Integration of Airway Inflammation and Remodeling Mechanisms Specific to Eosinophilic Asthma Through Differential Co-Expression of Genes in Bronchial Brush Biopsy Samples

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Abstract

Background

Heterogeneity of asthma complicates search for targeted treatment against airway hyperresponsiveness and remodeling. We conducted a systems biology approach study to establish differential co-expression of genes (DCG) in eosinophilic and non-eosinophilic asthma patients and infer their role in the disease.

Materials and Methods

N = 40 Caucasian adult moderate to severe non-smoking asthma patients (half with eosinophilic asthma) undergone bronchial brush biopsy sampling for mRNA expression using hybridization to cDNA microarray. Mechanistic interpretation of DCG was inferred from existing literature.

Results

Differentially co-expressed genes bear significance in airway viral infection (ATP1B1, EPS15), arachidonic acid metabolism (CLC, FADS6), cell migration (EPS8L1, STOML3, RhoBTB2), surface receptors endocytosis (STRN4, EPS15, ATP1B1) or decreased expression (CCT7), oxidative stress (DIO3, RhoBTB2), decreased adhesion (ATP1B1, RAPH1, STOML3), epithelial-mesenchymal transition (ASB3, RADX, CCT7, MRPL14, PPP2R3B, RPS13, SLC19A1), myofibroblast differentiation (CCT7), smooth muscle proliferation (ASB3, ATP1B1), airway hyperreactivity (RECK, STOML3, ATP1B1, OR52I1), extracellular matrix remodeling (FBN3, RECK), angiogenesis (GPI, RhoBTB2) and neuronal pathogenesis of asthma (OR52I1, STRN4, TTC3P1, GPI, CABP5) and were linked to asthma in genome- (MRPL14, ASB3, RPS13) and epigenome-wide (CLC, EPS15, GPI, SSCRB4, STRN4) association studies. Signaling pathways involved (especially TGF-β/Smad2/3) are inferred from the co-expression pattern.

Conclusion

Activity of genes and pathways of known or tentative role in asthma pathogenesis was established in regard to a condition cognizable in clinical practice.

Introduction.

Asthma is a prevalent chronic inflammatory disease of the airways with variable etiology, mechanistic pathways involved (i.e. endotype), clinicopathological presentation (i.e. phenotype), severity, reaction to treatment and prognosis. Quarter of all asthma patients and half of those with severe asthma develop progressive and irreversible airway obstruction, leading to persistent symptoms despite the exhaustion of therapeutic measures. Such course of the disease is attributed to airway remodeling, a process of structural changes of bronchi including loss of epithelial apical-to-basolateral polarity, epithelial sheathing,

subepithelial fibrosis, epithelial-mesenchymal and fibroblast-to-myofibroblast transition, hyperplasia of smooth muscle and goblet cells, neovascularization and cartilage degeneration[1], leading to hyperresponsiveness and persistent airflow limitation. Current treatment options have limited influence on remodeling and do not target it directly as mechanisms underlying the process are still investigated[23]. Since no accurate biomarker or clinical predictor of ongoing remodeling was agreed upon to date, there are no methods of selecting patients who could benefit the most from future experimental e.g. biological therapies before the process becomes clinically symptomatic or apparent in imaging studies[4].

As eosinophilic asthma (EA) accounts for approximately half of the severe disease cases[5], search for therapeutic targets behind airway inflammation and remodeling specific to this phenotype presents an important scientific challenge.

In the present hypothesis-generating study we utilized systems biology approach to identify genes with coordinated expression in the airways of EA and non-EA patients, established networks of their binary interactions and regulation using curated resources and conducted a systematic review of the relevant literature to infer their possible role in the disease pathogenesis in search for novel regulators, biomarkers and mechanisms of airway inflammation and remodeling.

1. Materials And Methods.

1.1 Patient selection

The study included 40 non-smoking patients aged 20-70 years with at least 10-year history of asthma diagnosed by a physician in accordance with Global Initiative for Asthma (GINA) 2018 guidelines. The exclusion criteria were pregnancy or breastfeeding, any acute illness, history of congestive heart failure, atrial fibrillation (paroxysmal or persistent), myocardial infarction or stroke, neoplastic disease (past or current), thyroid disease, liver injury and chronic kidney disease (stage 3 or above) and smoking. Past smokers who stopped smoking at least 5 years before and accumulated less than 7 pack-years were permitted in the study. Asthma medications, except for biological treatment, were permitted, with oral corticosteroids at a daily dose ≤10 mg of prednisolone equivalent unless the doses were unchanged during the preceding 3 months.

Half of the subjects (N=20) were diagnosed with eosinophilic asthma defined as having at least 1% of eosinophils in bronchoalveolar lavage fluid (BALF), thus comprised the EA group. The remaining individuals formed the non-eosinophilic asthma (non-EA) group (N=20).

1.2 Spirometry studies

Spirometry with bronchial reversibility test using 400 mg of albuterol and body plethysmography after bronchodilator with assessment of the residual volume (RV) and total lung capacity (TLC) were carried out to assess differences in lung function between eosinophilic and non-EA patients using a Jaeger MasterLab spirometer (Jaeger-Toennies GmbH; Hochberg, Germany).

1.3 Lung computed tomography (CT) with evaluation of the airway cross-sectional geometry and percent of low attenuation area (LAA%)

CT indices of bronchial remodeling (parameters of airway cross-sectional geometry) and air trapping (LAA%) were measured to compare EA and non-EA patients. Chest CT scans was performed 1 hour after administration of 400 mg albuterol using 64-raw multidetector computed tomography (Aquilion TSX-101A, Toshiba Medical Systems Corporation, Otawara, Japan) in a helical scanning mode (CT parameters: 64 x 0.5 mm collimation, helical pitch of 53 and 0.5 second per rotation with standard radiation dose [150±50 mAs and 120 kVp]). Automated program AW Server (General Electric Healthcare, Wauwatosa, WI, USA) was used to quantify the airway cross-sectional geometry in four different bronchi: the RB1, the right lower lobe basal posterior bronchus (RB10), the left apicoposterior bronchus (LB1+2) and the left lower posterior basal bronchus (LB10). Scans were obtained with patients in the supine position at maximal inspiration with their arms held over their heads. Airway-geometry parameters were measured using methods previously described[4]. The LAA% was quantified automatically using the Volume Viewer 11.3 software (General Electric Healthcare, Wauwatosa, WI, USA) with 1 mm soft tissue reconstruction algorithm.

1.4 Bronchofiberoscopy

Bronchofiberoscopy was performed according to the guidelines of the American Thoracic Society[6] using the bronchofiberoscope BF 1T180 (Olympus, USA) with local anesthesia (2% lidocaine) and in mild sedation (0.05-0.1 mg fentanyl and 2.5-5 mg midazolam, both intravenously). Brochoalverolar lavage (BAL) was performed with 200 ml of 0.9% saline given to the right middle lobe bronchus and 2-3 bronchial brush biopsy (BBB) specimens were taken from the right lower lobe (the carina between B9 and B10). First 10 ml of the obtained BAL fluid (BALF) was discarded.

1.5 RNA acquisition.

Total RNA was extracted from BBB samples with TRIzol®, fractioned in gravity gradient, isolated in chromatographic columns and stored at -80°C. The quality of each sample was assessed using Qiagen® QIAxel and RNA integrity was verified using agarose gel electrophoresis. The resulting RNA was reverse-transcribed into cDNA library using Syngen® UniversalScript Reverse Transcriptase. The product was purified by Syngen ® PCR ME Mini Kit and fluorescently labeled and purified using Kreatech ® ULS Platinum Bright Red/Orange Kit. Hybridization to microarrays occurred on the Human Genomic 49K Mi ReadyArray (a Human Exonic Evidence Based Oligonucleotide array [HEEBO]; Microarray Inc., Huntsville, AL, USA) at 37°C for 24 h, subsequently washed.

1.6 Laboratory investigations

Fasting blood samples were drawn from the antecubital vein using minimal stasis between 8:00 and 11:00 A.M. into serum tubes and centrifuged, similarly to BALF at 2,000 g for 20 minutes. The supernatant was frozen in aliquots and stored at -70°C until analysis.

Immunoenzymatic assay (ELISA) was used to measure serum and BALF levels of interleukin (IL)-4, IL-5, IL-6, IL-10, IL-12p70, IL-17A, IL-23 and interferon gamma (INF-γ) (eBiosciencea, Vienna, Austria), serum periostin (Phoenix Pharmaceuticals, Burlingame, CA, USA) and serum disintegrin and metalloproteinase domain-containing protein 33 (ADAM 33) (Cloud-Clone Corp., Katy, TX, USA).

Basic statistical analysis was conducted using Statsoft® Statistica, v. 10.

1.7 Microarray data retrieval.

Microarrays were scanned with a InnoScan 900 Microarray Scanner and hybridization signals were detected using Mapix software (v.6.0.1; Innopsys, Carbonne, France).
The software failed to perform microarray gridding correctly in some cases. Since gridding is crucial for image analysis as an error occurring during this process may be propagated to further stages[7], a custom gridding algorithm to avoid grid displacement was developed. The coordinates of positioning markers to establish sub-grids were found using an MSER[8]-based method. Computed results were verified manually and corrected if needed, with overall gridding performance exceeding that of the commercial software, ultimately resulting in expression profiles of 33519 gene products. Microarray data preprocessing (background correction and normalization) was done by limma R software using the established methods[9,10]. Genomic data were analyzed with Bioconductor v.3.7. software of the R environment v.3.5.0.

1.8 Differential gene expression and co-expression identification and analysis.

A list of differentially expressed genes (DEGs) was established using limma package to assess for possible confounding with differential co-expression[11]; false discovery rate (FDR) was assessed with Benjamini and Hochberg procedure. A list of differentially co-expressed genes (DCGs) was established using CoXpress[12] R plugin. In this method, groups of genes which expression levels are highly correlated in one set of experiments (i.e. EA), but not significantly correlated in a second set of experiments (i.e. non-EA) are found. A summary statistic of gene expression correlation matrix is made for each sample group and compared to distributions of n=1000 similar summary statistics generated by selecting groups of random genes of the same size from the same data set. The summary statistic used is the t-statistic taken from a test of the unique, pairwise, Pearson correlation coefficients against zero. The proportions of random statistics above the observed statistic are used to create a “probability of randomness” for each group in the two sample groups. A group of genes which distribution of pairwise correlations is found to be random in non-EA ($p \geq 0.05$), and non-random in EA ($p < 0.05$), is said to be differentially co-expressed between the two groups.

1.9 Gene set enrichment, creation of interaction networks and their topological robustness analysis.

Associations between DCGs, airway inflammation and remodeling were investigated using the following workflow:

1. Gene set enrichment analysis (GSEA) was performed to establish histone modifications and cell types matching DCGs’ expression pattern as well as associated biological processes, molecular functions

and signaling pathways. The analysis was performed using Enrichr tool[13] using curated datasets (**Tab. 1**).

2. Based on curated resources (**1**), we established (1) lung-specific binary protein-protein interactions of DCGs, (2) DCGs' upstream regulators and (3) DCG-microRNA-transcription factor interactions. Semi-automated analytics platforms Cytoscape 3.7.1 and NetworkAnalyst 3.0 were used to construct interaction networks as undirected graphs, where individual molecular entities formed the nodes and their interactions the edges.
3. Topological robustness analysis of the resulting graphs was performed by the previously described principle of topological attack on biological network[14]. Betweenness centrality was used to identify nodes with highest network-disruptive potential as possible targets for pharmacological interventions.
4. Betweenness centrality of the nodes was plotted against MEDLINE literature coverage using the keyword "asthma" to identify already established and candidate molecular entities of potential relevance. GenClip 3.0 sofware was used for automated literature mining.
5. Both individual DCGs and their first-degree interactors were investigated in an attempt at mechanistic explanation of their role in the disease by literature mining using the keyword "asthma" and either of allergy and airway inflammation-related or airway remodeling-related keywords:

(1) allergy and airway inflammation: Th1, Th2, Th17, dendritic cell, antigen-presenting, granulocyte, neutrophil, eosinophil, basophil, mastocyte, mast cell, monocyte, macrophage, lymphocyte, inflammation, thromboxane, prostaglandin, leukotriene, oxidative stress, allergy, allergic, atopy

(1) bronchial remodeling: airway, remodeling, epithelium, sheathing, basal lamina, basement membrane, extracellular matrix, subepithelial, fibrosis, fibroblast, myofibroblast, epithelial-mesenchymal, smooth muscle, goblet, mucus, neovascularization, cartilage, hyperresponsiveness, hypertrophy, mucus, cilia, cilium.

1.10 Funding.

This study was funded by the National Science Centre based on decision No. DEC-2013/09/B/NZ5/00758.

1.11 Bioethics committee approval.

The study was approved by the Ethics Committee of the Jagiellonian University (Skawinska 8 Str, 31-066 Krakow, Poland; phone number +48 12 433 27 39, e-mail: komisja_bioetyczna@cm-uj.krakow.pl) under approval number KBET/151/B/2013 and conducted in accordance with the Declaration of Helsinki. All participants gave written informed consent to participate in the study.

2. Results And Discussion.

2.1 Sample description.

Demographic, clinical and laboratory characteristics of the study subjects are provided in **Tables 2-4.** EA and non-EA patients were comparable regarding most demographic and clinical features except for BMI, which was higher in the latter.

While the studied groups did not differ significantly in regard to lung function parameters, EA patients had more prominent absolute indices of airflow limitation (lower forced expiratory volume in 1 second; FEV_1) and more favorable obstruction reversibility (higher $DFEV_1$), while non-EA patients had lower total lung capacity (TLC), possibly due to overrepresentation of obese subjects in this group. HRCT indices of airway remodeling were significantly more prominent in the EA group, consistent with the differences in lung function, suggesting computed tomography as superior to spirometry in detecting advancement of airway remodeling.

2.2 Identification and study of differentially expressed genes.

After background correction, quantile normalization and filtration for coefficients of gene expression variation between 0.3 and 10, 14823 annotated gene products were included in subsequent analyses. Expression levels of 20 genes differed significantly between EA and non-EA by unadjusted p-value. According to automated literature mining, at least one of them was described in the context of asthma alone in one paper, three in the context of inflammation-related keywords over 8 papers and 9 in the context of remodeling-related keywords over 80 papers (**Tab. 5, Fig. 1**).

After adjustment for false discovery rate, no gene's expression level was found to significantly differ between EA and non-EA, concluding DEG analysis and indicating unconfounded pure differential co-expression discovery[11].

2.3 Identification and study of differentially co-expressed genes.

Hierarchical cluster analysis revealed a total of 23 groups of DCGs. A single group consisting of 32 genes with the highest mean, pairwise difference between the correlation matrices of gene expressions in the two asthma groups was chosen for subsequent analyses **(Tab. 6).**

Only 5 of the 32 genes co-occurred with asthma over 9 papers, 6 with allergy/inflammation-related keywords over 98 papers and 15 with remodeling-related keywords over 133 papers (**Fig. 2**). Abundance of significant correlations between DCGs' expression levels within EA group may indicate its pathogenetic consistency, contrary to less correlated DCGs in the non-EA group (**Fig. 3**). The resulting list of candidate genes as well as their interactors and regulatory networks (**Fig. 4-6)** which may differentiate airway inflammation and remodeling mechanisms specific to EA and non-EA are discussed.

2.4 Gene set enrichment analysis (GSEA) of DCGs.

None of the DCGs were significantly enriched for ontology terms at adjusted p-value (false discovery rate) < 0.05, expectedly, given the small gene set size. The results were ranked based on combined p-value and z-score.

2.4.1 Cell type-specific histone modifications related to expression of DCGs.

Fifteen of the 32 DCGs (all up-regulated in the EA) matched differential expression pattern related to histone modifications H3K9ac and H3K27me3 of human lung fibroblast hg19, suggesting them as the likely source of the gene expression signal (**Tab. 7)**. H3K9ac and H3K27me3 are the two histone modifications of dominant and opposite role in regulation of neuroectodermal differentiation (H3K9ac) or pluripotency (H3K27me3)[15]. With H3K27me3 previously linked to epithelial-mesenchymal transition (EMT) and extracellular matrix degradation upon treatment of cell culture with TNF-a and TGF-b[16], up-regulation of the genes related to both histone modifications in our study could indicate their role in ongoing EMT in EA, consistent with the abundance of eosinophil-derived TGF-b1 in this disease phenotype[17].

2.4.2 Gene ontology associations.

Molecular functions and biological processes associated with DCGs by gene ontology are listed in **Tab. 8-9**. They include phenomena significant in the pathogenesis of asthma and airway remodeling, such as T-cell receptor binding, MHC II protein complex binding, immunoglobulin secretion, myeloid cell apoptosis, regulation of cellular response to growth factors (e.g. TGF-b) and metalloendopeptidase inhibitory activity, as further discussed in **section 3.5.**

2.4.3 Kinase perturbation studies.

Kinase perturbation studies included in Gene Expression Omnibus (GEO) database indicate differential expression of several DCGs upon knockout of the following kinases (**Tab. 10**):

(1)TGF-b receptor II (TGFBR2): down-regulation of CCT7, EPS15, MRPL14;

(2)Homeodomain-interacting protein kinase 1 (HIPK1): down-regulation of GPI, MAEA, STRN4;

(3) Inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKBKE): up-regulation of DGLUCY, RAPH1, ASB3.

These kinase-dependent DCGs might indicated differential activity of the related pathways and have pathogenetic role in EA. The ligand of **TGFBR2**, TGF-b, has well-established role in asthma and airway remodeling, as further discussed in **section 3.5**, while the receptor itself was found to be involved in T-cell differentiation. The role of **HIPK1** was to date only described in fetal angiogenesis activated by TGF-β-TAK1 pathway[18], apoptosis induced by TNF-a and prevention of MAP3K5-JNK activation. Our study links **HIPK1** with eosinophilic asthma through the pattern of DCGs, a suggestion reinforced by association between MAP3K5 and atopy[19]. **IKBKE** is a known target of the nuclear factor κB (NFκB), a transcription factor involved in chronic inflammation of established role in asthma pathogenesis, including remodeling.

2.5 Literature-based assessment of individual DCGs for mechanistic role in EA and bronchial remodeling.

Individual DCGs are discussed below in the following order: gene's function, possible involvement in the disease and interaction with other DCGs from our study (shown in **bold**).

2.5.1 Sodium/potassium-transporting ATPase subunit beta-1 (ATP1B1)

The sodium-potassium pump is a heterodimer composed of the catalytic subunit alpha and non-catalytic **subunit beta**. The latter may be expressed in several cell types and conditions involved in asthma pathogenesis:

(1) In the epithelia, the **beta-1 subunit** may be related to epithelial sheathing as it contributes to formation and stabilization of intercellular junctions. It regulates the number of pumps transported to plasma membrane through assembly of alpha/beta heterodimers, which can act as positive or negative regulator of intracellular adhesion, depending on its proportion to FXYD5, a membrane protein involved in chemokine up-regulation and loosening of cell adhesion through down-regulation of E-cadherin[20].

(2) In cytomegalovirus (CMV) infection, previously linked to asthma pathogenesis via promotion of Th2 response[2122], **subunit beta 1** was found to co-localize with viral UL136 protein suggesting involvement in cell-to-cell spread of the infection[23]. It is also upregulated in human neutrophils during respiratory syncytial virus (RSV) infection, a trigger of asthma exacerbations.

(3) In lymphocytes, **subunit beta 1** is known to be up-regulated in response to phytohemagglutinin, suggesting possible role as marker of their activation status[24] and **AT1B1** was among four genes upregulated in human lymphocytes under Th17-polarizing conditions.

(4) Decreased activity the pump elevates intracellular free calcium concentration,
a phenomenon linked to the activation of inflammatory cells and airway smooth muscles (ASM) resulting in increased proliferation, spreading, and eosinophil-attracting eotaxin-1 release in experimental conditions[25262728].

(5) In the goblet cells, IL-4 produced by Th2-lymphocytes causes up-regulation and translocation of ATP1B1 from basal to apical aspect of the cell, where it colocalizes with H+/K+-ATPase, ATP12A, being required for its function implied in mucous secretion in asthma and cystic fibrosis[29].

(6) **ATP1B1** is modulated by PXK, which is thought to participate in regulation of electrical excitability and synaptic transmission by, in part, epidermal growth factor (EGF) receptor ligand-induced internalization[30], both processes being postulated to play a role in airway hyperreactivity in asthma.

ATP1B1, CLC, FADS6, FBN3, RECK and **SLC19A1** share a common transcription factor Sp1[313233] involved in multiple cellular processes ranging from cell proliferation and differentiation to immune response, their specific function being highly dependent on post-translational modification. Sp1 expression and DNA binding activity are increased in CMV infection[34], a positive-feedback loop given **ATP1B1**'s role in cell-to-cell virus transmission. Furthermore, Sp1 has an established role in asthma pathogenesis as a transcription factor for (1) lipooxygenase ALOX5[35], which promoter region's polymorphism affects asthma control, and (2) vascular endothelial growth factor (VEGF), hypersecreted from ASM in asthma and

contributing to remodeling. Of note, both **ATP1B1** and **RECK** are implied to take part in airway remodeling in mustard lung[36], adding to evidence of Sp1-regulated genes in airway pathology.

ATP1B1 was one of the genes included in transcriptomic cluster TAC3 of patients with moderate to high sputum eosinophilia in the U-BIOPRED cohort of Th2-dependent asthma[37], with our study being the second to indicate the link.

Of note, as **ATP1B1** is involved in kidney proximal tubule bicarbonate reclamation, aldosterone-regulated sodium reabsorption and kidney stone formation, common regulation of **ATP1B1** in both bronchi and renal tubule may underlie apparent higher incidence of nephrolithiasis and chronic kidney disease in asthma patients[3839].

2.5.2 Charcot-Leyden crystal protein (CLC)

Charcot-Leyden crystal protein or **galectin-10 (Gal-10)** is an atypical galectin preferentially binding b-mannosides. It takes part in sequestration and vesicular transport of eosinophil granule cationic ribonucleases: eosinophil-derived neurotoxin and eosinophil cationic protein[40] and was found to poses lysolecithin acylhydrolase (lysophospholipase; phospholipase B) activity. Released upon degranulation, it is ubiquitous in sputum of EA patients and considered a marker of eosinophilic inflammation. It is thought to regulate immune responses through the recognition of cell-surface glycans and may possess IgE-binding capability. Furthermore, through lysophospholipase activity, it may hydrolyze 1) phospholipids, producing arachidonic acid for synthesis of eicosanoids of important role in asthma pathogenesis and 2) surfactant phosphatidylcholine, leading to surfactant dysfunction and small airway closure[41].

Recently, the existence of regulatory CD16-high eosinophils with distinct suppressor function on T-cell proliferation has been suggested, with their function likely mediated by **Gal-10** in immune synapses between eosinophils and lymphocytes[42]. Furthermore, **Gal-10** was recently found to be expressed in human CD4(+)CD25(+)Foxp3(+) T regulatory cells (Treg), while nearly absent in resting and other activated CD4(+) T cells. In Treg cells this lectin is essential to limit proliferation and suppressive function, similarly to the above CD16-high eosinophils[43].

CLC shares transcription factor Sp1 with **ATP1B1, RECK, FBN3, FADS6** and **SLC19A1**, which points to a possible involvement in a common biological process.

Consequently, **CLC** was included in transcriptomic cluster TAC1 of patients with high sputum eosinophilia in the abovementioned U-BIOPRED asthma study[37] and associated with asthma in an epigenome-wide association study (EWAS)[44].

Since galectins can frequently act as both positive and negative modulators of the same processes, possibly depending on isoform plasticity, posttranslational modifications, localization or cofactors[45], further research is needed to assess if galectin-10 can be influenced to act as an anti-inflammatory factor, similar to galactin-9 (Gal-9). This galectin, released by various cell types plays a substantial role in control

of effector cells and Th1/Th2 balance. It is known to be upregulated upon CMV infection through induction of IFN-b[46] and released upon calcium-mediated exocytosis[47]. It is able to reduce Th2-associated airway inflammation and airway hyperresponsiveness through binding with T cell immunoglobulin mucin domain 3 (Tim-3), persistently expressed on functional T-cells during chronic viral infections. By inducing maturation of monocyte-derived dendritic cells it promotes Th1-associated immune response. It also triggers apoptosis of mature Th1 cells by aforementioned Tim-3 receptor, preventing degranulation of mast cells by forming complex with IgE[48] and inhibits interaction-dependent migration of inflammatory cells via CD44 pathway [49–52]. These properties elicit galectin-9 and possibly galectin-10 as potential candidates for development of novel protein drugs for the treatment of asthma.

2.5.3 Pregnancy-specific beta-1-glycoprotein 2 (PSG2)

PSG2 stimulates transcription of FOXP3 in mononuclear cells and CD4+ T cells, thus providing signal for Treg and Th17 differentiation, a process consistent with **CLC/Gal-10** expression on CD4(+)CD25(+)Foxp3(+) Treg cells, with loss of FOXP3 expression causing Th2-differentiation[53]. Human PSG proteins are able to activate latent TGF-b bound to the extracellular matrix and cell membrane, thus enabling its pleiotropic action[54] involved in suppression of innate immunity, Th17 differentiation, expression of T-cell regulatory phenotype but also up-regulation of periostin, a component of subepithelial fibrosis in asthma[55].

Co-expression of the two genes involved in limitation of Th2- and induction of Th17-differentiation, **CLC** and **PSG2**, in EA patients suggests both a counterweight mechanism to limit Th2-response and a possible role behind heterogeneity of Th2-high asthma, recently proposed to be divided into IL-5-high/IL-17F-high asthma (with mixed granulocytic infiltration) and IL-4/IL-13-high asthma (with eosinophilic infiltration alone)[56].

2.5.4 EPS8 like 1 (EPS8L1)

EPS8L1 encodes a protein related to the epidermal growth factor receptor kinase substrate 8 or Eps8 involved in actin remodeling. **EPS8L1** itself is known to play a role in T-cell receptor binding, membrane ruffling and remodeling of actin cytoskeleton through F-actin organization by stimulating guanine exchange of SOS1, thus taking part in regulation of cell locomotion. In a murine knockout models, **EPS8L1** was required for EGF-dependent membrane ruffling [57] and Eps8 was required for maintaining front-to-back polarity of dendritic cell, both features required for cell migration[58]. Furthermore, Eps8 takes part in Cdc42–IRSp53–Eps8 pathway involved in formation of filopodia and cell migration, with **EPS8L1** being the only Eps8-related protein associating with IRSp53 and thus possibly involved in the process[59].

No direct link between EPS8L1 an asthma was made to date.

Cell migration is a regulated by Rho GTPases, of which **RhoBTB2** is an example, although no associations between Eps8 or **EPS8L1** exist to date.

2.5.5 Rho related BTB domain containing 2 (RHOBTB2)

RHOBTB2 is an atypical member of the Rho family of small GTPases which control cell migration, invasion and cycle and is required for expression of CXC motif ligand 14 (CXCL14). CXCL14 is a chemoattractant that controls dendritic cell activation, leukocyte migration and angiogenesis and an autocrine growth factor for fibroblasts facilitating their migration[60] and which expression is lost through unknown mechanisms in a wide range of epithelial cancers[61]; e.g., loss of **RhoBTB2** expression correlates with downregulation and loss of CXCL14 secretion by head and neck squamous cell carcinoma cell lines, whereas reintroduction of **RhoBTB2** restores CXCL14 secretion[62].

In a genome-wide association study conducted in Australia, CXCL14 was one of the six most-associated loci with the asthma susceptibility [63]. Moreover, effects of exposure to tobacco smoke pollution are thought to be mediated through the CXCL14. The experimental data suggests differential effects of occasional[64] and chronic[65] tobacco exposure on CXCL14 expression, as well as differential effects of tobacco smoking on airway eosinophilia (elevated in chronic smokers, but attenuated by occasional exposure in non-smokers).

While CXCL14 receptor is not yet identified, migration of antigen-presenting cells (APCs) towards CXCL13, the ligand of CXCR5, is significantly potentiated in the presence of CXCL14, possibly contributing to mucosal recruitment of inflammatory cells[66], including APCs and Th17 lymphocytes expressing CXCR5 as well. Regulation by **RhoBTB2** suggests its role in disease mediated through CXCL14 and resulting migration of leukocytes, dendritic cells and possibly epithelial-mesenchymal transition of fibroblasts.

Furthermore, **RhoBTB2** is a substrate adaptor for Cul3-based ubiquitin ligase complex known to play a role in a Keap1-guided degradation of antioxidative Nrf transcription factor implicated in asthma pathogenesis[67]. As Keap1 is believed to compete with other BTB proteins for culling-3 binding, down-regulation of **RhoBTB2** may leave E3 ubiquitin ligase open for adaptor Keap1-BTB-mediated ubiquitination and degradation of antioxidative Nrf2, thus suppressing its transcriptional activity and promoting oxidative stress. Recent studies indicate that **RhoBTB2** is involved in the Hippo signaling pathway through LKB1 regulation, with loss of **RhoBTB2** leading to ubiquitination and loss of LKB1 as well as increased YAP activity of role in asthma pathogenesis. Available GEO dataset indicates correlation between **RhoBTB2** expression and asthma exacerbation, with significant difference in expression between pools of peripheral blood mononuclear cells from exacerbated and convalescent asthma patients (GDS3615[68]). Furthermore, **RhoBTB2** was one of the genes which expression changed significantly after bronchial thermoplasty[69].

RhoBTB2's GTPase activity as well as interaction with Cul3 requires Hsp90, a chaperone regulated by **CCT7**[70]. Its role in membrane ruffling makes a possible link with aforementioned **EPS8L1.**

2.5.6 Ras association (RalGDS/AF-6) and pleckstrin homology domains (RAPH1)

RAPH1 is a member of Mig10/Rap1-interacting family of adaptor proteins regulating actin dynamics. As a mediator of localized membrane signaling implicated in the regulation of lamellipodial dynamics it

regulates cell migration, and its internalization negatively regulates cell adhesion, possibly contributing to epithelial sheathing. Phosphorylation of **RAPH1** by C-abl oncogene takes part in its coordination of actin remodeling in response to external stimuli[61]. As a target gene of regulatory miR-203, it facilitates keratinocyte migration and wound healing[71]. While no direct role of **RAPH1** in airways was reported, miR-203 is known to inhibit proliferation in ASM cells through attenuation of c-Abl expression and phosphorylation by ERK1/2, a pathway involved in epithelial-mesenchymal transition in airways, of which **RAPH1** is a phosphorylation target. Furthermore, both ERK1/2 and **RAPH1** are affected by histone demethylase KDM2A, implicated in lung carcinogenesis[72]. Because of its place at the intersection of KDM2A, miR-203, c-Abl and ERK1/2, **RAPH1** upregulation in EA patients suggests its role in ASM proliferation, as well as epithelial sheathing and epithelial-mesenchymal transition.

2.5.7 Signal recognition particle receptor beta subunit (SRPRB)

SRPRB is one of two subunits of the signal recognition particle receptor (SRP) required for co-translational targeting of secretory and membrane proteins. With the alpha subunit targeting SRP-ribosome-nascent polypeptide complexes to translocon, the beta subunit is a transmembrane GTPase which anchors the alpha subunit to the endoplasmic reticulum (ER).

Protein-protein interaction studies suggest SRPRB as involved in trafficking of proteins relevant for asthma pathogenesis: (1) beta-2-adrenergic receptor, (2) caspase-4 (a protein of increased expression in alveolar macrophages implied in inflammatory cell death contributing to tissue injury in asthma (https://www.nature.com/articles/s41467-020-14945-2), (3) TNFRSF14 (a receptor for TNF superfamily member TNFSF14 found to promote Th2 lymphocytes and airway remodeling) https://www.nature.com/articles/ncomms13696 and (4) glutathione S-transferase kappa 1 (implied in susceptibility to childhood asthma)[73].

ILK1, an integrin-linked kinase responding to signals from the extracellular matrix, including injury, and regulating basal stem cell activity and ASM differentiation[74] links **SRPRB** to **CCT7** through affinity capture protein-protein interaction of unknown biological significance.

2.5.8 Ankyrin repeat domain 26 pseudogene 1 (ANKRD26P1)

ANKRD26P1 is a pseudogene of little known function. It interacts with MAGEA-6[75], a protein encoding epitopes presented with HLA-DRB1*0401. Presentation of these epitopes recognized by CD4(+) T-cells from patients with melanoma or renal cell carcinoma enhances T-cell response against cells expressing both proteins in HLA-DRB1*0401 patients[76]. The same haplotype was previously associated with severity of asthma, incidence of rheumatoid arthritis and eosinophilic granulomatosis with polyangiitis, an autoimmune disease resulting in eosinophilic tissue infiltrates and EA phenotype in its clinical presentation[77,78].

MAGEA-6 is up-regulated upon exposure of cell culture to macrophage migration inhibitory factor (MIF), a pro-inflammatory factor known to play a role in asthma and chronic obstructive pulmonary disease[79].

These findings suggest that genetic factors such as human leukocyte antigens may be associated with susceptibility to EA and that it may be mediated through T-cell response against cells expressing **ANKRD26P1** and MAGEA-6, possibly in response to MIF and resulting in T-cell production of IL-4 and IL-13 that drive eosinophilic inflammation.

2.5.9 Chaperonin containing TCP1 subunit 7 (CCT7)

CCT7 is a molecular chaperon, member of chaperonin containing TCP1 complex (CCT; TRiC) assisting folding of proteins like actin and tubulin and regulating Hsp90 chaperone.
Its expression increases in fibrotic wound healing and is essential for the accumulation of α-smooth muscle actin (α-SMA) in fibroblasts and cell differentiation to myofibroblasts[80]. **CCT7** was recently found to coimmunoprecipitate with thromboxane A2 receptor as well as β2-adrenergic receptor from human HEK 293 cells and its depletion resulted in reduced cell surface expression of both receptors[81]; a fact significant given the momentous role of both G-protein coupled receptors in asthma pathogenesis. Two-hybrid screening revealed interaction between **CCT7** and ANXA1, which deficiency causes spontaneous airway inflammation and hyperresponsiveness in murine studies[82].

Notable is the **CCT7**'s central position in the PPI network of multiple DCGs and their interactors of high asthma literature coverage (**Fig. 4**) and involvement in epithelial-mesenchymal transition, implying regulatory function of the CCT/TRiC complex in asthma pathogenesis, involving e.g. Hsp90-dependent GTPase activity of **RhoBTB2**.

2.5.10 Deiodinase iodothyronine type III (DIO3)

DIO3 product catalyzes the inactivation of thyroid hormone by inner ring deiodination of the prohormone thyroxine (T4) and the bioactive hormone 3,3',5-triiodothyronine (T3) to inactive metabolites, 3,3',5'-triiodothyronine (RT3) and 3,3'-diiodothyronine (T2), respectively. **DIO3** is thought to prevent fetal tissues from exposure to adult-level concentrations of thyroid hormones.

TGF-b is known to induce **DIO3** expression in various cell types, including lung fibroblasts. Of note, deiodinase type III is a selenoprotein while selenium deficiency has been suggested to take part in inflammatory diseases including asthma[83] through contribution to oxidative stress, a mechanism thought to underlie asthma exacerbations in hyperthyroidism. As oxidative stress is implied to induce **DIO3** expression, asthma exacerbations in selenium deficiency are aggravated through impaired **DIO3** function and increased tissue exposure to active thyroid hormones. No direct link between **DIO3** and asthma has been made to date, however, its upregulation might be a response to oxidative stress, activation of fetal genes due to tissue repair (along with **FBN3** and **GPI**, below) or adult overexpression of genes hypermethylated prenatally, contributing to the hypothesis of prenatal epigenetic changes as predisposing to asthma development[52]. Promotor of the above-mentioned **ATP1B1** has sequences binding thyroid hormones (TRE), forming a link to upregulation of both **ATP1B1** and **DIO3** in our study.

2.5.11 Epidermal growth factor receptor pathway substrate 15 (EPS15)

EPS15 is a protein involved in the clathrin-dependent internalization of ligand-inducible tyrosine kinase receptor (RTK) types (including EGFR, TGF-b receptors and integrin b-1) as well as cell adhesion molecules (integrins, E-cadherin), receptors relevant for bronchoconstriction (β2-adrenergic and M3-muscarinic receptors) and antigen-presenting major histocompatibility class II proteins.

EPS15 is also necessary for non-clathrin-dependent endocytosis of EGFR and TGF-b, with knockout models resulting in attenuated TGF-β-induced Smad2 phosphorylation[84] of role in fibroblast to myofibroblast transition[85].

EPS15 forms PARK2-EPS15-EGFR complex with parkin, an E3 ubiquitin ligase influenced by IL-13 and known to enhance airway mitochondrial DNA release contributing to inflammation[86]. Since IL13-stimulated parkin aids mono-ubiquitination of **EPS15** thus preventing RTKs internalization and potentializing the effects of TGF-b, it forms a link between the two pro-fibrotic cytokines as well as mtDNA-mediated inflammation.

EPS15L1, a protein of suggested role in the process of receptor endocytosis, coimmunoprecipitates with **EPS15** and is one of the genes associated with asthma in an EWAS study[44] and **EPS15** transcript is upregulated in human ASM cells upon their stimulation[87].

EPS15 and TTC3 (a gene homologous with **TTC3P1**) are potential binding partners of Eps15 homology domain-containing 1 (EDH1), a protein involved in endocytic receptor recycling[88].

2.5.12 Fatty acid desaturase 6 (FADS6)

FADS6 is an peroxisomal enzyme of oxidoreductase activity taking part in aerobic very long chain-polyunsaturated fatty acids (PUFAs) biosynthesis. By desaturating linoleic acid to g-linoleic acid, it contributes to synthesis of arachidonic acid, precursor of eicosanoids of established role in asthma pathogenesis, in a similar fashion to FADS1 and FADS2 implicated in asthma pathogenesis[8990919293]. **FADS6** is regulated by hsa-miR-331-3p, a micro-RNA post-transcriptional regulator associated with lung function in asthma, with our study supplying evidence for the mechanism of such association being mediated through **FADS6**[94]. **FADS6** promoter binds factors reported to influence asthma risk: EZH2, as well as abovementioned Sp1, a transcription factor for **ATP1B1, FBN3, RECK** and **SCL19A1**.

2.5.13 Fibrillin 3 (FBN3)

Fibrillins are components of extracellular calcium-binding microfibrils, which occur either in association with elastin or in elastin-free bundles and act as structural support important for extracellular matrix integrity. Like PSG2, fibrillins may be involved in regulation of TGF-b activity through association with latent TGF-b binding proteins. Physiological **FBN3** expression was described only in fetal developing bronchi[9596], but its mutations have been found in lung cancer[97]. It shares a common transcription factor Sp1 with **ATP1B1, FADS6, RECK, SLC19A1**[33]. Up-regulation of the **FBN3** gene in the current gene set might both reinforce the hypothesis of fetal program execution during bronchial remodeling (together with **GPI**

and **DIO3**), regulation of signaling through latent TGF-b and add to a weak association between lung cancer and asthma[98].

2.5.14 Glucose-6-phosphate isomerase (GPI)

GPI expresses differential function depending on location, being (1) an intracellular glycolytic enzyme that interconverts glucose-6-phosphate and fructose-6-phosphate (thus taking part in glycolysis, gluconeogenesis and pentose phosphate pathway), (2) an extracellular neurotrophic factor neuroleukin that promotes survival of skeletal motor neurons and sensory neurons in fetal development, (3) a lymphokine that induces immunoglobulin secretion and (4) an autocrine motility factor (AMF) acting as an angiogenic factor.

GPI has a proven pathogenetic role in rheumatoid arthritis, acting as an autocrine synovial fibroblast proinflammatory cytokine resulting in proliferation, apoptosis inhibition and secretion of tumor-necrosis factor (TNF)-a and IL-1β[99]. Immunization of murine CD4(+) T-cells against **GPI** causes experimental model polyarthritis with production of proinflammatory TNF-a, IL-17 and IL-6 and anti-GPI autoantibodies are detectable in some human rheumatoid arthritis patients. **GPI** was found to be a direct regulator VEGF-mediated angiogenesis in rheumatoid arthritis, with both proteins being up-regulated by HIF-1a, a transcription factor involved in airway remodeling. Though rheumatoid arthritis is classically considered as Th1-dependent disease, it seems to share genetic risk factors and elements of pathogenesis with asthma[100]. Further studies are needed to assess if anti-GPI is present in sputum of asthma patients similarly to anti-eosinophil peroxidase and anti-IgE autoantibodies and if VEGF-mediated airway remodeling is regulated by GPI in similar fashion to that seen in rheumatoid arthritis.

As an enzyme taking part in glycolysis, it may affect glucose metabolism in the airway epithelia, where hyperglycemia and impaired glucose transport induce cell proliferation[101]. Association with asthma in an EWAS[44] adds evidence of its pathogenetic role. Its role in fetal development along with **DIO3** and **FBN3** may be indicative of fetal genetic program execution in asthmatic airways.

2.5.15 Mitochondrial ribosomal protein L14 (MRPL14)

MRPL14 is a nuclear gene that encodes part of two intersubunit bridges in the assembled mitochondrial ribosome required for mitochondrial translation. Related to asthma in a genome-wide association study[102] and controlled by MYC transcription factor similarly to **DGLUCY**, it may be involved in asthma-specific mitochondrial biogenesis and accumulation in ASM.

2.5.16 Olfactory receptor family 52 subfamily I member 1 (OR52I1)

OR52I1 is an olfactory receptor functioning by CaBP/Cam-dependent inhibition of calcium channel, a function which may be influenced by **CABP5** due to functional replaceability with calmodulin. While no link between **OR52I1** and asthma was found to date, other olfactory receptors like OR1D2 and OR2AG1 are known to be expressed in ASM cells and their activation by amyl butyrate was shown to inhibit histamine-

induced ASM contraction. **OR52I1** and **CABP5** may thus be involved in pathology of airway hyperreactivity in asthma of undetermined significance.

2.5.17 Protein phosphatase 2 regulatory subunit B beta(PPP2R3B)

PPP2R3B might modulate its substrate selectivity and catalytic activity and direct the localization of the catalytic enzyme to a particular subcellular compartment. Also known as PR48, it regulates cell cycle progression likely by controlling initiation of DNA replication[103]. It's downregulation in EA may indicate increased airway or inflammatory cell proliferation.

2.5.18 Reversion inducing cysteine rich protein with kazal motifs (RECK)

RECK is a membrane-anchored negative regulator of matrix metalloproteinase-9 (MMP)-9 able to suppress its secretion and enzymatic activity. MMP-9, a gelatinase, is known to be secreted by epithelial cells, neutrophils and eosinophils in response to allergen challenge or TNF-a and was previously linked to both eosinophilic infiltration into the bronchial wall and remodeling as a major MMP involved in asthma[104]. Its activity is increased in sputum of asthmatic patients and correlates with loss of FEV1 in response to allergens[105]. Furthermore, **RECK** down-regulation by oncogenic signals may facilitate tumor invasion and metastasis through regulation of MMP-2 and MT1-MMP, further linking asthma and carcinogenesis. It shares a common transcription factor Sp1 with **ATP1B1, FADS6, FBN3** and **SLC19A1**[32].

2.5.19 Scavenger receptor cysteine rich family member with 4 domains (SSCRB4D)

SSCRB4D belongs to the scavenger receptor cysteine-rich (SRCR) superfamily of highly conserved proteins involved in the development of the immune system and regulation of immune responses. **SSCRB4D** may be associated with asthma by being one of the target genes of BACH1, involved in the response to oxidative stress and previously associated with asthma in an EWAS study[44].

2.5.20 Stomatin like 3 (STOML3)

STOML3 modulates mechanotransduction channels and acid-sensing ion channels (ASIC) proteins and potentiates PIEZO1 and PIEZO2 function by increasing their sensitivity to mechanical stimulations. Studies in animal models suggest that mechanotransduction may play a role in hyperresponsiveness of the airways in asthma, while PIEZO1 deficiency decreases cell adhesion and increases migration of lung epithelial cells[106], a feature of asthmatic bronchial remodeling[107].

2.5.21 Striatin-4 (STRN4)

STRN4 belongs to a family of proteins binding calmodulin in a calcium-dependent matter, which may function as scaffolding or signaling protein. It is known to co-localize with protein phosphatase 2A (PP2A) acting as a regulatory subunit of the STRIPAK kinase-phosphatase complex involved in multiple cellular processes. Animal studies suggest that STRIPAK might be involved in cell cycle control, cell adhesion, migration, epithelial integrity and epithelial-to-mesenchymal transition.

STRN4, by inhibition of MAP4K4 kinase, is considered the key regulator inhibiting the Hippo pathway involved in asthma pathogenesis[108]. In T-cells, deficiency of MAP4K4 results in Th17 differentiation with IL-6 and IL-17 expression[109]. In a study of asthmatic patients, miRNAs expressed in the airways frequently targeted MAP kinases including MAP4K4[110], suggesting their involvement in the disease.

STRN4 itself was also proposed as a part of mechanism to control dendritic spine morphology, adding to possible neuronal involvement in airway disease.

STRN4 is one of the genes associated with asthma in an EWAS[44].

Through **STRN4**, STRIPAK interacts with Mob3, NDPK, dynamin and abovementioned **EPS15** to take part in regulation of clathrin-dependent endocytosis (e.g. of EGF, TGF-b or b_2-adrenergic receptors)[111].

2.5.22 Tetratricopeptide repeat domain 3 pseudogene 1 (TTC3P1)

TTC3P1 is a pseudogene resembling TTC3, a E3 ubiquitin-protein ligase that mediates ubiquitination of phosphorylated Akt (AKT1, AKT2 and AKT3) as part of negative feedback required to control Akt levels after pathway activation. TTC3 contributes to TGF-b1-induced epithelial-mesenchymal transition any myofibroblast differentiation, forming a direct link with bronchial remodeling[112]. TTC3 may also play a role in neuronal differentiation inhibition via its interaction with CIT, associated with asthma in *GWASdb* database[102], a fact of potential significance for the neuronal involvement in the disease[113]. As an apparently transcribed pseudogene, **TTC3P1** may regulate TTC3 function. Membership in a E3 ligase complex associated with CUL3 links TTC3 to **RhoBTB2** while involvement in Akt pathway makes an association with **ASB3**.

2.5.23 Calcium binding protein 5 (CABP5)

CABP5 product inhibits calcium-dependent inactivation of L-type calcium channel, thus increasing cell excitability. While primarily involved in the transmission of light sensation in the retina, it was reported in T-cells including Th1, Th17, mucosa-associated invariant T (MAIT) cells and B-cells, being differentially expressed in allergic asthma patients[114]). Together with the involvement in stimulating neurite outgrowth and vesicle exo- as well as endocytosis in PC12 cells, these facts suggest that **CABP5** plays a role in immune reactions, neuronal growth (a feature of airway remodeling) and vesicle handling. Able to functionally replace calmodulin, it may potentially interact with striatin in clathrin-dependent membrane receptor endocytosis, thus relating **CABP5** to **STRN4** and **EPS15**[115]**.**

2.5.24 Ribosomal protein S13 (RPS13)

RPS13 is a component of the 40S ribosomal subunit essential for eukaryotic protein synthesis and regulated through redundant mechanisms, hence described as a reference housekeeping gene. However, it is known to take part in G1 to S cell phase transition and was featured in cluster 1 of asthma-linked modules obtained from the combination of differential gene expression and GWAS study of the U-BIOPRED project[116]. Furthermore, it forms TNF-a/NF-Kappa B signaling complex of established role in asthma[67].

These data provide arguments for viewing **RPS13** as an active player in asthma pathogenesis and reevaluate it as a reference housekeeping gene of seemingly stable expression across biological conditions. Affinity capture studies indicate interactions of **RPS13**, **CCT7** and **EPS15** with VCAM1, ITGA4, FN1 proteins involved in cell-cell and cell-extracellular matrix adhesion, with VCAM and FN1 both binding to ITGA4, as subunit of integrin a4b1. While the nature of the interaction between proteins involved in extracellular matrix and endocytosis, regulation of chaperones and ribosome structure is unknown.

2.5.25 Ankyrin Repeat And SOCS Box Containing 3 (ASB3)

ASB3 is part of ASB gene family involved in Erk1/2 and PI3K/Akt signal transduction pathways by regulation of MAP kinase and Akt phosphorylation, both implicated in smooth muscle proliferation in asthma[117].

It may regulate lymphocyte differentiation through TNF-a receptor (TNFR) 2 ubiquitination by recruitment of E3 ubiquitin ligase adaptors: elongins-B/C[118]. While TNFR2 initiates immune modulation and tissue regeneration, signaling through TNFR1 triggers pro-inflammatory pathways[119]. Loss of TNFR2 signaling can impair expansion and stability of Tregs and decrease their sensitivity to IL-2. Moreover, through reciprocal PI3K/Akt pathway activation and phosphorylation of STAT5, **ASB3** impairs Th17 differentiation and may impair IL-12 signaling through JAK2/STAT4 to favor Th2 rather than Th1 differentiation[120], resulting in predominantly eosinophilic, rather than neutrophilic inflammation.

Recent GWAS study has associated three SNPs in a region of chromosome 2 near **ASB3** and SOCS with degree of bronchodilation following inhalations of albuterol in asthma patients[121], with our study indicating **ASB3** as an agent in the disease pathogenesis.

2.5.26 Solute carrier family 19 member 1 (SLC19A1)

SLC19A1, also referred to as reduced folate carrier protein, is a bidirectional membrane anion transporter enabling both folate and anti-folate medications, e.g. methotrexate (MTX) influx into the cell. Folate is required for synthesis of purines and thymine, homocysteine-methionine metabolism, both homocysteine-dependent and independent production of nitric oxide and reactive oxygen species (ROS) as well as DNA methylation, exerting both anti-inflammatory and pro-inflammatory properties, possibly depending on chronicity of an inflammatory disease[122].

Folate deficiency was previously linked to asthma severity[123] as well as risk of developing the disease in children of folate-deficient mothers[124]. It affects fibroblast expression of genes related to cytoskeleton remodeling, extracellular matrix and signaling through Wnt pathway (DKK1, WISP1 and WNT5A) [125] and may affect collagen metabolism in concomitant ascorbic acid deficiency. Folate deficiency increases the CD4/CD8 ratio and murine models indicate its role in maintaining CD4(+)Foxp3(+) regulatory T-cells, decreased in human asthma patients[126].

Furthermore, as a recently discovered major transporter of immunoreactive cyclic dinucleotides (CDN) of autologous or microbial origin[127], **SLC19A1** may take part in dysregulation of the cGAS-STING pathway which overactivation can drive inflammatory lung diseases, including asthma[128].

A genetic risk factor of atopy, an SNP *rs12483377-A* is mapped to **SLC19A1** and COL18A1, both functionally implied in the process[19].

Up-regulation of **SLC19A1** may be driven by transcription factors linked to asthma (CEBPB, USF1, SP1), Th2-differentiation (CEBPB)[122] and asthma-specific mitochondrial biogenesis (NRF-1). Furthermore, it is up-regulated by vitamin D[129], which deficiency was previously linked to asthma control. Differential co-expression in EA may indicate both activity of the above transcription factors, cell proliferation status or impaired reaction to folate deficiency, a condition under which **SLC19A1** is considered cytotoxic, since it may aggravate folate deprivation by expulsion of folate monoglutamates[130]. These facts indicate that **SLC19A1** may have pathogenetic role in asthma, being affected by both transcription factors involved in asthma and nutritional status linked to asthma risk and severity.

We hypothesize that ill-regulated overexpression of **SLC19A1** by activity of transcription factors to asthma pathogenesis in a bystander effect may sensitize cells to pro-inflammatory effects of self- and microbial DNA or aggravate cell folate deprivation, activating cGAS-STING pathway or creating a vicious cycle of abnormal DNA methylation and exhibition of folate's pro-inflammatory properties.

Co-expression of **SLC19A1** in EA patients may have therapeutic implications, since MTX is consideration as steroid-sparing medication for severe asthma patients and its transport may affect treatment outcomes.

2.5.27 MAEA (macrophage erythroblast attacher, E3 ubiquitin ligase).

MAEA is an ubiquitin ligase and part of CTLH complex of significance in TNF-a-mediated apoptosis, as well as erythropoiesis and macrophage maturation. An EWAS found association between trans-CpG site in MAEA and IL1RL1a, an isoform of IL1RL1 associated with multiple immune reactions in the lung, including Th2-response[131]. **MAEA** methylation positively correlated to birth weight in an observational study[132], suggesting possible mechanistic association between obesity, **MAEA** and IL1RL1a activity in asthma. As an apoptosis-related gene it is up-regulated upon exposure to cigarette smoke extract, possibly due to circulating TNF-a, placental growth factor or loss of VEGF signaling, as well as RSV exposure, along with MMP-9[133].

2.5.28 DGLUCY (D-glutamate cyclase; C14orf159).

C14orf159 is a nuclear gene encoding mitochondrial protein recently identified as D-glutamate cyclase (**DGLUCY)** converting D-glutamate to 5-oxy-proline (pyroglutamic acid).

DGLUCY can be linked to asthma through (1) impact on E-cadherin expression (involved in to epithelial integrity in asthma), possibly through inhibition of ERK and P90RSK phosphorylation[134], (2) its suppression by miRNA-199b (overexpressed during bacterial but not viral infections)[135], (3) possibly

related differential expression in neutrophilia[136], (4) strong differential expression implied in seasonal remodeling of the immune system (reinforcing its role in immune system function and possibly regulation)[137].

Expression of **DGLUCY** is influenced by signaling through oestrogen receptor alpha[137], possibly adding to mechanisms of different asthma phenotypes between sexes. Similarly to abovementioned **MRPL14**, it is a downstream target of MYC, a transcription factor promoting mitochondrial biogenesis and oxidative metabolism in preparation for mitosis and obligatory for cell cycle entry and progression, with NRF-2 and MYC-interacting YY1 being the other transcription factor involved. Invertebrate studies of potential human application, given highly conservative nature of **DGLUCY**, identify MYC as regulated by TOR, FOXO and PI3K/Akt pathway in response to nutrient deprivation, linking nutrient availability with macromolecular synthesis enabled by mitochondrial biogenesis[138].

2.5.29 RADX (RPA1 related single stranded DNA binding protein, X-linked; CXorf57)

RADX is one of the genes upregulated upon culture of pericytes under conditions differentiating them into mesenchymal stromal cells[139]. It is recognized, that migration of subepithelial microvascular pericytes contribute to airway remodeling; if involved in asthma, pericytes can form a source of mesenchymal cells for myofibroblast differentiation as in the murine model[140] and **RADX** may indicate or mediate such transition.

2.5.30 Asthma ignorome:

We couldn't find relevant associations with asthma, inflammation, airway remodeling or other DCGs for **GOLGA2P3Y, RP11-321E2.2** and **RP3-473B4.3.** They will become part of asthma ignorome[141] of currently unknown role until proven otherwise by future studies.

2.6 Regulatory networks of differentially co-expressed genes and inferred pathways.

Regulatory networks of DCGs are presented in **Figures 5-6**. Associated transcription factors were plotted against betweenness centrality of combined regulatory network to identify highly connected nodes with low literature representation and thus presenting research opportunities (**Fig. 7**). An enrichment analysis of the transcription factors revealed multiple signaling pathways listed in **Tab. 11**. Exceptional enrichment of **SMAD2/3** nuclear signaling regulation corresponds to profibrotic effects of TGF-β/Smad2/3 pathway involved in fibroblast to myofibroblast transition[85]. **Activator protein-1** pathway is necessary for transcription of Th2-profile cytokine IL-4 and indicated as therapeutic target in asthma[142]c. The **E2F/Rb** and **Wnt/b-catenin** pathways are both implied in airway smooth muscle hypertrophy[143,144]. This concordance with current literature validates our results and suggests other less studied pathways as important for future studies.

3. Summary.

Systems biology approach allowed us to place 29 of the 32 differentially co-expressed genes in a pathogenetic network with molecular entities previously described as involved in asthma pathogenesis, thus indicating novel candidates for asthma research and highly-connected network disruptors of potential therapeutic applications.

Genes differentially co-expressed in our study bear significance in airway viral infection (ATP1B1, EPS15), arachidonic acid metabolism (CLC, FADS6), cell migration (EPS8L1, STOML3, RHOBTB2), surface receptors endocytosis (STRN4, EPS15, ATP1B1) or decreased expression (CCT7), oxidative stress (DIO3, RHOBTB2), decreased adhesion (ATP1B1, RAPH1, STOML3), epithelial-mesenchymal transition (ASB3, RADX, CCT7, MRPL14, PPP2R3B, RPS13, SLC19A1), myofibroblast differentiation (CCT7), smooth muscle proliferation (ASB3, ATP1B1), airway hyperreactivity (RECK, STOML3, ATP1B1, OR52I1), extracellular matrix remodeling (FBN3, RECK), angiogenesis (GPI, RHOBTB2) and neuronal pathogenesis of asthma (OR52I1, STRN4, TTC3P1, GPI, CABP5) and were associated with asthma in genome-wide (MRPL14, ASB3, RPS13) and epigenome-wide (CLC, EPS15, GPI, SSCRB4, STRN4) association studies.

Study of their regulatory networks allowed us to infer associated pathways involved in asthma, both concordant with current literature (TGF-β/Smad2/3) and novel to human studies.

Future research should verify the tentative role of DCGs and associated pathways in EA pathogenesis and feasibility of their use as biomarkers of active remodeling and therapeutic targets.

Declarations

Data availability.

The datasets generated and analyzed during the current study are available from the corresponding author upon request.

Author contribution statements.

Concept and design: Stanislawa Bazan-Socha, Jan G. Bazan, Pawel Kozlik

- Acquisition of funding: Stanislawa Bazan-Socha
- Application for bioethics committee approval: Stanislawa Bazan-Socha
- Patient recruitment: Stanislawa Bazan-Socha, Joanna Zuk, Jerzy Soja
- Acquisition of clinical and spirometry data: Stanislawa Bazan-Socha, Joanna Zuk, Jerzy Soja
- Acquisition and analysis of imaging data: Jacek Zarychta
- Acquisition of tissue samples: Jerzy Soja
- Acquisition of genomic data from tissue samples: Aleksander Myszka, Sylwia Buregwa-Czuma, Izabela Zawlik
- Statistical analysis of clinical and spirometry data: Stanislawa Bazan-Socha, Joanna Zuk, Jerzy Soja, Pawel Kozlik

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- Topological analysis, literature mining and ontology enrichment analysis of genomic data: Pawel Kozlik, Andzelika Siwiec
- Drafting the article: Pawel Kozlik
- Preparation of figures and tables: Pawel Kozlik, Andzelika Siwiec
- Critical revision of the article for important intellectual content and final approval of the version to be submitted: Pawel Kozlik, Sylwia Buregwa-Czuma, Izabela Zawlik, Aleksander Myszka, Joanna Zuk, Andzelika Siwiec, Jacek Zarychta, Krzysztof Okon, Lech Zareba, Jerzy Soja, Michał Kępski, Jan G. Bazan, Stanislawa Bazan-Socha

Competing interests.

The authors (Pawel Kozlik, Sylwia Buregwa-Czuma, Izabela Zawlik, Aleksander Myszka, Joanna Zuk, Andzelika Siwiec, Jacek Zarychta, Krzysztof Okon, Lech Zareba, Jerzy Soja, Michał Kępski, Jan G. Bazan and Stanislawa Bazan-Socha) declare no competing interests.

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Tables

Tab. 1. Curated resources used in gene set enrichment analysis and construction of interaction networks.

Interactome	Description
Libraries used for gene set enrichment analysis	
ENCODE histone modifications	Database of histone modification probed by ChIP-seq, ChIP-chip and qChIP studies[145]
GO Biological process	Library of gene associated with biological programs accomplished by multiple molecular activities[146,147]
GO molecular function	Library of genes associated with molecular-level activities[146,147]
PID	The Pathway Interaction Database collection of curated and peer-reviewed pathways composed of human molecular signaling and regulatory events and key cellular processes[148]
Interactomes used for network construction	
ENCODE	Transcription factor and gene target data derived from the ENCODE ChIP-seq data[149,150]
JASPAR	Transcription factor targets derived from the JASPAR TF binding site profile database[151]
ChEA	Transcription factor targets database inferred from integrating literature curated Chip-X data[152]
TF-miRNA coregulatory interactions	RegNetwork repository[153]

Tab. 2. Sample's demographics and clinical features.

Characteristic	EA group	Non-EA group	Difference p-value***
Sample size	N=20	N=20	n/a*
Gender, %female	13 (65%)	16 (80%)	0.240
Age, years**	58 (16)	52 (23)	0.096
BMI, kg/m^2**	**25.4 (3.1)**	**30.4 (11.4)**	**0.020**
Atopy, N (%)	11 (58%)	9 (53%)	0.515
Allergic rhinitis, N (%)	15 (88%)	17 (89%)	0.655
Gastroesophageal reflux disease, N (%)	8 (44%)	10 (58%)	0.305
Nicotinism, current, N (%)	0 (0%)	3 (18%)	0.114
Nicotinism, past, N (%)	13 (65%)	16 (80%)	0.563
ACT, points**	14 (8)	16 (9)	0.227
GINA, class (mode)	4 (45%)	3 (40%)	0.569
Severe asthma (GINA class 4), n (%)	9 (45%)	7 (35%)	0.519

***** not applicable, ****** median (interquartile range), ******* Mann-Whitney U-test or Pearson's chi-squared tests, where applicable; differences of statistical significance at <0.05 are **in bold.**

Tab. 3. Sample's spirometry and high-resolution computed tomography (HRCT) proxies of airway remodeling.

FEV1 - forced expiratory volume in 1 second, VC – vital capacity, TLC – total lung capacity, RV – residual volume.

Characteristic	EA group	Non-EA group	Difference p-value*
Spirometry (with reversibility test)			
FEV1, L	2.1 (0.79)	2.28 (1.26)	0.196
FEV1, %predicted	77.4 (40.2)	89.7 (31.1)	0.421
VC, L	3.33 (1.78)	3.36 (1.11)	0.292
FEV1/VC	62.86 (16.86)	67.8 (17.1)	0.267
DFEV1, L	0.21 (0.23)	0.17 (0.22)	0.506
DFEV1, %	0.118 (0.143)	0.07 (0.13)	0.419
TLC, L	6.65 (2.13)	5.63 (1.51)	0.419
TLC, % predicted	114.25 (18.5)	111.9 (22.5)	0.295
RV%TLC	30.63 (3.68)	30.45 (11.48)	0.913
High-resolution computed tomography remodeling proxies (at RB1 bronchus)			
Wall thickness (mm, average)	**1.216 (0.32)**	**1.078 (0.22)**	**0.033**
Lumen diameter (mm, average)	2.719 (0.84)	2.344 (0.47)	0.077
Airway diameter (mm, average)	**5.238 (1.58)**	**4.593 (0.68)**	**0.044**
Wall thickness/airway diameter	14.692 (2.2)	14.817 (1.94)	0.879
Wall area (mm^2)	**25.365 (12.81)**	**18.708 (6.56)**	**0.033**
Wall area ratio	44.24 (4.99)	45.290 (4.98)	0.676

***Mann-Whitney U-test; differences of statistical significance at <0.05 are **in bold.**

Tab. 4. Sample's serum and bronchoalveolar lavage fluid (BALF) cytokine concentrations, cytology and IgE levels.

Serum cytokines (pg/mL)	EA group (mean±SD)	Non-EA group (mean±SD)	Difference p-value*
ADAM33 (ng/mL)	2.229±1.612	1.462±0.944	0.126432
IFN-g	0.575±1.406	0.405±1.355	0.490335
IL – 4	0.005±0	0.005±0	0.989209
IL – 5	0.005±0	0.946±4.210	0.797198
IL – 6	2.058±2.059	1.366±1.508	0.386709
IL – 10	**1.260±1.074**	**0.602±0.807**	**0.017294**
IL – 12p70	1.941±3.777	1.255±2.640	0.946084
IL – 17A	0.188±0.579	0.171±0.450	0.860431
IL – 23	15.81±25.11	48.53±98.36	0.323482
Periostin	1.284±3.038	0.329±0.086	n/a**
Blood count (cells/ml)			
Neutrophils	3580 (2100)	3480 (1260)	0.370
Lymphocytes	2240 (1110)	1930 (730)	0.132
Eosinophils	**480 (880)**	**140 (260)**	**0.008**
Basophils	**40 (30)**	**20 (20)**	**0.012**
Monocytes	**700 (370)**	**505 (110)**	**0.020**
Other			
IgE (IU/mL)	161 (582)	32 (124)	0.018

BALF cytokines (pg/mL)	EA group (mean±SD)	Non-EA group (mean±SD)	Difference p-value*
Periostin	0.741±0.210	**0.911±0.143**	**0.010721**
IL – 4	0.645±1.832	0.379±1.276	0.930612
IL – 5	0.342±1.509	0.005±0	0.342113
IL – 6	0.586±0.583	0.832±0.624	0.197439
IL – 10	0.005±0	0.005±0	n/a
IL – 12p70	0.068±0.021	0.063±0.033	0.684814
IL – 17A	0.005±0	0.005±0	n/a
IL – 23	0.245±1.076	0.005±0	0.342113
IFN-g	0.005±0	0.005±0	n/a

***Mann-Whitney U-test; differences of statistical significance at a=0.05 are **in bold.**

****not available

Table 5. Genes differentially expressed in bronchial brush biopsy samples of eosinophilic and non-eosinophilic asthma patients.

logFC – log2 fold change (eosinophilic/non-eosinophilic asthma); p-value is unadjusted.

Differentially expressed gene		Gene statistics		Literature coverage by keywords (papers)		
gene symbol	gene name	logFC	p-value	asthma	inflammation	remodeling
LRRN2	leucine rich repeat neuronal 2	0.752832	0.000398	-	-	-
DLX3	distal-less homeobox 3	0.7525317	0.001541	-	-	13
SERP2	stress associated endoplasmic reticulum protein family member 2	-0.57667	0.003001	-	-	-
PYURF	PIGY upstream reading frame	-0.54608	0.003259	-	-	-
RUVBL2	RuvB like AAA ATPase 2	-0.53847	0.004246	-	1	47
RAPH1	Ras association (RalGDS/AF-6) and pleckstrin homology domains 1	0.736463	0.004406	-	-	5
C1QB	complement C1q B chain	-0.71181	0.005344	-	-	3
TMEM207	transmembrane protein 207	0.553981	0.005435	-	-	-
DOLK	dolichol kinase	0.529385	0.006096	-	-	-
HCG22	HLA complex group 22 (gene/pseudogene)	0.536178	0.006125	1	-	-
CARD6	caspase recruitment domain family member 6	-0.44055	0.006864	-	-	1
CFAP299	cilia and flagella associated protein 299	0.690009	0.00689	-	-	-
GNGT1	G protein subunit gamma transducin 1	0.45062	0.007328	-	-	-
GLRA2	glycine receptor alpha 2	0.618096	0.0074	-	-	2
DTD2	D-aminoacyl-tRNA deacylase 2	0.470056	0.00858	-	-	-

KLK15	kallikrein related peptidase 15	0.475823	0.008796	-	-	3
SPACA3	sperm acrosome associated 3	-0.68256	0.008854	-	5	3
NFXL1	nuclear transcription factor, X-box binding like 1	0.536674	0.009328	-	-	-
PERP	p53 apoptosis effector related to PMP22	-1.24111	0.0097	-	2	3
GTF2E2	general transcription factor IIE subunit 2	0.496494	0.009859	-	-	-

Table 6. Genes differentially co-expressed in bronchial brush biopsy samples of eosinophilic and non-eosinophilic asthma patients. logFC – log2 fold change (eosinophilic/non-eosinophilic asthma). All p-values for differential expression ≥0.01.

Differentially co-expressed genes		Gene statistics	Literature coverage by keywords (papers)		
gene symbol	Entrez ID	logFC	asthma	inflammation	remodeling
Up-regulated in eosinophilic asthma					
ATP1B1	481	0.533982929	1	1	8
STRN4	29888	0.480691965	-	-	-
GPI	2821	0.348522395	-	16	33
ANKRD26P1	124149	0.259044209	-	-	-
RAPH1	65059	0.24468644	-	-	5
RP3-473B4.3	340581	0.145016395	-	-	-
CLC	1178	0.123772953	5	75	9
RADX	55086	0.107286105	-	-	-
RECK	8434	0.083728976	1	1	45
C14orf159	80017	0.076215968	-	-	-
SLC19A1	6573	0.067933101	1		5
TTC3P1	286495	0.060221639	-	-	-
RP11-321E2.2	202201	0.052235123	-	-	-
MAEA	10296	0.048993296	1	4	8
FBN3	84467	0.034589284	-		1
PSG2	5670	0.030748795	-	1	3
PPP2R3B	28227	0.024124236	-	-	-
GOLGA2P3Y	84559	0.023073362	-	-	-
STOML3	161003	0.024668921	-	-	-
DIO3	1735	0.018910174	-	-	6
FADS6	283985	0.017092506	-	-	-
EPS8L1	54869	0.011524955	-	-	1
RPS13	85388	0.013323104	-	-	1
ASB3	388949	0.00830234	-	-	1
MRPL14	64928	0.008790486	-	-	-
Down-regulated in eosinophilic asthma					

EPS15	2060	-0.004142139	-	-	3
RHOBTB2	23221	-0.010079354	-	-	-
SRCRB4D	136853	-0.01620345	-	-	-
OR52I1	390037	-0.097623097	-	-	-
CCT7	10574	-0.141075317	-	-	4
SRPRB	58477	-0.161702668	-	-	-
CABP5	51476	-0.601734939	-	-	-

Tab. 7. Cell-type enrichment analysis of histone ChIP-seq peak marks of human cell lines annotated within the Encyclopedia of DNA Elements (ENCODE).

Note: adjusted p-values >0.05.

Histone modification and cell line	p-value	Affected genes
H3K9ac fibroblast of lung hg19	0.035726	ASB3; RADX; CCT7; MRPL14; PPP2R3B; RPS13; SLC19A1
H3K27me3 fibroblast of lung hg19	0.045305	CLC; RADX; DIO3; EPS8L1; FADS6; FBN3; SLC19A1; SRCRB4D; STOML3
H3K36me3 myocyte mm9	0.054552	ASB3;RPS13
H3K27me3 SK-N-SH hg19	0.122431	FBN3;FADS6;EPS8L1;DIO3;CLC;RADX
H3K9ac K562 hg19	0.166164	FADS6;RAPH1;MRPL14;PPP2R3B;CCT7; ATP1B1;SRCRB4D;SLC19A1
H3K4me1 G1E mm9	0.209147	FADS6;RAPH1;EPS8L1;SRPRB;SRCRB4D; RECK
H3K9me3 A549 hg19	0.211366	CABP5;ANKRD26P1;TTC3P1;DIO3; ATP1B1
H3K4me1 CD14-positive monocyte hg19	0.211366	GPI;FADS6;EPS8L1;CCT7;RECK
H2AFZ skeletal muscle myoblast hg19	0.211366	FADS6;RAPH1;ASB3;SLC19A1;RHOBTB2
H3K9ac mammary epithelial cell hg19	0.211366	EPS8L1;MRPL14;PPP2R3B;ASB3;RHOBTB2
H3K27me3 T-cell acute lymphoblastic leukemia hg19	0.211366	GPI;STOML3;EPS8L1;SRCRB4D;STRN4
H3K27ac HepG2 hg19	0.211366	GPI;MAEA;RADX;CCT7;RPS13
H3K27ac GM12878 hg19	0.211366	RAPH1;PPP2R3B;CCT7;ATP1B1;SLC19A1
H3K4me3 skeletal muscle myoblast hg19	0.211366	MRPL14;PPP2R3B;RADX;CCT7;RPS13
H3K27ac astrocyte hg19	0.211366	C14ORF159;ASB3;STRN4;RPS13;RHOBTB2
H3K27me3 myotube hg19	0.211366	FBN3;CABP5;EPS8L1;CLC;SRCRB4D
H3K9me1 keratinocyte hg19	0.211366	GPI;MAEA;CCT7;RECK;STRN4
H3K9ac CD14-positive monocyte hg19	0.211366	RAPH1;EPS8L1;MRPL14;PPP2R3B;CCT7

Tab. 8. Biological processes associated with genes differentially co-expressed between eosinophilic and non-eosinophilic asthma in bronchial brush biopsy samples.

Gene ontology derived from GO Biological Process 2018 by Gene Ontology Consortium.

Note: adjusted p-values >0.05.

Biological process	Accession number	Genes	p-value	z-score	combined log(p-value)*z-score
Rho protein signal transduction	GO:0007266	EPS8L1 RHOBTB2	0.00591	-39.98	89.1
regulation of T cell tolerance induction	GO:0002664	CLC	0.00956	-239.9	484
negative regulation of myeloid cell apoptotic process	GO:0033033	MAEA	0.00956	-239.9	484
regulation of cytokine production involved in immune response	GO:0002718	CLC	0.00956	-239.9	484
hemostasis	GO:0007599	GPI	0.01115	-205.6	401
folic acid transport	GO:0015884	SLC19A1	0.01115	-205.6	401
positive regulation of protein localization to Cajal body	GO:1904871	CCT7	0.01273	-179.9	341
regulation of protein localization to Cajal body	GO:1904869	CCT7	0.01273	-179.9	341
regulation of T cell mediated immunity	GO:0002709	CLC	0.01273	-179.9	341
positive regulation of establishment of protein localization to telomere	GO:1904851	CCT7	0.01431	-159.9	295
regulation of immunoglobulin secretion	GO:0051023	GPI	0.01431	-159.9	295
positive regulation of immunoglobulin secretion	GO:0051024	GPI	0.01431	-159.9	295
potassium ion import across plasma membrane	GO:1990573	ATP1B1	0.01431	-159.9	295
thyroid hormone generation	GO:0006590	DIO3	0.01589	-143.9	259
regulation of establishment of protein localization to telomere	GO:0070203	CCT7	0.01589	-143.9	259
cardiac muscle cell membrane repolarization	GO:0099622	ATP1B1	0.01746	-130.8	230
membrane repolarization during action potential	GO:0086011	ATP1B1	0.01746	-130.8	230
import across plasma membrane	GO:0098739	ATP1B1	0.01746	-130.8	230

relaxation of muscle	GO:0090075	ATP1B1	0.01746	-130.8	230
positive regulation of sodium ion transmembrane transporter activity	GO:2000651	ATP1B1	0.01746	-130.8	230
relaxation of cardiac muscle	GO:0055119	ATP1B1	0.01746	-130.8	230
positive regulation of protein localization to chromosome, telomeric region	GO:1904816	CCT7	0.01746	-130.8	230
positive regulation of potassium ion transmembrane transporter activity	GO:1901018	ATP1B1	0.01904	-119.9	206
positive regulation of establishment of protein localization	GO:1904951	CCT7	0.01904	-119.9	206
positive regulation of ruffle assembly	GO:1900029	EPS8L1	0.01904	-119.9	206
sodium ion export	GO:0071436	ATP1B1	0.02061	-110.7	187
cellular potassium ion homeostasis	GO:0030007	ATP1B1	0.02061	-110.7	187
sodium ion export from cell	GO:0036376	ATP1B1	0.02061	-110.7	187
regulation of T cell cytokine production	GO:0002724	CLC	0.02061	-110.7	187
membrane repolarization	GO:0086009	ATP1B1	0.02061	-110.7	187
transmembrane transport	GO:0055085	STOML3 ATP1B1 SLC19A1	0.02215	-11.42	18.9
modified amino acid transport	GO:0072337	SLC19A1	0.02218	-102.8	170
cell communication by electrical coupling involved in cardiac conduction	GO:0086064	ATP1B1	0.02218	-102.8	170
clathrin coat assembly	GO:0048268	EPS15	0.02218	-102.8	170
establishment or maintenance of actin cytoskeleton polarity	GO:0030950	RHOBTB2	0.02218	-102.8	170
positive regulation of telomerase RNA	GO:1904874	CCT7	0.02374	-95.94	156

localization to Cajal body					
amide transport	GO:0042886	SLC19A1	0.02374	-95.94	156
cofactor transport	GO:0051181	SLC19A1	0.02374	-95.94	156
cellular sodium ion homeostasis	GO:0006883	ATP1B1	0.02374	-95.94	156
negative regulation of RNA metabolic process	GO:0051253	RPS13	0.02374	-95.94	156
membrane repolarization during cardiac muscle cell action potential	GO:0086013	ATP1B1	0.02374	-95.94	156
positive regulation of potassium ion transmembrane transport	GO:1901381	ATP1B1	0.02374	-95.94	156
protein stabilization	GO:0050821	CCT7 ATP1B1	0.02452	-18.94	30.5
cellular monovalent inorganic cation homeostasis	GO:0030004	ATP1B1	0.02687	-84.65	133
positive regulation of calcium ion transmembrane transporter activity	GO:1901021	ATP1B1	0.02687	-84.65	133
viral entry into host cell	GO:0046718	EPS15	0.02687	-84.65	133
regulation of cellular response to growth factor stimulus	GO:0090287	FBN3	0.02842	-79.95	124
negative regulation of RNA splicing	GO:0033119	RPS13	0.02842	-79.95	124
regulation of telomerase RNA localization to Cajal body	GO:1904872	CCT7	0.02842	-79.95	124
drug transport	GO:0015893	SLC19A1	0.02998	-75.74	115
folic acid metabolic process	GO:0046655	SLC19A1	0.02998	-75.74	115
positive regulation of immunoglobulin production	GO:0002639	GPI	0.02998	-75.74	115
Golgi to endosome transport	GO:0006895	EPS15	0.03153	-71.96	108
regulation of ruffle assembly	GO:1900027	EPS8L1	0.03308	-68.53	101

regulation of cardiac muscle contraction by calcium ion signaling	GO:0010882	ATP1B1	0.03308	-68.53	101
vitamin transport	GO:0051180	SLC19A1	0.03618	-62.57	90.2
folic acid-containing compound metabolic process	GO:0006760	SLC19A1	0.03618	-62.57	90.2
positive regulation of ion transmembrane transporter activity	GO:0032414	ATP1B1	0.03618	-62.57	90.2
glycolytic process through glucose-6-phosphate	GO:0061620	GPI	0.03926	-57.56	80.9
canonical glycolysis	GO:0061621	GPI	0.03926	-57.56	80.9
regulation of potassium ion transmembrane transporter activity	GO:1901016	ATP1B1	0.03926	-57.56	80.9
potassium ion homeostasis	GO:0055075	ATP1B1	0.03926	-57.56	80.9
glucose catabolic process to pyruvate	GO:0061718	GPI	0.03926	-57.56	80.9
sodium ion homeostasis	GO:0055078	ATP1B1	0.0408	-55.35	76.9
endocytic recycling	GO:0032456	EPS15	0.04234	-53.3	73.2
regulation of actin polymerization or depolymerization	GO:0008064	EPS8L1	0.04387	-51.4	69.8
cardiac muscle cell action potential	GO:0086001	ATP1B1	0.04541	-49.62	66.6
dicarboxylic acid transport	GO:0006835	SLC19A1	0.04541	-49.62	66.6
regulation of activated T cell proliferation	GO:0046006	CLC	0.04541	-49.62	66.6
regulation of protein polymerization	GO:0032271	EPS8L1	0.04694	-47.97	63.7
ion transmembrane transport	GO:0034220	STOML3 ATP1B1	0.04775	-13.14	17.4
Ras protein signal transduction	GO:0007265	EPS8L1 RHOBTB2	0.04932	-12.91	16.9
positive regulation of telomere maintenance via telomerase	GO:0032212	CCT7	0.04999	-44.97	58.5
purine ribonucleoside triphosphate metabolic process	GO:0009205	ATP1B1	0.04999	-44.97	58.5

Tab. 9. Molecular functions associated with genes differentially co-expressed between eosinophilic and non-eosinophilic asthma in bronchial brush biopsy samples.

Gene ontology derived from GO Molecular Function 2018 by Gene Ontology Consortium.

Note: adjusted p-values >0.05.

Molecular function	Accession number	Genes	p-value	z-score	combined log(p-value)*z-score
T cell receptor binding	GO:0042608	EPS8L1	0.00956	-239.85	484.4
small ribosomal subunit rRNA binding	GO:0070181	RPS13	0.01115	-205.59	401.5
signal recognition particle binding	GO:0005047	SRPRB	0.01273	-179.89	340.9
armadillo repeat domain binding	GO:0070016	STRN4	0.01431	-159.9	294.9
potassium-transporting ATPase activity	GO:0008556	ATP1B1	0.01746	-130.83	230
sodium:potassium-exchanging ATPase activity	GO:0005391	ATP1B1	0.01746	-130.83	230
sodium ion binding	GO:0031402	ATP1B1	0.01904	-119.93	206.3
metalloendopeptidase inhibitor activity	GO:0008191	RECK	0.01904	-119.93	206.3
potassium ion binding	GO:0030955	ATP1B1	0.01904	-119.93	206.3
Rac guanyl-nucleotide exchange factor activity	GO:0030676	EPS8L1	0.02218	-102.79	170
alkali metal ion binding	GO:0031420	ATP1B1	0.0253	-89.945	143.6
MHC class II protein complex binding	GO:0023026	ATP1B1	0.0253	-89.945	143.6
cysteine-type endopeptidase activity involved in apoptotic process	GO:0097153	CLC	0.0253	-89.945	143.6
MHC protein complex binding	GO:0023023	ATP1B1	0.02998	-75.743	115.4
lysophospholipase activity	GO:0004622	CLC	0.02998	-75.743	115.4
protein phosphatase 2A binding	GO:0051721	STRN4	0.03153	-71.956	108
mRNA 5'-UTR binding	GO:0048027	RPS13	0.03463	-65.414	95.5
ATPase activator activity	GO:0001671	ATP1B1	0.03618	-62.57	90.2

Tab. 10. Impact of kinase perturbations in animal studies on genes differentially expressed between eosinophilic and non-eosinophilic asthma in bronchial brush biopsy samples. Kinase perturbation studies

data retrieved from Gene Expression Omnibus using Enrichr software. Only terms with p-value <0.05 are shown; p-values adjusted by Fisher's exact test >0.05.

Affected kinase and study type	GEO accession number	Affected genes	p-value	z-score	combined log(p-value) *z-score
Genes up-regulated due to kinase perturbation					
HIPK1 (knockout)	GSE39253	GPI MAEA STRN4	0.0120	-14.39	27.63348
TGFBR2 (knockout)	GSE22989	MRPL14 CCT7 EPS15	0.0120	-14.39	27.63348
Genes down-regulated due to kinase perturbation					
IKBKE (siRNA-mediated knockdown)	GSE27869	C14ORF159 RAPH1 ASB3	0.0120	-14.39	27.63348

Tab. 11. Signaling pathways associated with transcription factors from the regulatory network of genes differentially co-expressed between eosinophilic and non-eosinophilic asthma in bronchial brush biopsy samples.

Signaling pathway	Genes	p-value	q-value	z-score	combined log(p-value) *z-score
Regulation of nuclear SMAD2/3 signaling	CEBPB; HDAC1; GATA3; NR3C1; FOXO3; SIN3B; HNF4A; MYC; EP300; E2F4; E2F5; SMAD2; JUN; SMAD4; SMAD3; VDR; TFE3; ESR1; RUNX2; RUNX1; AR; NCOR1; CREB1; TFDP1; SP1; SP3; TCF3; ATF3	8.73E-37	1.82E-34	-83.78	3021.193231
AP-1 transcription factor network	EGR1; JUN; NR3C1; GATA2; ETS1; HIF1A; ESR1; FOSL1; CREB1; CCND1; SP1; MYC; EP300; CTNNB1; TP53; ATF3	2.86E-18	2.99E-16	-56.20	986.0307212
E2F transcription factor network	HDAC1; TFE3; BRCA1; HIC1; YY1; TFDP1; TRIM28; SP1; MYC; E2F1; EP300; MYBL2; HBP1; E2F4; E2F5; E2F6	6.00E-18	4.18E-16	-53.86	927.6012899
Notch-mediated HES/HEY network	HDAC1; STAT3; ARNT; GATA4; RBPJ; HIF1A; GATA1; RUNX2; YY1; AR; NCOR1; E2F1; EP300; TCF3	1.09E-17	5.70E-16	-70.69	1199.092417
Validated targets of C-MYC transcriptional repression	SMAD2; SMAD4; SPI1; SMAD3; NFYA; HDAC1; NFYC; BRCA1; FOXO3; CREB1; CCND1; SP1; MYC; EP300	7.48E-16	3.13E-14	-53.86	814.7221182
Glucocorticoid receptor regulatory network	EGR1; JUN; SPI1; HDAC1; GATA3; NR3C1; RELA; NFKB1; PBX1; SMARCA4; NR4A1; CREB1; IRF1; EP300; TP53	1.51E-15	5.27E-14	-44.34	657.0726358
Regulation of nuclear beta catenin signaling and target gene transcription	JUN; HDAC1; LEF1; MITF; KLF4; SMARCA4; AR; CCND1; MYC; SALL4; EP300; CTNNB1; HBP1; TCF4	2.18E-14	6.51E-13	-42.95	586.7956942
Regulation of retinoblastoma protein	CEBPB; JUN; SPI1; HDAC1; MITF; RUNX2; SMARCA4; TFDP1; CCND1; E2F1; EP300; PPARG; E2F4	2.92E-14	7.62E-13	-49.23	666.3598742
Regulation of Telomerase	JUN; SMAD3; HDAC1; ESR1; NFKB1; CCND1; SIN3B; WT1; SP1; MYC; IRF1; SP3; E2F1	5.50E-14	1.28E-12	-47.03	623.584993
FOXA1 transcription factor	FOXA1; AR; CEBPB; JUN; NFIA; SP1; NFIC; EP300;	2.48E-13	5.18E-12	-60.59	763.8201229

network	BRCA1; ESR1; FOXA2				
C-MYB transcription factor network	CEBPB; SPI1; NCOR1; CCND1; TRIM28; SP1; MYC; LEF1; EP300; GATA3; ETS1; GATA1	1.77E-11	3.36E-10	-35.47	381.3941217
Validated nuclear estrogen receptor alpha network	CEBPB; JUN; SMAD4; NCOR1; CCND1; HDAC1; MYC; EP300; BRCA1; ESR1; ESR2	2.04E-11	3.55E-10	-41.66	445.3469976
Signaling events mediated by HDAC Class I	YY1; NCOR1; SIN3B; HDAC1; STAT3; EP300; PPARG; GATA2; GATA1; RELA; NFKB1	2.90E-11	4.66E-10	-40.40	425.6891794
HIF-1-alpha transcription factor network	JUN; SMAD4; CREB1; SMAD3; SP1; HNF4A; EP300; ARNT; GATA2; ETS1; HIF1A	2.90E-11	4.33E-10	-40.40	425.6891794
Direct p53 effectors	FOXA1; JUN; NFYA; VDR; NFYC; HIC1; SMARCA4; TFDP1; SP1; E2F1; EP300; TP53; TP63; ATF3	4.65E-11	6.48E-10	-24.95	257.7948324
Regulation of Androgen receptor activity	AR; EGR1; JUN; HDAC1; EP300; SRY; NR3C1; NR2C2; GATA2	1.35E-09	1.76E-08	-41.95	372.1071412
Signaling mediated by p38-alpha and p38-beta	ATF1; CEBPB; JUN; CREB1; MITF; HBP1; ESR1; TP53	2.19E-09	2.70E-08	-51.03	441.8400253
p73 transcription factor network	YAP1; WT1; SP1; MYC; HSF1; EP300; FOXO3; GATA1; TP63; RELA	3.39E-09	3.94E-08	-31.07	263.1731164
Calcineurin-regulated NFAT-dependent transcription in lymphocytes	FOSL1; EGR1; JUN; IRF4; E2F1; PPARG; GATA3; FOXP3	1.10E-08	1.21E-07	-42.15	335.5324137
RXR and RAR heterodimerization with other nuclear receptor	SREBF1; NR4A1; VDR; RARA; PPARG; PPARD	7.93E-08	8.29E-07	-60.59	430.2696264
Notch signaling pathway	YY1; NCOR1; CCND1; HDAC1; MYC; EP300; GATA3; RBPJ	8.39E-08	8.35E-07	-32.86	232.5624802
FOXA2 and FOXA3 transcription factor networks	FOXA1; CEBPB; CREB1; SP1; HNF4A; NR3C1; FOXA2	2.09E-07	1.99E-06	-37.70	251.8464888
Presenilin action in Notch and Wnt	JUN; CCND1; HDAC1; MYC; CTNNB1; RBPJ; PPARD	2.45E-07	2.22E-06	-36.88	243.8556084

signaling					
HIF-2-alpha transcription factor network	SP1; EP300; ARNT; ETS1; ELK1; POU5F1	7.32E-07	6.38E-06	-42.77	262.4281795
Validated transcriptional targets of AP1 family members Fra1 and Fra2	FOSL1; JUN; CCND1; SP1; EP300; USF2	8.77E-07	7.33E-06	-41.55	251.6791655
ATF-2 transcription factor network	JUN; CREB1; CCND1; EP300; BRCA1; ESR1; ATF3	1.25E-06	1.00E-05	-29.25	172.697571
FOXM1 transcription factor network	CCND1; SP1; MYC; EP300; FOXM1; ESR1	1.99E-06	1.54E-05	-36.36	207.2522483
Validated transcriptional targets of deltaNp63 isoforms	YAP1; VDR; HBP1; POU2F2; TP63; RUNX1	4.07E-06	3.03E-05	-32.32	174.2178406
IL6-mediated signaling events	CEBPB; JUN; MYC; IRF1; STAT3; MITF	4.64E-06	3.34E-05	-31.61	168.6197375
Validated transcriptional targets of TAp63 isoforms	TFAP2C; VDR; SP1; EP300; HBP1; TP63	1.08E-05	7.50E-05	-27.44	136.3231851
Signaling events mediated by HDAC Class II	BCOR; NR3C1; GATA2; GATA1; ESR1	1.63E-05	1.10E-04	-35.64	170.6272481
IL2 signaling events mediated by PI3K	MYC; E2F1; FOXO3; RELA; NFKB1	1.89E-05	1.23E-04	-34.63	163.549873
BCR signaling pathway	JUN; POU2F2; ETS1; ELK1; RELA; NFKB1	3.22E-05	2.04E-04	-22.72	102.0670923
Integrin-linked kinase signaling	JUN; ZEB1; CREB1; CCND1; CTNNB1	6.59E-05	4.05E-04	-26.93	112.6035437
LKB1 signaling events	SMAD4; CREB1; MYC; ESR1; TP53	8.15E-05	4.86E-04	-25.78	105.4357932
Validated targets of C-MYC transcriptional activation	FOSL1; SMAD4; SMAD3; MYC; EP300; TP53	1.06E-04	6.17E-04	-18.41	73.15299554
Retinoic acid receptors-mediated signaling	HDAC1; VDR; RARA; EP300	1.35E-04	7.63E-04	-34.63	133.9714725
TGF-beta receptor	YAP1; SMAD2; SMAD4;	1.59E-04	8.75E-04	-22.44	85.23980904

signaling	SMAD3; CTNNB1				
CD40/CD40L signaling	JUN; MYC; RELA; NFKB1	1.78E-04	9.55E-04	-32.32	121.156723
Coregulation of Androgen receptor activity	AR; CCND1; CTNNB1; TCF4; BRCA1	2.42E-04	0.001267	-20.54	74.26029048
IL4-mediated signaling events	CEBPB; SPI1; IRF4; SP1; ETS1	2.63E-04	0.001338	-20.20	72.32655319
Trk receptor signaling mediated by PI3K and PLC-gamma	EGR1; CREB1; CCND1; FOXO3	3.66E-04	0.001823	-26.93	92.53414197
Downstream signaling in naive CD8+ T cells	EOMES; FOSL1; EGR1; JUN; ELK1	4.10E-04	0.001994	-18.36	62.1904905
ErbB1 downstream signaling	ATF1; EGR1; JUN; CREB1; STAT3; ELK1	5.02E-04	0.002383	-13.85	45.70010227
Regulation of cytoplasmic and nuclear SMAD2/3 signaling	SMAD2; SMAD4; SMAD3	5.20E-04	0.002416	-42.77	140.4574345
IFN-gamma pathway	CEBPB; IRF1; STAT3; EP300	5.52E-04	0.002508	-24.24	78.96896859
Hypoxic and oxygen homeostasis regulation of HIF-1-alpha	ARNT; HIF1A; TP53	6.20E-04	0.002757	-40.40	129.57832
Angiopoietin receptor Tie2-mediated signaling	ETS1; ELK1; RELA; NFKB1	0.001108	0.004823	-20.20	59.69672704
Signaling events mediated by HDAC Class III	EP300; FOXO3; TP53	0.001868	0.007967	-27.97	76.31116825
p53 pathway	YY1; TRIM28; EP300; TP53	0.002105	0.008797	-17.01	45.53025547

Figures

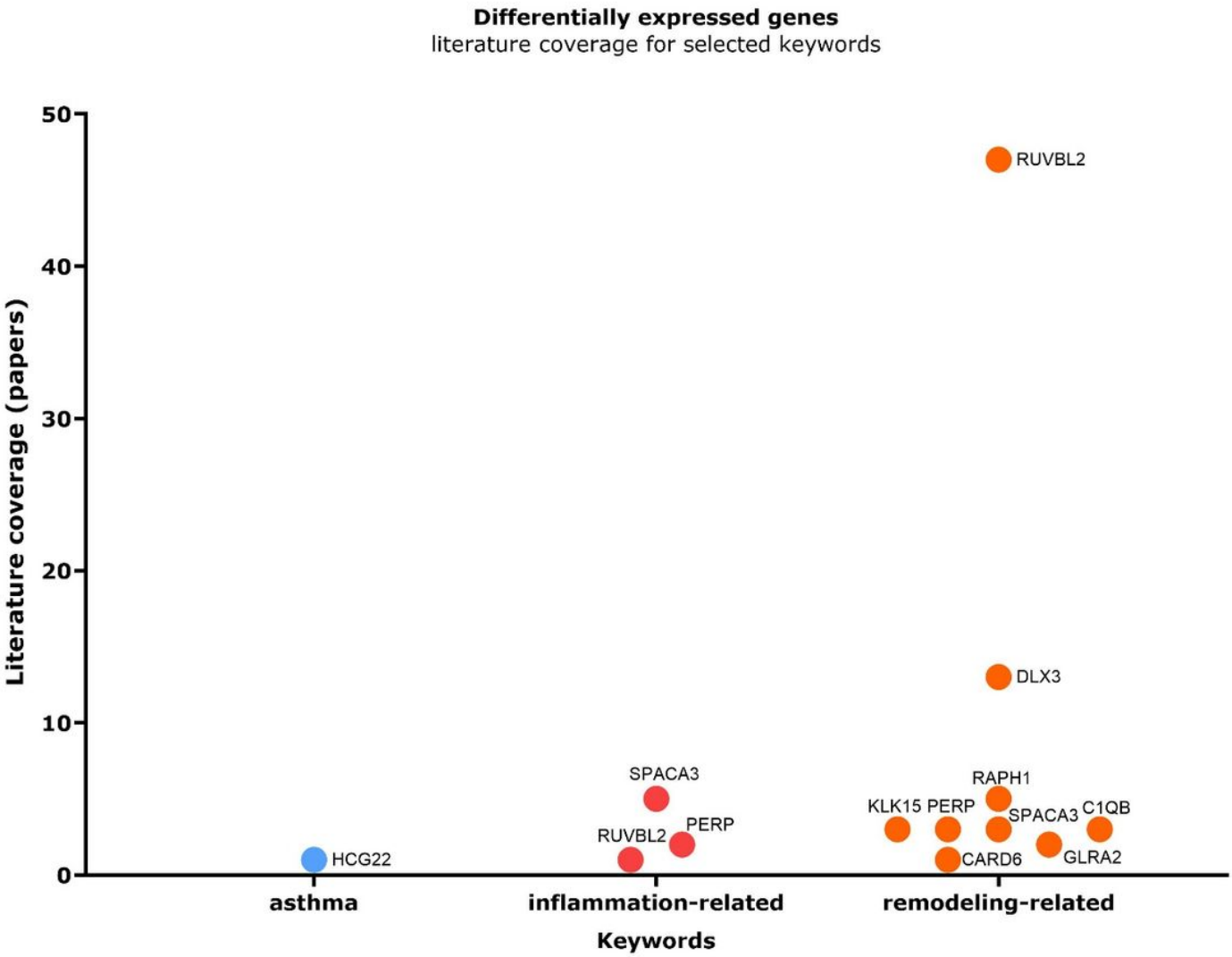

Figure 1

Literature coverage of differentially expressed genes for asthma, inflammation-related and remodeling-related keywords. All p-values for differential expression ≥0.01. Automated literature mining performed using GenClip 3.0.

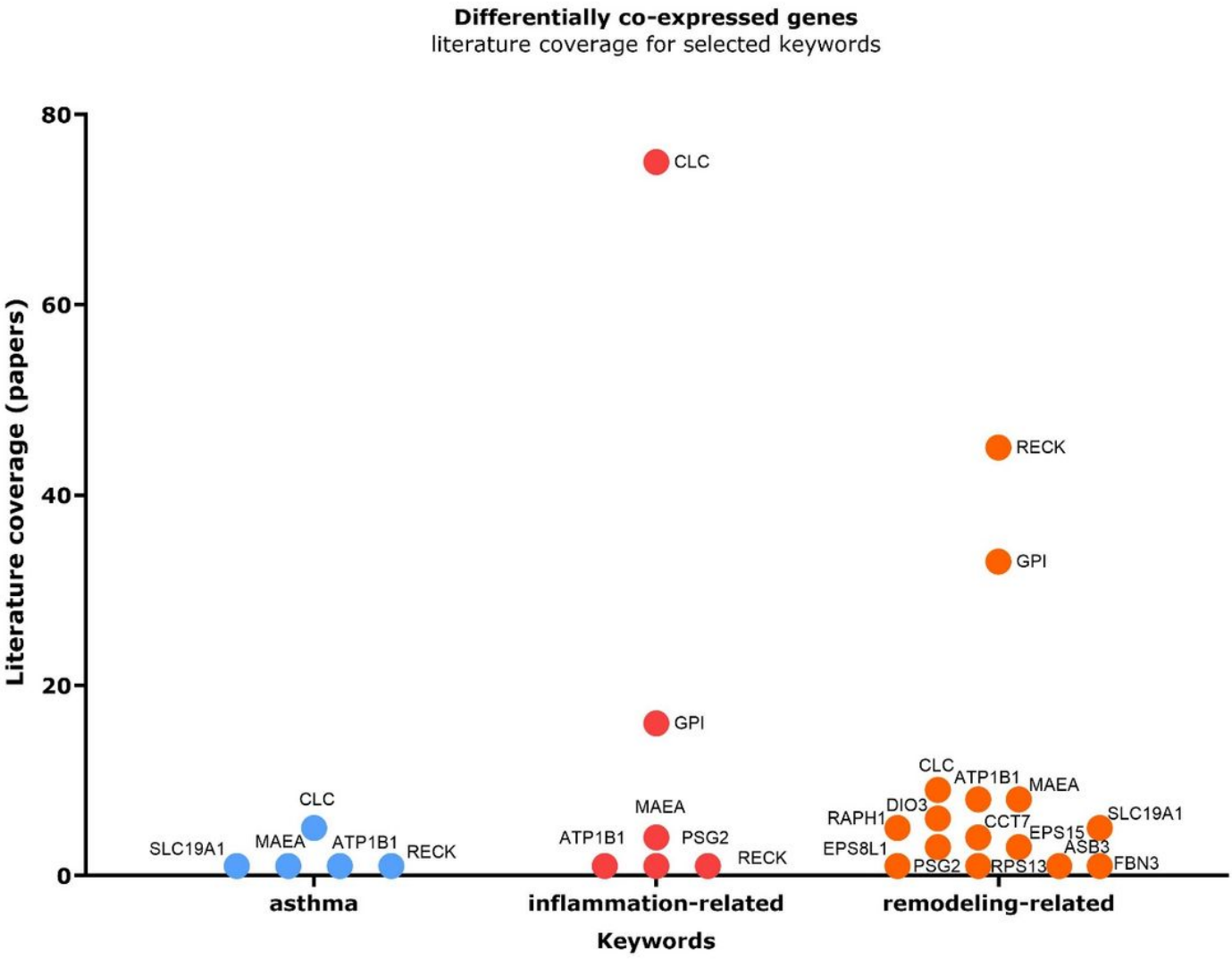

Figure 2

Literature coverage of differentially co-expressed genes for asthma, inflammation-related and remodeling-related keywords. All p-values for differential expression ≥0.01. Automated literature mining performed using GenClip 3.0.

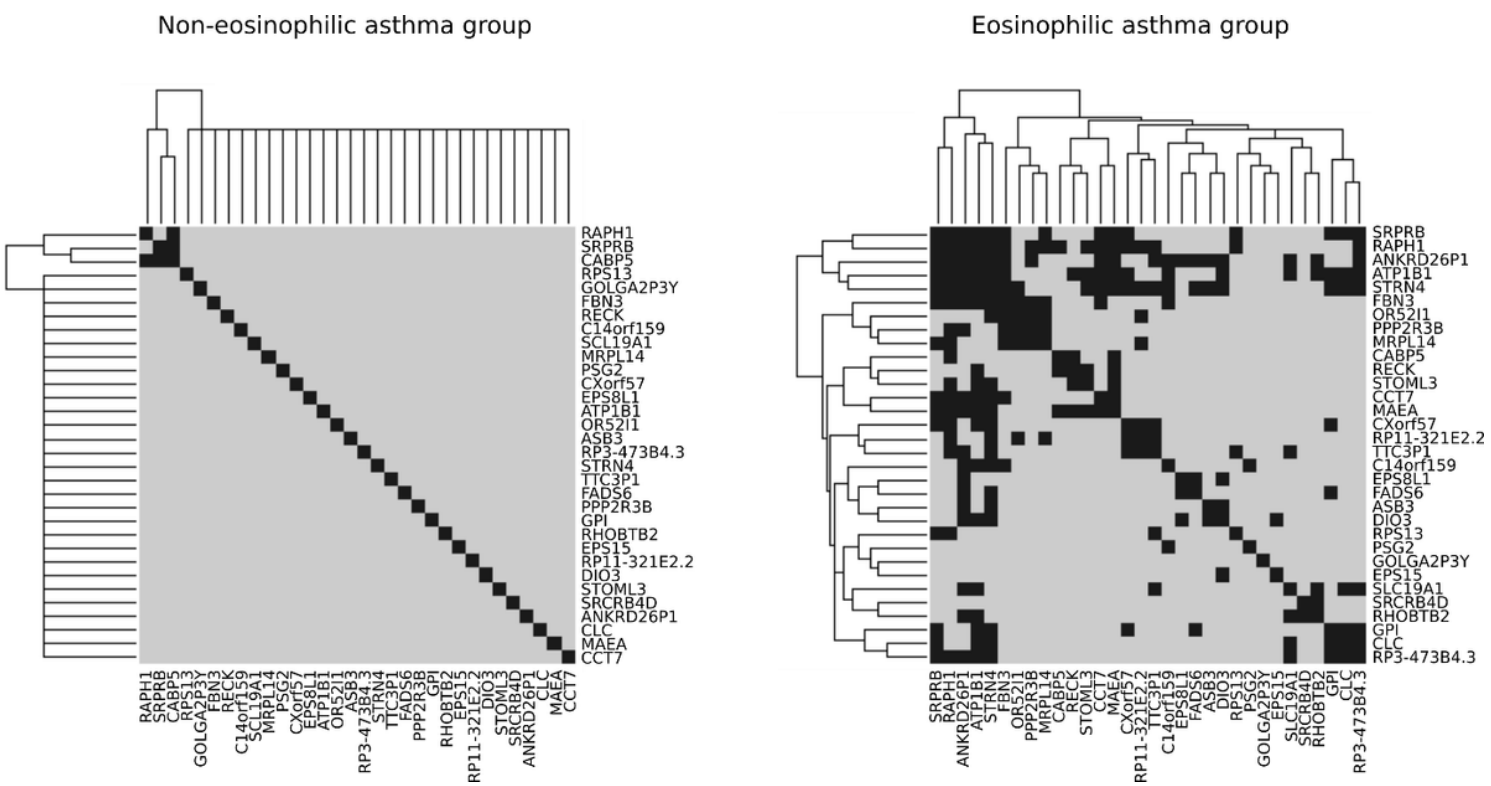

Figure 3

Structural patterns of gene differential co-expression (adjacency matrices). Black color indicates gene-gene correlation exceeding r = 0.8.

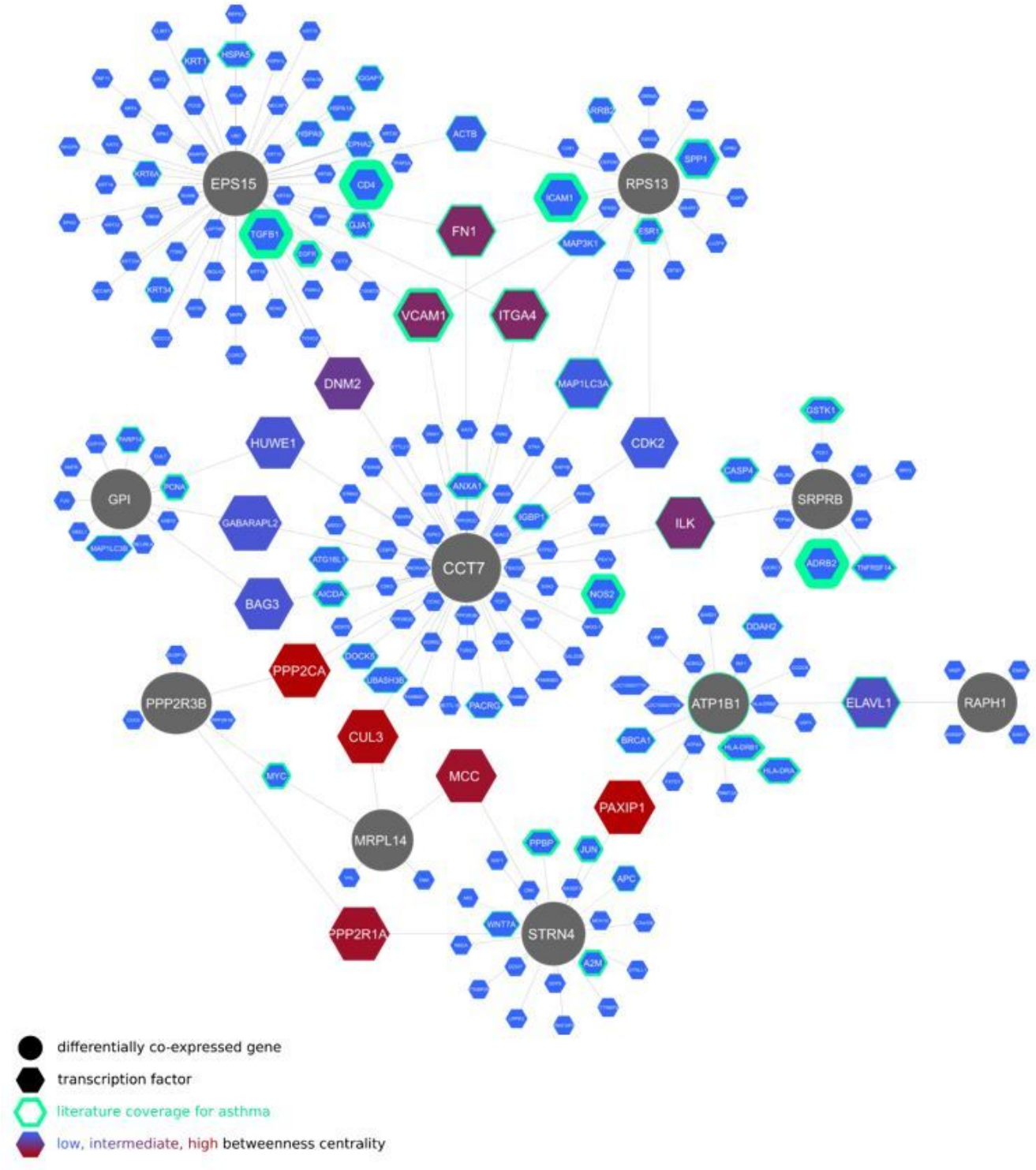

Figure 4

Lung-specific protein-protein interactions of genes differentially expressed between eosinophilic and non-eosinophilic asthma patients in bronchial brush biopsy samples. Interactions retrieved from curated DifferentialNet database.

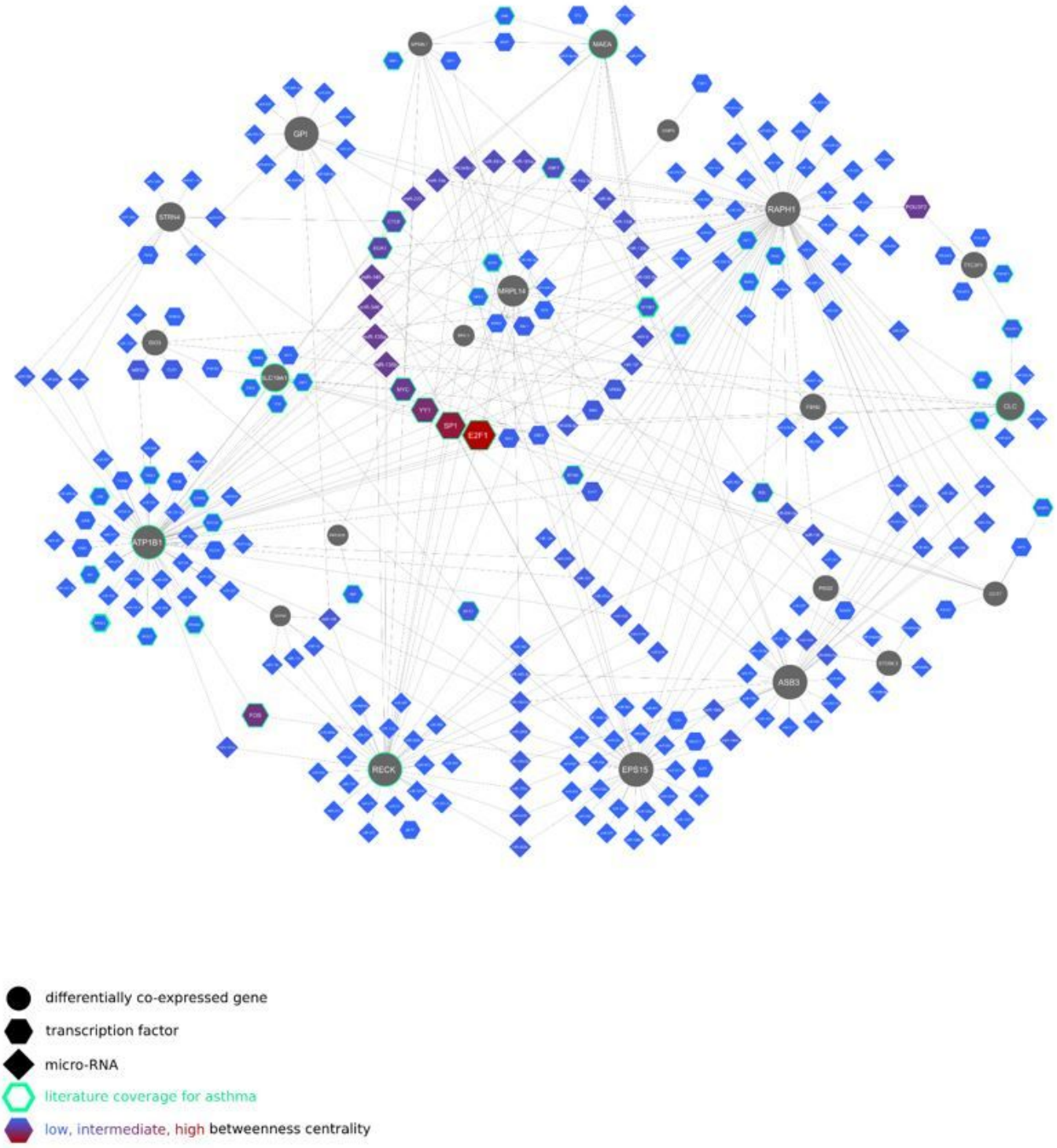

Figure 5

Coregulatory network of miRNA, transcription factors and genes differentially expressed between eosinophilic and non-eosinophilic asthma patients in bronchial brush biopsy samples. Interactions retrieved from the RegNetwork repository.

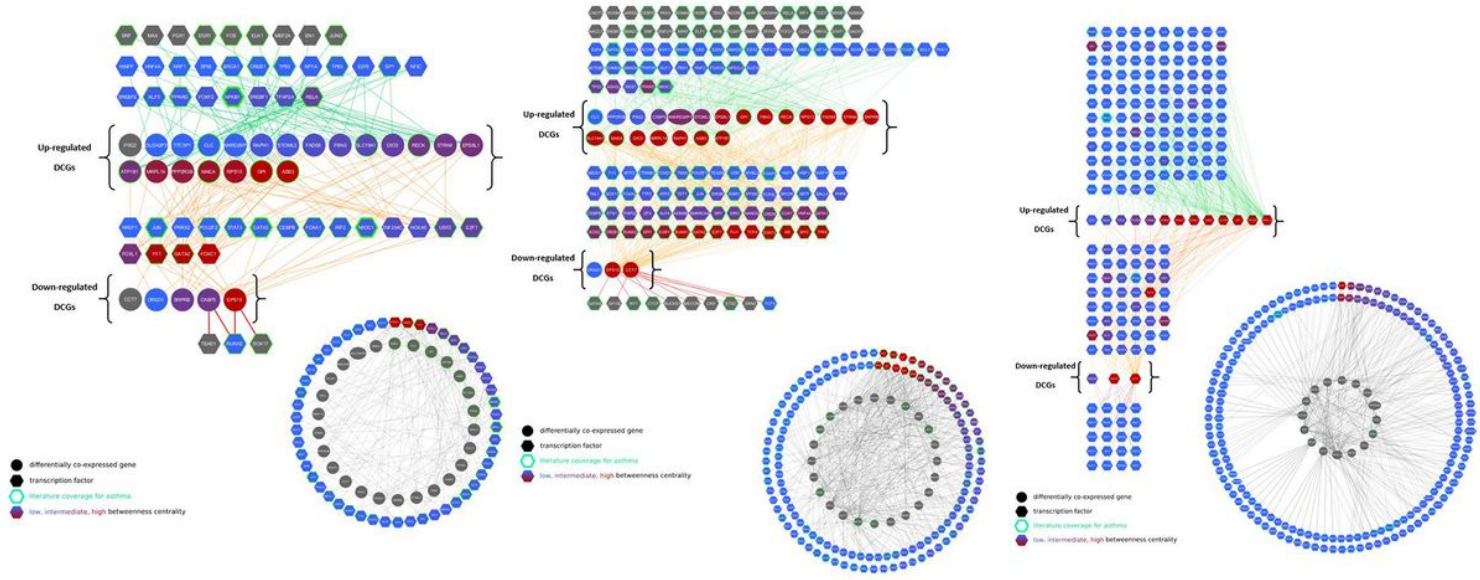

Figure 6

A. Gene regulatory network – transcription factor-gene interactions. Transcription factor targets database inferred from integrating literature curated JASPAR data. Green edges link to TFs targeting only DCGs up-regulated in EA, orange edges to TFs targeting DCGs upregulated in both EA and non-EA, red edges – TFs targeting only DCGs upregulated in non-EA. B. Gene regulatory network – transcription factor-gene interactions. Transcription factor targets inferred from Chip-X curated database. C. Gene regulatory network – transcription factor-gene interactions. Transcription factor and gene target data derived from the ENCODE ChIP-seq data. Peak intensity signal <500, predicted regulatory potential score <1 (BETA minus algorithm).

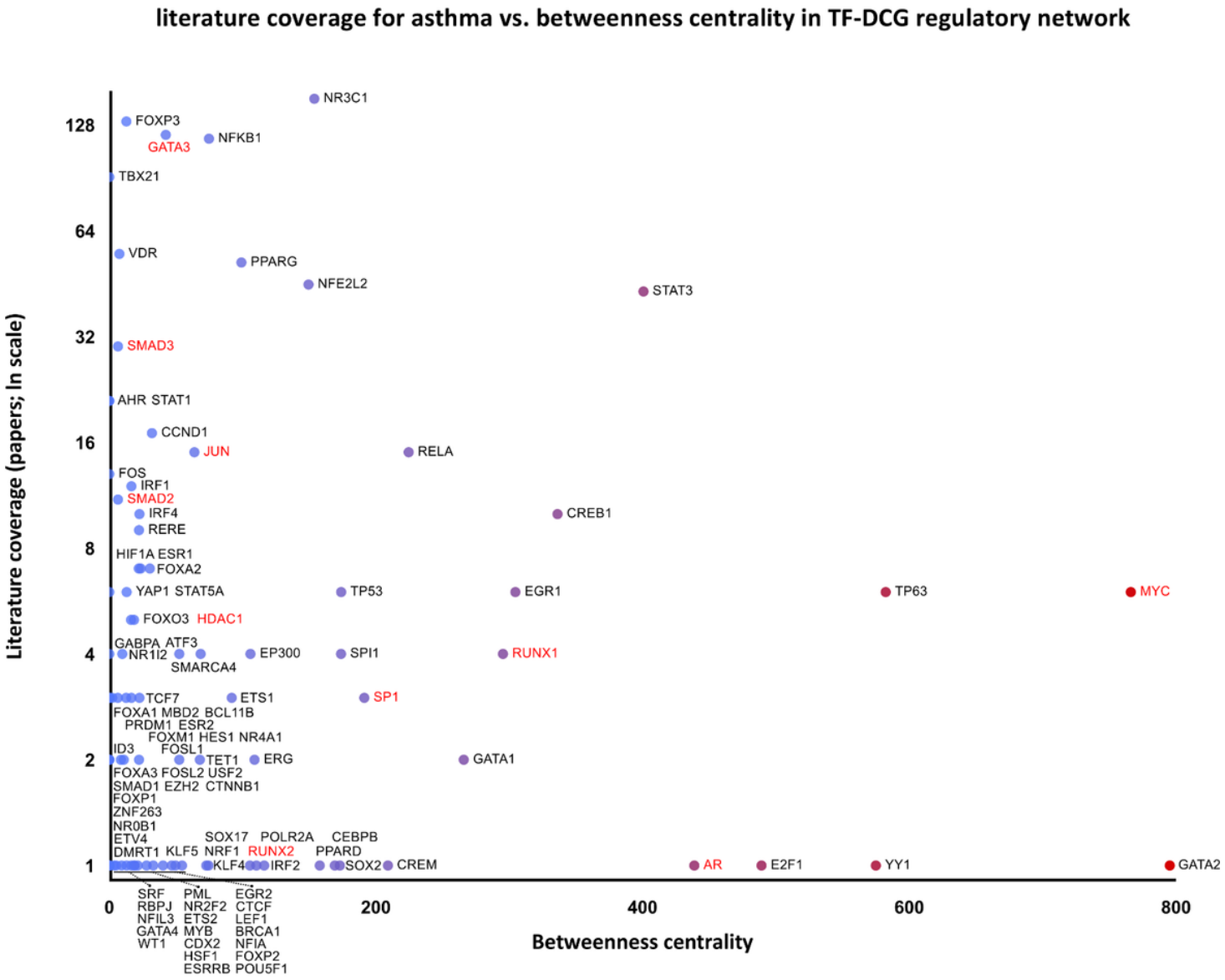

Figure 7

Literature coverage for asthma against betweenness centrality in transcription factor-differentially co-expressed genes. Transcription factors combined from Figures 6A-C. Genes labeled in red form the SMAD2/3 signaling pathway.