functional interactions of NGF/NTRK1 and IL-13 pathways in the pathogenesis of allergic inflammation.

Unlike mouse models [16, 47], we could not detect increased level of EGR1 in response to IL-13 stimulation. Yet our findings that decreased EGR1 expression correlated with diminished IL-13 response suggest that EGR1 synergizes with IL-13 in induction of early target genes presumably by interacting with STAT6. Cooperative interaction of EGR1 with other transcription factors, such as nuclear factor of activated T-cells (NFAT) and nuclear factor kappa-B (NFkB) has been previously reported in T cells [48]. Similarly, synergistic interaction between STAT6 and NFkB in IL-4—induced transcription has been shown [49]. Moreover, EGR1 has been implicated in the formation of chromatin loops required for its synergistic interaction with other transcriptional factors [50], substantiating the potential role of epigenetics in allergic transcriptional response. Notably, EGR1 was required for efficient IL-13 induction of some but not all genes, suggesting that other factors are required for synergistic activation of target genes by IL-13 and NGF during propagation of allergic inflammation.

Epigenetics is considered as a possible mechanism involved in the development of many disorders, including allergic diseases [51, 52]. The most common epigenetic mechanisms include DNA methylation, histone modifications, and noncoding RNAs, all of which can affect gene transcription through effects on DNA structure and inducing gene silencing. We have previously demonstrated that these mechanisms are involved in IL-13–mediated allergic inflammation [53–55]. Accordingly, IL-13–mediated *NTRK1* induction was accompanied by increased levels of activating epigenetic modifications. Notably, we have found high levels of activating marks in the promoter of *NTRK1* prior to IL-13 stimulation, suggesting that *NTRK1* is epigenetically poised for induction [56–58]. Similarly, elevated levels of histone acetylation were observed in the *CCL26* promoter, collectively suggesting that epigenetic poising may be a common mechanism of induction for early IL-13 targets. Interestingly, the level of the H3K4me3 mark, which is commonly present at the promoters of transcribed genes [59], was relatively low on the *CCL26* promoter compared to *NTRK1*. A low level of H3K4me3 has been observed previously in ~20% of expressed genes and the percentage of genes with the H3K4me3 mark was not increased following cell activation [56].

The low level of H3K4me3 in the promoter of the *CCL26* gene may reflect a more "enhancer like" signature of this region, which is consistent with the known signature of active enhancers (low H3K4me3, high H3K27Ac) [60]. Alternatively, it is possible that the peak of H3K4me3 in the promoter of *CCL26* is shifted relatively to other histone marks and therefore is not detected by RT-PCR. Performing ChIP-sequencing experiments should differentiate between these possibilities. The ultimate goal of future studies will be the comprehensive characterization of the IL-13–mediated epigenome by ChIP-sequencing and utilizing this knowledge in developing new diagnostic and therapeutic strategies in allergy.

Among the 90 RTKs identified in the human genome [36], NTRK1 was the only one induced both transcriptionally and epigenetically by IL-13 in esophageal epithelial cells. Remarkably, we did not observe significant changes in the NGF level in esophageal biopsies, where it was detected independent of disease status. Transcriptional regulation of NTRKs has been widely investigated [61]; however, to the best of our knowledge, these data are the first example of regulating NTRK1 signaling by modulating the level of the receptor rather than the ligand in human epithelial cells. One previous study described down regulation of NTRK1, but not NGF, in the cerebellum of rat pups exposed to ethanol [62]. On the basis of our findings, we propose an NTRK1/IL-13 axis is involved in the propagation of allergic inflammation (Figure 9). In this model, early transcriptional targets of IL-13 in epithelial cells are epigenetically poised for expression, as evident from the presence of activating epigenetic marks on the promoters of genes prior to IL-13 stimulation. IL-13 causes increased expression of *NTRK1* together with other early transcriptional targets (*CCL26*) accompanied by augmenting epigenetic changes in the promoters of these genes. Subsequently NTRK1 becomes activated by the extracellular pool of NGF molecules, which in turn leads to NTRK1-mediated transcriptional responses (e.g. increased *EGR1*), as well as synergistic interaction with IL-13 in eliciting transcription and secretion of CCL26 and other mediators of the allergic response.

In summary, we identify NTRK1 as an early epigenetic and transcriptional target of IL-13 in human epithelial cells. We provide evidence that NTRK1, but not its ligand NGF, is dramatically up regulated in the biopsies of patients with active EoE, establishing a mechanism wherein the receptor, but not the ligand, is the limiting checkpoint in the pathway. We demonstrate synergistic induction of the critical allergic mediator CCL26 by IL-13 and NGF/NTRK1 signaling, supporting the role of NTRK1 in the pathogenesis of mucosal allergic inflammation.

Our finding that NTRK1 is highly increased in human esophageal biopsies from EoE patients and synergizes with IL-13 in propagating allergic inflammation provides the rationale for pharmacological targeting of NTRK1 signaling. The increasingly recognized link between NTRK1 signaling and cancer progression [40, 63–65] as well as the critical role of NTRK1 in pain sensitivity [32, 66, 67] has stimulated development of highly specific NTRK1 inhibitors, such as AR-786, AR-256 and AR-618 [68]. Additionally, the wide range of existing tyrosine kinase inhibitors specific to a number of protein kinases including NTRK1, such as lestaurtinib (CEP-701) and crizotinib, used in our study can be utilized to block NTRK1 activity. Some of these inhibitors have been approved for use in human subjects primarily for cancer treatment[41, 69]. Importantly, because NTRK1 is the only protein tyrosine kinase

dramatically induced by IL-13 in epithelial cells, even the use of tyrosine kinase inhibitors not specifically developed for NTRK1 may be beneficial for decreasing allergic inflammation. Our experiments provide initial support for the feasibility of this approach.

Materials and Methods

Cell culture and treatment

Primary esophageal epithelial cells were cultured as previously described [10]. The squamous esophageal epithelial cell line TE-7 (a kind gift of Dr. Hainault, France), which was originally selected from human esophageal tumors [17], was maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum (FCS). The esophageal epithelial cell line (hTERT-immortalized EPC2 line) was a kind gift from Dr. Anil Rustgi (University of Pennsylvania). EPC2 cell monolayers were grown on 0.4-μm pore–size polyester permeable supports (Corning Incorporated, Corning, NY) in keratinocyte serum-free media (K-SFM) (Life Technologies, Grand Island, NY). Once confluent, medium was switched to high-calcium ($[Ca^{2+}]$ = 1.8 mM) K-SFM for an additional 3–5 days. Epithelial differentiation was then induced by removing culture media from the inner chamber of the permeable support and maintaining the esophageal epithelial cells for 5–7 days at the air-liquid interface (ALI). Normal human primary bronchial epithelial cells were purchased from Lonza (Lonza, MD, CC-2540) and cultured as previously described [70]. IL-13 (Peprotech, Rocky Hill, NJ) and NGF (human β–NGF; Cell Signaling Technology, MA) were added to the culture media at indicated concentrations 0f 1, 10 or 100 ng/ml for the indicated periods of time. Tyrosine kinase inhibitors crizotinib (PZ0191) and lestaurtinib (CEP-701 hydrate, C7869) were purchased from Sigma-Aldrich (Sigma-Aldrich Corp. St. Louis, MO) and were dissolved in DMSO to stock concentration of 10 mM. Cells were pre-treated with the inhibitors at 0.1 μM for 15 min prior to stimulation with NGF at 10 ng/ml for 2 hr.

Antibodies

Rabbit antibodies against TrkA (14G6) (#2508), phospho-TrkA (Tyr674/675)/TrkB (Tyr706/707) (#C50F3); phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (#9101), p44/42 MAPK (ERK1/2) (137F5) (#4695); phospho-STAT6 (Tyr641) (C11A12); EGR1 (44D5) (#4154); EGR3 (#2559), p38 MAPK (D13E1) XP® (#8690); mouse monoclonal antibody against p44/42 MAPK (Erk1/2) (3A7, #9107) and anti-rabbit IgG, HRP-linked (#7074) were purchased from Cell Signaling (Cell Signaling Technology, MA). STAT6 (S-20, sc-621) was from Santa Cruz (Santa Cruz Biotechnology, TX). Monoclonal anti-GAPDH-peroxidase clone GAPDH-71.1 (#G9295) was from Sigma (Sigma-Aldrich Corp. St. Louis, MO). For chromatin immune precipitation (ChIP) H3K4me3 (ab8580), H3K9Ac (ab10812) and H3K27Ac (ab4279) were from Abcam (Abcam, Cambridge, MA). Some ChIP experiments were performed with H3K27Ac antibody from Diagenode (pAb-196-050, Diagenode Inc., Denville, NJ) and H3K4me3 antibody from Millipore (17–614, Billerica, MA). For immunofluorescence goat antrabbit Alexa Fluor 647 (A-21244) and goat ant-mouse Alexa Fluor 488 (A11029) were from Invitrogen (Invitrogen, Carlsbad, CA).

STAT6 gene silencing in TE-7 cells

Lentiviral shRNA vector against *STAT6* (MISSION® shRNA, Sigma-Aldrich, clone ID NM_003153.3-3231s1c1, TRCN0000019409) was used to produce lentiviral particles by the Cincinnati Children's Hospital Medical Center (CCHMC) Lenti-shRNA Library Core. A vector that targets no known mammalian genes was used as a control (SHC002 SIGMA MISSION® pLKO.1-puro Non-Mammalian shRNA Control). TE-7 cells were grown in RPMI medium with 5% FCS and infected on 6-well plates in the presence of polybrene at 5 μg/ml by centrifuging the plate at 2000 × *g* for 1 hr at room temperature. The following day, puromycin selection was applied at 2 μg/ml for at least 7 days. shSTAT6 stably infected cells were maintained in puromycin at 1 μg/ml. Puromycin was removed 24 hr prior to the start of experiments.

EGR1 and *NTRK1* gene silencing in TE-7 cells

For *EGR1* silencing, TE-7 cells were grown on 12 well plates in RPMI with 1% FCS medium and transfected with SMART pool ON-TARGET plus EGR1 siRNA (L-006526-00-0005; GE Dharmacon, Lafayette, CO) using Dharmafect 1 transfection reagent (GE Dharmacon). Final siRNA concentration was 30nM. Following 36hr of incubation, cells were stimulated with IL-13 at 1 ng/ml for the indicated periods of time without medium change. For *NTRK1* silencing pLVX control and NTRK1 stable pools were grown in RPMI medium with 5% FCS. SMART pool ON-TARGET plus NTRK1 siRNA (L-003159-00-0005, GE Dharmacon, Lafayette, CO) was used at final concentration of 30nM. For control pool silencing was performed for 18 hr prior to induction with IL-13 at 100 ng/ml for 24hr followed by NGF stimulation. For NTRK1 pools silencing was performed for 36 hr prior to NGF stimulation.

Preparation of stable pools of TE-7 cells expressing NTRK1

Homo sapiens neurotrophic tyrosine kinase, receptor, type 1 (NTRK1), transcript variant 1, mRNA (NM_001012331.1) was amplified from the pCMV-Sport6 vector (MHS1010-202736585, clone ID 5200930; Fisher Scientific, PA) and cloned between EcoRI and XbaI sites in pLVX-IRES-Puro vector using the In-fusion method (Clontech, CA). Lentiviral particles were produced by transfecting HEK 293T cells according to the protocol recommended by Clontech. TE-7 cells were grown in RPMI medium with 5% FCS and infected on 6-well plates in the presence of polybrene at 5 μg/ml by centrifuging the plate at 2000 × *g* for 1 hr at room temperature. The following day, puromycin selection was applied at 1 μg/ml for at least 7 days. NTRK1 stably infected cells were maintained in puromycin at 1 μg/ml. Puromycin was removed 24 hr prior to the start of experiments. Two independent transductions were performed to generate NTRK1 (1) and NTRK1 (2) pools. Expression levels were validated by Western blotting and immunofluorescence analysis.

Immunofluorescence of TE-7 cells

For immunofluorescence, pLVX Control and NTRK1 pools cells were grown on round cover slips placed in the well of the 6 well plate. Cells were fixed with cold methanol for 10 min on ice and washed three times with PBS for 5 min each wash. Blocking was performed in 10% goat serum for 30 min at RT and cover slips were incubated with primary antibodies

against NTRK1 (rabbit) and ERK1/2 (mouse) at 1/250 dilution for 2 hr at RT. After 3 additional washes with PBS secondary antibodies goat anti rabbit Alexa Fluor 647 and goat anti mouse Alexa Fluor 488 were added for 1 hr at RT at 1/500 dilution. Cells were washed 3 times with PBS with 4′,6-diamidino-2-phenylindole (DAPI, D3571, Molecular Probes, Life Technology) added to the first wash at 0.5 μg/ml for 20 min at RT. Cover slips were mounted on the polysine microscope slides on Prolong Gold mounting medium (P36930, Molecular Probes, Life Technology). Images were acquired using an Apotome upright confocal microscope (Zeiss, Thornwood, NY) under the same settings.

mRNA extraction, quantitative RT-PCR, and RNA-sequencing analysis

Total RNA was isolated from cells with the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. For RNA-sequencing experiments, RNA was treated with On-Column DNase Digestion kit (Qiagen, Valencia, CA) according to the supplied protocol. cDNA was synthesized with the iScript™ synthesis kit (BioRad, Hercules, CA). RT-PCR was performed using a 7900HT Fast Real-Time PCR system from Applied Biosystems (Life Technologies Grand Island, NY) with FastStart Universal SYBR Green Master mix (Roche Diagnostics Corporation Indianapolis, IN). Next Generation RNA-sequencing was performed by the CCHMC Genetic Variation and Gene Discovery Core Facility using Illumina TruSeq kits and sequenced on the Illumina HiSeq2000. For RNA-sequencing analysis, Fastq files from the Illumina pipeline were aligned by TopHat [71] with –T –G parameters (http://tophat.cbcb.umd.edu/manual.html). RefSeq annotation from the University of California Santa Cruz (UCSC) genome browser [72] for hg19 genome was used. The –T parameter is used to align reads to the human transcriptome only, and the –G parameter is used to provide transcriptome annotation. To estimate the difference between treated and untreated experiments, the differential expression analysis for sequence count data (DESeq) package was applied [18]. For heat map generation, Cluster 3.0 was used for clustering data using Euclidian distance with average linkage (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm). For visualization, the Java Treeview program was used (http://jtreeview.sourceforge.net/). All the genes expressed below RPKM 1 in IL-13–treated cells were excluded from the analysis. RNA-sequencing data files were uploaded to the GEO database under the accession number GSE57637.

Chromatin immunoprecipitation (ChIP)-PCR analysis

We fixed 10–20×10^6 TE-7 cells with 0.8% formaldehyde by adding 1 ml of 10X fixation buffer (50 mM Hepes-KOH, pH 7.5; 100 mM NaCl; 1 mM EDTA; 0.5 mM EGTA; 8% formaldehyde) to 9 ml of growth medium for 8 minutes at room temperature with shaking. The reaction was stopped by adding glycine to a final concentration of 125 mM for an additional 5 minutes. After washing with PBS, pellets were frozen at −80°C for at least overnight. Nuclei were prepared by re-suspending pellets in 1 ml of L1 buffer (50 mM Hepes-KOH, pH 7.5; 140 mM NaCl; 1 mM EDTA; 10% glycerol; 0.5% NP-40; 0.25% Triton X-100) and incubated at 4°C for 10 min. Nuclei were pelleted and re-suspended in 1 ml of L2 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 rotated for 10 min at room temperature. Nuclei were briefly washed with sonication buffer (Tris-EDTA [TE] buffer + 0.1% SDS) and re-suspended in 1 ml of sonication buffer. All buffers were supplemented with complete EDTA-free protease

inhibitors (Roche Diagnostics Corporation Indianapolis, IN). Sonication was performed by a Covaris S220 focused ultrasonicator (Covaris, Inc. Woburn, MA) at 175W Peak Incident Power, 10% output, 200 bursts for 10 min in 12×12-mm, round-bottom glass tubes. Efficient DNA fragmentation was verified by agarose gel electrophoresis. ChIP was performed by SX-8G IP-Star® Automated System (Diagenode Inc., Denville, NJ) in RIPA buffer (TE + 0.1% SDS, 1% Triton X-100, 150 mM NaCl, 0.1% sodium deoxycholate) following the protocol of the manufacturer with 2–4 μg of indicated antibodies (see "Antibodies" section). The levels of histone modifications on the promoters were assessed by Prime Time Taqman qPCR assay (Integrated DNA Technologies, Inc. Coralville, Iowa) and normalized to the levels of modifications in the input sample.

Western blotting

Proteins from cell cultures were extracted with RIPA buffer (50 mM Tris-HCl pH 8; 150 mM NaCl; 1% Igepal; 0.5% sodium deoxycholate; 0.1% SDS, 1 mM EGTA, protease and phosphatase inhibitors), loaded on a 4–12% gel (Invitrogen), and subjected to Western blot analysis with the indicated antibodies.

Procuring and processing of esophageal biopsies

This study was performed with the approval of the CCHMC Institutional Review Board. Informed consent was obtained from patients or their legal guardians to donate tissue samples for research and to have their clinical information entered into the Cincinnati Center for Eosinophilic Disorders (CCED) database. Patients with no history of EoE or other eosinophilic gastrointestinal disorders (EGID) and with a current biopsy indicating 0 eosinophils/X400 high power field (HPF) in the distal esophagus and taking no form of glucocorticoid treatment at time of biopsy served as normal controls. Patients with active EoE were defined as those having 15 or more eosinophils/HPF at the time of biopsy and not receiving swallowed glucocorticoid or diet treatment at time of endoscopy. Fluticasone propionate (FP) responders (FPR) had a history of EoE, but their current distal esophageal biopsy count was 0 or 1 eosinophil/HPF, and they were receiving swallowed FP at the time of endoscopy. Total RNA was isolated from distal esophageal biopsy specimens using the miRNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. For RT-PCR analysis, total RNA (500 ng) was used to synthesize cDNA using Superscript II Reverse Transcriptase (Invitrogen) or iScript (Bio-Rad) using the protocols suggested by the manufacturer. For Western blot, esophageal biopsy protein lysates were prepared on ice by sonication in M-PER reagent (Thermo Fisher Scientific Inc., Waltham, MA USA) supplemented with 1X protease inhibitor cocktail (Roche Diagnostics Corporation Indianapolis, IN) according to the manufacturer's protocol, and soluble proteins were isolated by centrifugation at 10,000 × *g* for 10 min at 4°C. Immunohistochemical staining of biopsies was performed by the Pathology Research Core at CCHMC. Images were acquired using an Apotome upright confocal microscope (Zeiss, Thornwood, NY).

The enzyme-linked immunosorbent assay (ELISA) for CCL26 and NGF in cells and biopsies

For detection of secreted CCL26, cells were seeded on 12-well plates in RPMI medium containing 5% FCS. After 24 hrs, the medium was changed, and cells were allowed to grow for an additional 24 hr prior to stimulation with IL-13 and/or NGF at a final concentration of 100 ng/ml for 24 hr. At the time of the stimulation, cells were 50% confluent. NaCl was added to the supernatant of epithelial cells to a final concentration of 100 mM prior to collection. For detecting CCL26 and NGF in biopsies, esophageal biopsies were washed twice with 0.5 ml of PBS and sonicated for 2 min in 130 μl of SuperB buffer (Covaris, Inc. Woburn, MA) with addition of EDTA and protease and phosphatase inhibitors in a microTUBE in a S220 sonicator. Sonication conditions were used as recommended by the manufacturer (10% output, 70W Peak Incident Power, 200 cycles per burst). Samples were spun down at 10,000 × *g* for 15 min, and supernatant was collected for analysis. The BCA protein assay was used to determine the protein concentration of each sample (Thermo Fisher Scientific Inc., Waltham, MA USA). Samples were diluted 4 times in PBS prior to ELISA. Some samples were also used for Western blotting. Human CCL26/Eotaxin-3 and NGF DuoSet ELISA kits were used according to the manufacturer's recommendations (R&D Systems, Minneapolis, MN).

Statistical analysis

Statistical analysis was performed using Prism 6.0 software. Comparison among groups was done by the Kruskal-Wallis and ANOVA tests; *t*-test was applied for comparing two groups of data. A statistical probability of $p < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by the NIAID (R01AI083450 and R37AI045898), the CURED (Campaign Urging Research for Eosinophilic Disease) Foundation, the Food Allergy Research & Education (FARE), and the Buckeye Foundation. We thank Shawna Hottinger for editorial assistance; the Cincinnati Digestive Health Center Integrative Morphology Core for tissue processing, sectioning, histology, and immunohistochemical staining (NIH P30 DK078392); and Betsy DiPasquale for assistance with immunohistochemical stains.

Nonstandard abbreviations

H3K4me3	histone H3 lysine 4 trimethylation
H3K9Ac	histone H3 lysine 9 acetylation
H3K27Ac	histone H3 lysine 27 acetylation
RPKM	reads per kilobase per million reads
EoE	eosinophilic esophagitis
TSS	transcriptional start site

RTK	receptor tyrosine kinase
NGF	nerve growth factor
NTRK	neurotrophic tyrosine kinase receptor 1
ALI	air-liquid interface

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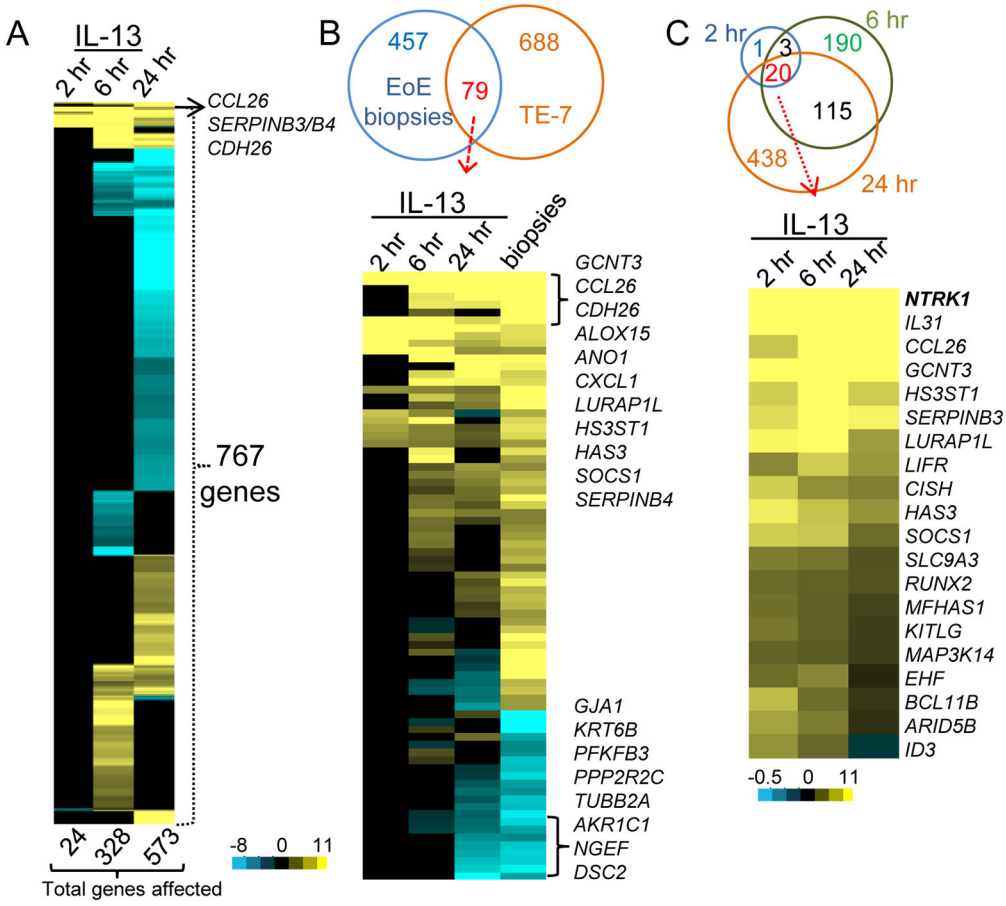

Figure 1. Kinetics of IL-13 response in TE-7 epithelial cells

In **A**, the heat map represents hierarchical clustering of the fold change difference of 767 genes significantly altered by IL-13 treatment in the TE-7 cell line compared to untreated control (DESeq $p < 0.05$). In **B**, the Venn diagram shows the overlap of genes significantly affected by IL-13 in TE-7 cells at all assessed time points and genes differentially expressed in human esophageal biopsies of patients with active EoE ($p < 0.05$). The heat map represents the log(2) fold change of 79 commonly affected genes compared to untreated TE-7 cells or control biopsies. The most highly up- and down regulated genes are indicated. In **C**, the Venn diagram represents the overlap of genes significantly affected by IL-13 after 2 hr, 6 hr, or 24 hr of stimulation. The heat map shows the kinetics of the transcriptional response for 20 genes affected at all IL-13 treatment time points. Note the increased expression of *NTRK1* throughout IL-13 stimulation. For all heat maps, clustering was performed using Euclidean distance and average linkage parameters. Yellow and blue colors correspond to increased or decreased expression, respectively, and black color indicates non-significant change compared to controls.

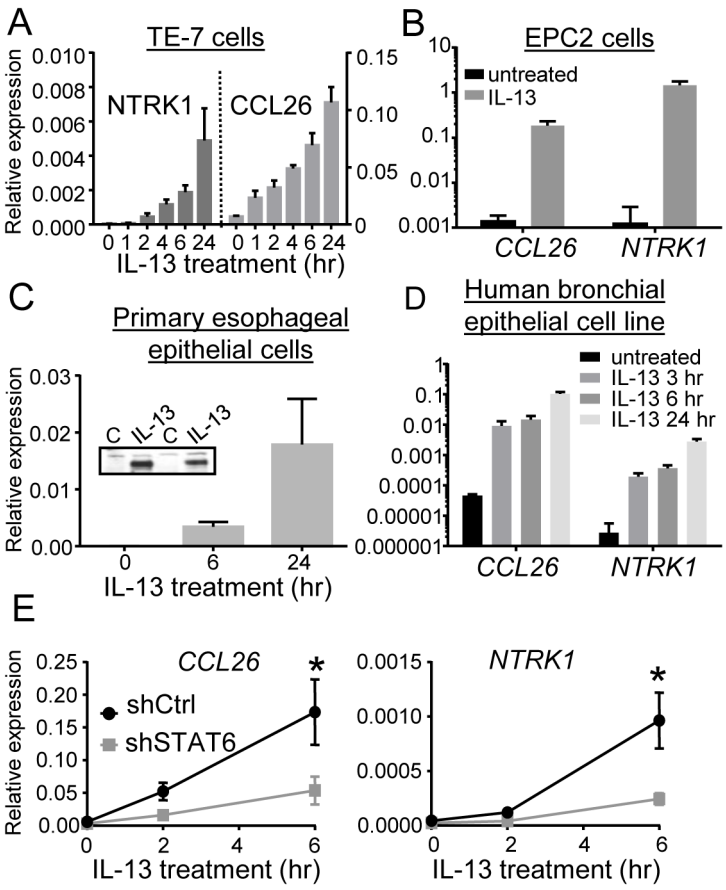

Figure 2. IL-13–stimulated *NTRK1* induction in epithelial cells and effect of *STAT6* gene silencing

In **A–D**, shown is quantitative RT-PCR analysis of *NTRK1* and *CCL26* transcription in TE-7 cells, EPC2 epithelial cell ALI culture, primary esophageal epithelial cells, and human bronchial epithelial cells. Cells were treated with IL-13 at 100 ng/ml for the indicated periods of time or for 6 days for EPC2 cells. The inset in **C** shows Western blot for NTRK1 in two independent cultures of primary esophageal epithelial cells stimulated with IL-13. In **E**, the effect of *STAT6* gene silencing by shRNA on *NTRK1* and *CCL26* induction in TE-7 cells was quantified by RT-PCR. TE-7 cells were stimulated with IL-13 (1 ng/ml) for the indicated periods of time. shCtrl, control shRNA; shSTAT6, shRNA against *STAT6*. Data for 3 to 4 independent experiments are presented as mean values for gene expression normalized to the level of *GAPDH* with standard error measurements.

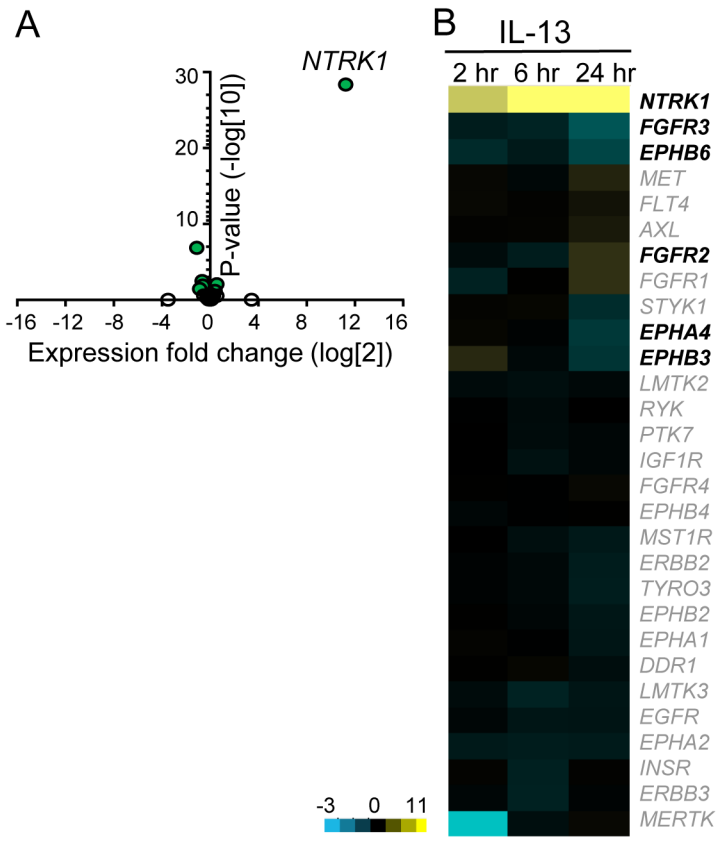

Figure 3. Effect of IL-13 on transcription of receptor tyrosine kinases (RTKs)

In **A**, volcano plot represents the expression of 29 RTKs with detectable expression in TE-7 cells (RPKM >1) after IL-13 stimulation for 24 hr. The x axis shows log (2) fold change of expression, and the y axis shows negative log (10) p-value for each gene. Green dots represent RTKs displaying significant change in expression in response to IL-13 in at least one time point (DESeq<0.05). In **B**, the heat map shows the kinetics of the transcriptional response for 29 RTKs after IL-13 stimulation. RTKs displayed as green dots in A are indicated in bold. Yellow and blue colors correspond to increased or decreased expression, respectively. The list of RTKs was compiled from Robinson *et al* [73] and filtered by the level of expression.

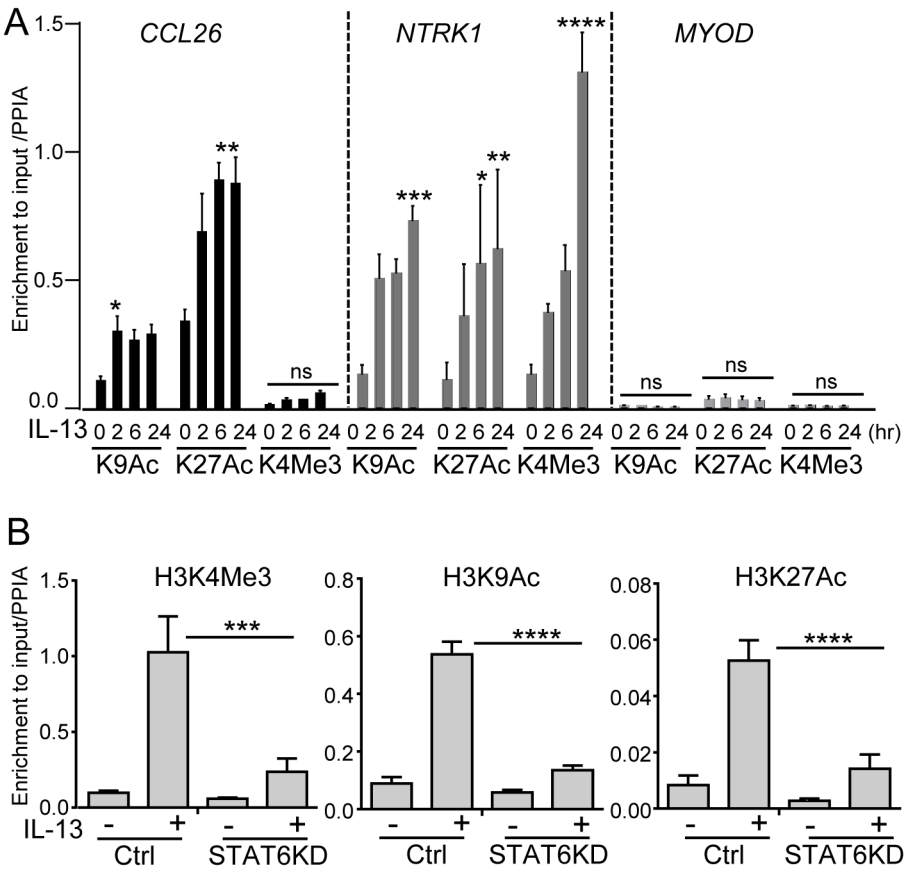

Figure 4. Effect of IL-13 stimulation and STAT6 silencing on epigenetic status of *NTRK1*

In **A**, levels of H3K9Ac, H3K27Ac, and H3K4me3 in the promoters of *CCL26*, *NTRK1*, and *MYOD* after IL-13 stimulation were quantified by ChIP-RT-PCR. Data from 3 independent experiments calculated as percentage of signal in input DNA normalized to the level of signal in PPIA gene are presented as mean values with standard error measurements. In **B**, levels of histone modification in the *NTRK1* promoter following induction with IL-13 for 24 hr were quantified by RT-PCR in control (Ctrl) and TE-7 cells where the STAT6 gene was silenced by shRNA (STAT6KD). Combined data for 2 independent experiments are shown. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns, not significant.

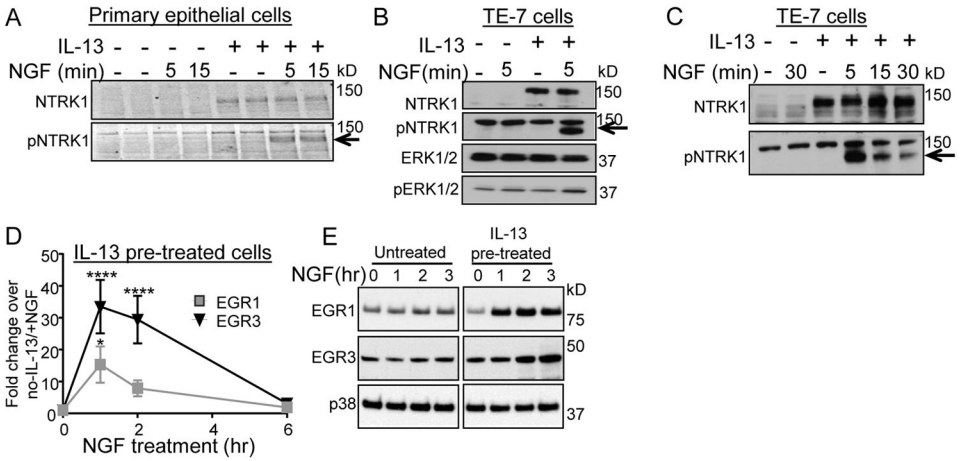

Figure 5. NTRK1 confers epithelial cell responsiveness to NGF

In **A**, shown is a Western blot analysis of primary esophageal epithelial cells pre-treated with IL-13 for 24 hr and then treated with recombinant human NGF for 0, 5, or 15 min. pNTRK1 indicates phosphorylated protein (arrow). In **B**, shown is Western blot analysis of TE-7 cells pre-treated with IL-13 for 24 hr and then treated with NGF for 5 min. pNTRK1 and pERK1/2 indicate phosphorylated proteins, arrow points at pNTRK1. In **C**, the kinetics of NTRK1 phosphorylation were assessed by Western blot. Cells were pre-treated with IL-13 for 24 hr and then treated with NGF for 5, 15 or 30 min. For **A–C**, phosphorylation was assessed at tyrosine residues Tyr674/675 in the catalytic domain of NTRK1. In **D**, kinetic analysis of *EGR1* and *EGR3* mRNA in TE-7 cells pre-treated with IL-13 for 24 hr followed by treatment with NGF for 1, 2, or 6 hrs was performed by RT-PCR. Fold change indicates increase over untreated (no IL-13) cells stimulated with NGF (+NGF). NGF was used at the concentration of 100 ng/ml. Data for 3 independent experiments are presented as mean value with standard error measurements; ****$p < 0.0001$, *$p < 0.05$. In **E**, EGR1 and EGR3 protein levels in TE-7 cells pre-treated with IL-13 for 24 hr followed by treatment with NGF were analyzed by Western blot; p38 serves as a loading control.

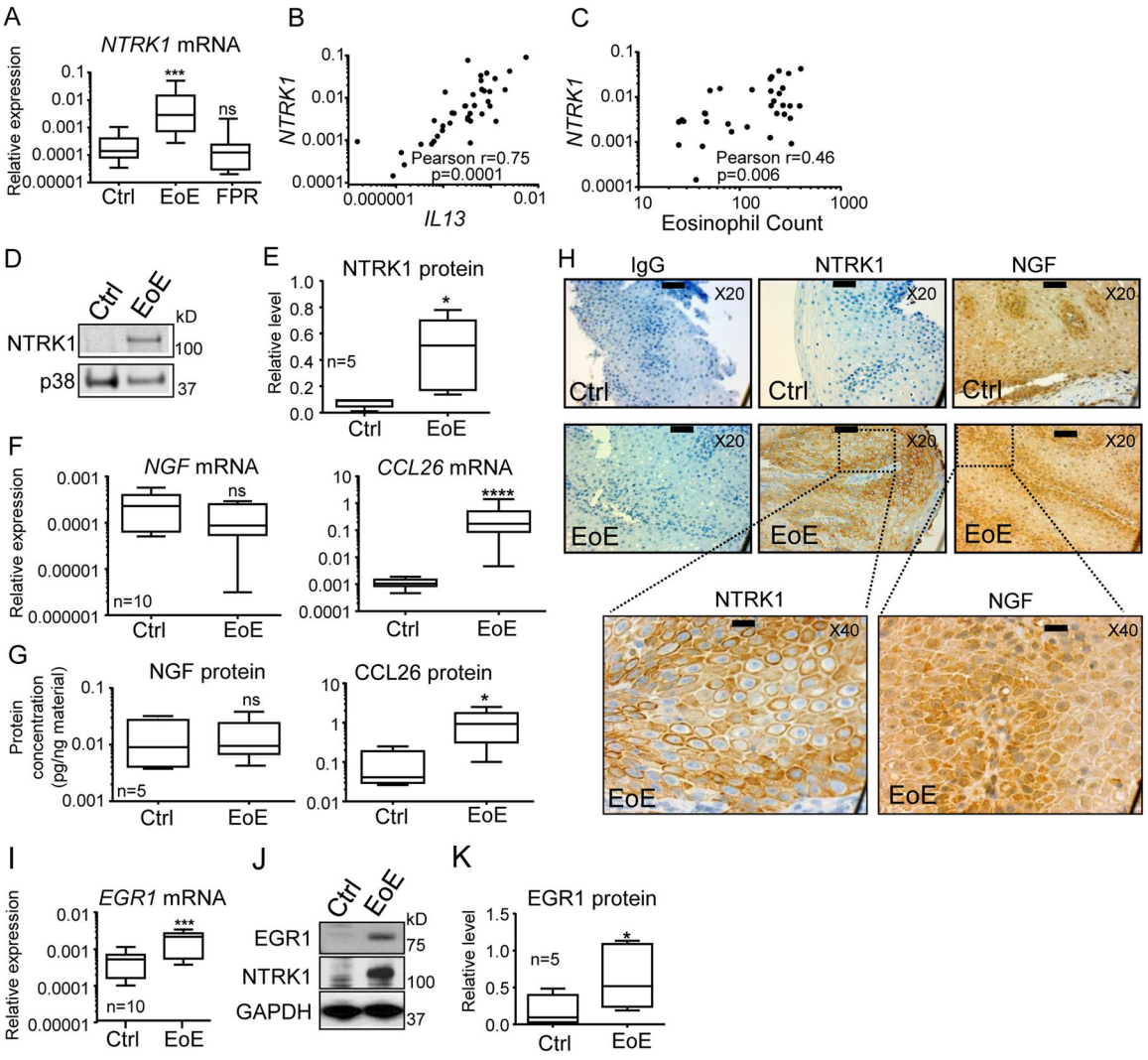

Figure 6. NTRK1, NGF, and EGR1 expression in human allergic inflammation

In **A**, RT-PCR analysis of *NTRK1* in biopsies was performed in unaffected control patients (Ctrl, n = 13), patients with active EoE (EoE, n = 12), and EoE patients who responded to swallowed steroid treatment (fluticasone responders [FPR], n = 13); ***p < 0.001; ns, not significant. In **B** and **C**, the graphs show Pearson correlation of *NTRK1* expression with *IL-13* levels and peak eosinophil counts in EoE biopsies. In **D**, a representative Western blot shows expression of NTRK1 in a control and EoE biopsy; p38 serves as a loading control. In **E**, quantification of NTRK1 in control and active EoE biopsies is shown (n = 5 each); *p < 0.05. Expression level was normalized to the level of GAPDH. In **F**, RT-PCR analysis of *NGF* and *CCL26* was performed in control and EoE biopsies (n = 10 each); ****p < 0.0001; ns, not significant. In **G**, amount of NGF and CCL26 protein was measured by ELISA in control and active EoE biopsies (n = 5 each); *p < 0.05; ns, not significant. In **H**, immunohistochemistry for NTRK1 and NGF in control (Ctrl, no eosinophils present in biopsy) and representative active EoE biopsy (EoE, 199 eosinophils per high-power field [HPF]) is shown. The bar is 20 μm for X20 images (top 6 panels) and 50 μm for X40 images

(bottom 2 panels). A total of 9 control and 10 active EoE biopsies were stained. In **I**, *EGR1* mRNA was measured by RT-PCR in esophageal biopsies of unaffected control (Ctrl) patients and patients with active EoE (n = 10 each). ***$p < 0.001$. In **J**, shown is a representative Western blot of EGR1 and NTRK1 in control and active EoE biopsies. GAPDH serves as a loading control. In **K**, relative level of EGR1 expression in biopsies is shown (n = 5 each); *$p < 0.05$. Expression level was normalized to the level of p38 protein. For A, F and I, mean values of gene expression were normalized to the level of *GAPDH*. For A, E, F, G, I and K, data are presented as box and whiskers plot.

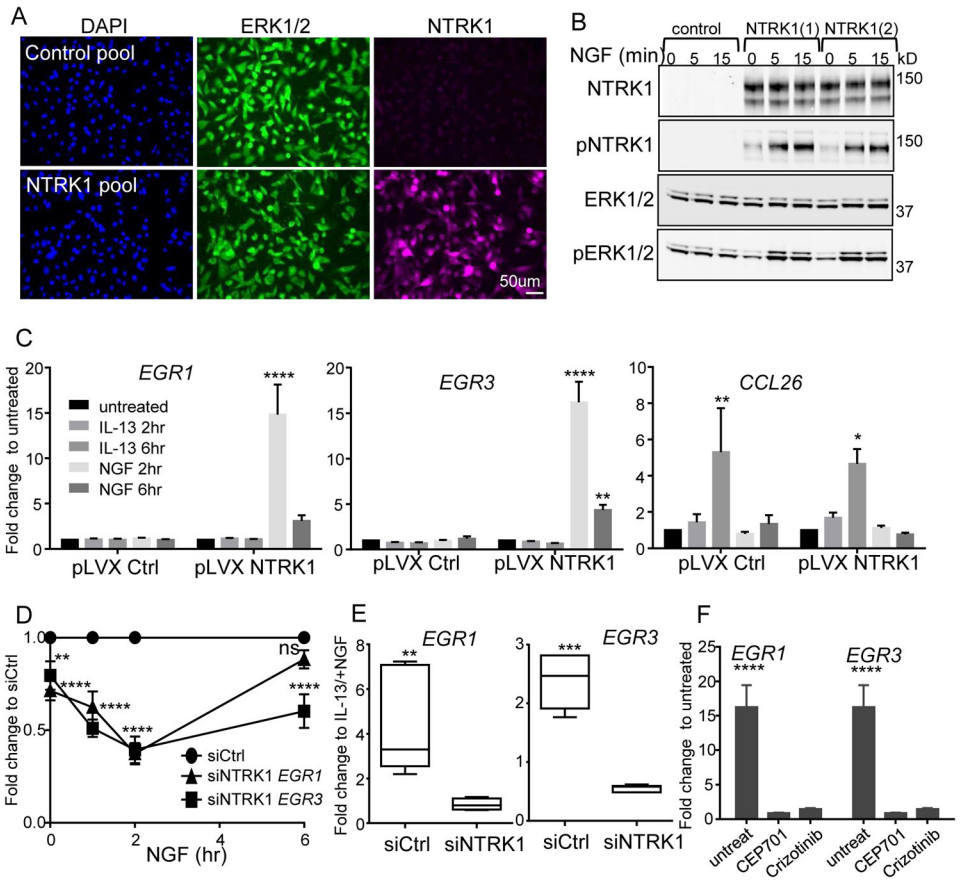

Figure 7. Characterization of TE-7 pools stably overexpressing NTRK1

In **A**, a representative immunofluorescent image of TE-7 cells infected with either empty virus (control pool) or virus expressing NTRK1 (NTRK1 pool) is shown. In **B**, shown is Western blot analysis of two independent pools of TE-7 cells stably expressing NTRK1 (NTRK1 (1) and NTRK1 (2)). Cells were treated with NGF (100 ng/ml) for the indicated times. pNTRK1 and pERK1/2 indicate phosphorylated proteins. In **C**, the effect of NGF and IL-13 stimulation on *EGR1*, *EGR3* and *CCL26* expression was assessed by RT-PCR in control (pLVX Ctrl) and NTRK1 stably expressing cells (pLVX NTRK1). In **D**, effect of NTRK1 down regulation in TE-7 cells stably expressing NTRK1 on *EGR1* and *EGR3* induction by NGF was assessed by RT-PCR. Cells were treated with siRNA for 36 hr followed by stimulated with NGF at 10 ng/ml for indicated periods of time. Expression level of EGRs relative to siCtrl-treated cells is shown. Data for 3 independent experiments are presented; error bars represent standard error measurements In **E**, the effect of decreased expression of NTRK1 in IL-13—pretreated TE-7 control pool on *EGR1* and *EGR3* induction by NGF was assessed by RT-PCR. Cells were treated with siRNA for 18 hr, induced with IL-13 at 10 ng/ml for 24 hr followed by stimulation with NGF at 10 ng/ml for 1 hr. siCtrl – control siRNA, siNTRK1 – siRNA against NTRK1. Data for 3 independent experiments are presented as box and whiskers plot. In **F**, the effect of pre-treatment of TE-7 cells stably expressing NTRK1 with tyrosine kinase inhibitors on *EGR1* and *EGR3* induction by NGF was assessed by RT-PCR. Cells were pre-treated with the inhibitors at 0.1 μM for 15 min

following stimulation with NGF at 10 ng/ml for 2 hr. Data for 2 independent experiments are shown. ****p < 0.0001, **p < 0.01, *p < 0.05, ns – not significant.

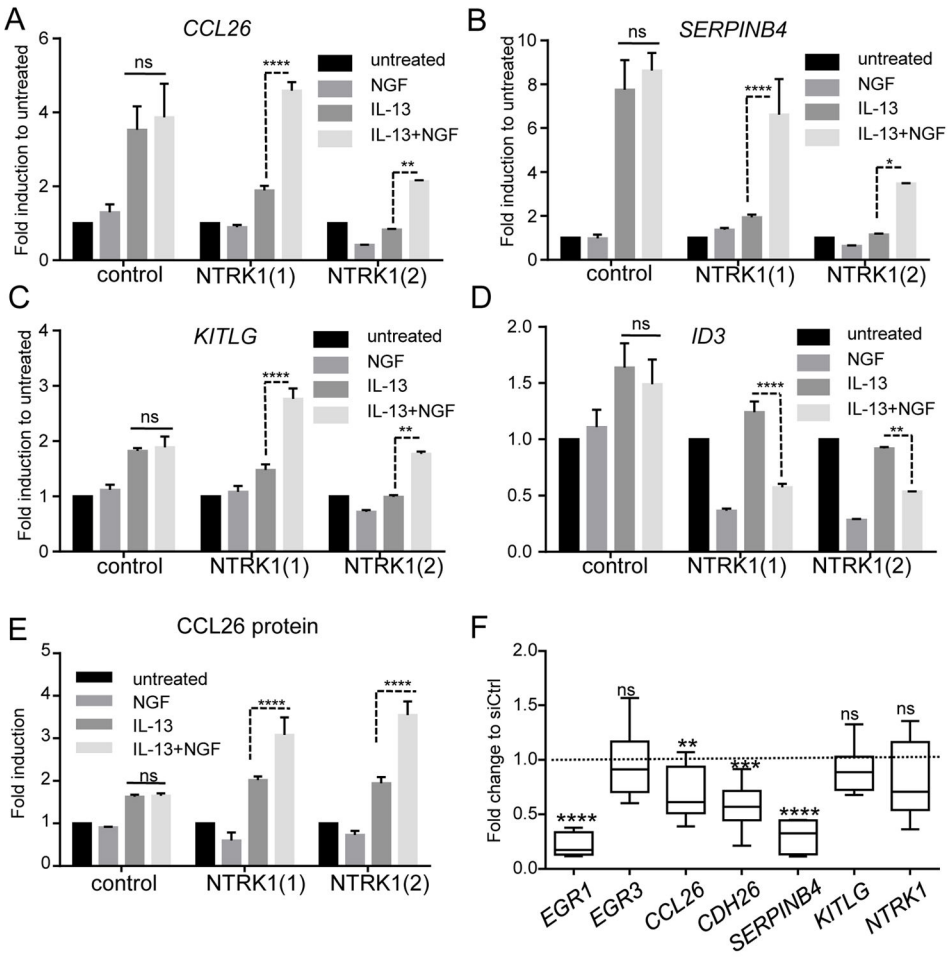

Figure 8. IL-13 and NGF/NTRK1 synergistically stimulate CCL26 production

In **A–D**, transcriptional levels of *CCL26*, *SERPINB4*, *KITLG* and *ID3* after IL-13 and/or NGF stimulation of TE-7 cells stably expressing NTRK1 were measured by RT-PCR. Induction was performed for 6 hr, IL-13 concentration was 1 ng/ml, NGF was used at 100 ng/ml. Expression was normalized to the level of *GAPDH*. In **E**, CCL26 protein level in cell supernatant was measured by ELISA. Cells were stimulated with IL-13 and NGF (100 ng/ml) for 24 hr; mean fold induction relative to untreated cells is shown. Error bars represent standard error of the mean for 4 independent experiments. In **F**, shown is the relative level of expression of the indicated genes following down regulation of *EGR1* by siRNA in TE-7 cells. Expression of genes in cells transfected with control siRNA was set as 1 (dotted line). Data for 4 to 5 independent experiments are presented as box and whiskers plot; ****p < 0.0001, ***p < 0.001, **p < 0.01, ns, not significant.

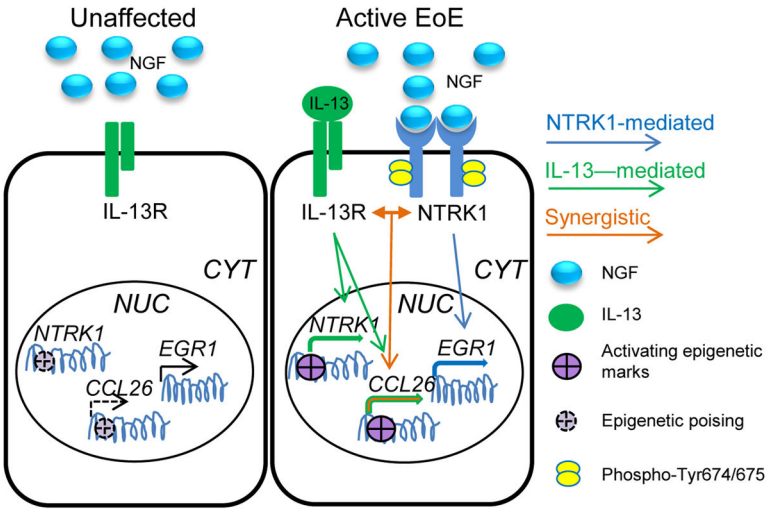

Figure 9. Model for propagation of IL-13–mediated allergic inflammation in epithelial cells

Prior to IL-13 stimulation (represented by unaffected cell), promoters of *CCL26* and *NTRK1* are epigenetically poised for activation (small light purple circles with broken lines), but genes are either not transcribed (*NTRK1*) or transcribed at a low level (*CCL26*, broken arrow). Early response gene *EGR1* is transcribed at a detectable level (represented by solid arrow). NGF is present in the environment but does not elicit a transcriptional response. During allergic inflammation (exemplified by active EoE), IL-13 initially induces epigenetic and transcriptional responses of its targets, as shown for *CCL26* and *NTRK1* (large purple circles with solid lines and green arrows) and increased expression of NTRK1. Subsequently, NGF binds to NTRK1 and elicits signal transduction (illustrated by phosphorylation of NTRK1 on kinase domain [yellow ovals]), which leads to an NGF/NTRK1-mediated transcriptional response (e.g. induction of *EGR1*, blue arrow), as well as a synergistic effect on transcription of key inflammatory genes (orange arrows, exemplified by *CCL26*). IL-13R, IL-13 receptor; *NUC*, nucleus; *CYT*, cytoplasm.