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Transcriptomic Analysis Identifies Phosphatases as Novel Targets for Adenotonsillar Hypertrophy of Pediatric OSA

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At a Glance Commentary:
Scientific Knowledge on the Subject: Untreated obstructive sleep apnea is associated with neurocognitive, metabolic and cardiovascular morbidities in children and can affect up to 3% of the pediatric population. Enlargement of adenotonsillar tissue is the key etiological event in this disorder, yet the pathophysiological mechanisms leading to lymphadenoid proliferation is poorly understood.
What This Study Adds to the Field: In this work, we propose a network-based paradigm to systematically identify transcriptional programs regulating tonsillar hypertrophy in children with sleep apnea and demonstrate that selective targeting of a network node, phosphoserine phosphatase, reverses the proliferative state of adenotonsillar tissue. Our findings may result in nonsurgical alternatives for the treatment of pediatric sleep apnea.

Online data supplement:
This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

ABSTRACT

Rationale: Obstructive sleep apnea (OSA) is a highly prevalent disorder in children, in which enlarged adenotonsillar tissues (AT) play a major pathophysiologic role. Mechanisms leading to the proliferation and hypertrophy of AT in children who subsequently develop OSA remain unknown, and surgical extirpation of AT is associated with potential morbidity and mortality.

Objective: We hypothesized that a computationally-based analysis of gene expression in tonsils from children with OSA and children with recurrent tonsillitis without OSA can identify putative mechanistic pathways associated with tonsillar proliferation and hypertrophy in OSA.

Methods and Results: Palatine tonsils from children with either polysomnographically-documented OSA or recurrent infectious tonsillitis were subjected to whole-genome microarray and functional enrichment analyses followed by significance score ranking based on gene interaction networks. The latter enabled identification and confirmation of a candidate list of tonsil-proliferative genes in OSA. *In vitro* studies using a mixed tonsil cell culture system targeting one of these candidates, phosphoserine phosphatase (PSPH), revealed that it was more abundantly expressed in tonsils of OSA children, and that pharmacological inhibition of PSPH led to marked reductions in T and B lymphocyte cell proliferation and increased apoptosis.

Conclusions: A systems biology approach revealed a restricted set of candidate genes potentially underlying the heightened proliferative properties of AT in children with OSA. Furthermore, functional studies confirm a novel role for protein phosphatases in AT hypertrophy, and may provide a promising strategy for discovery of novel, non-surgical therapeutic targets in pediatric OSA.

Word count: 240

Key words: sleep apnea, phosphatase, adenotonsillar, genetic network, microarray

INTRODUCTION

Obstructive sleep apnea (OSA) is a prevalent disorder affecting up to 2-3% of children, and imposes substantial neurocognitive, behavioral, metabolic, and cardiovascular morbidities (1, 2). This condition is characterized by repeated events of partial or complete obstruction of the upper airways during sleep, leading to recurring episodes of hypercapnia, hypoxemia, and arousal throughout the night (3). Adenotonsillar hypertrophy is the major pathophysiological contributor to OSA in children (4, 5). However, the mechanisms underlying the regulation of benign follicular lymphoid proliferation, hypertrophy, and hyperplasia are poorly understood, severely limiting the prediction of children who are at risk for developing adenotonsillar enlargement and OSA. Several epidemiological studies have demonstrated that factors such as environmental smoking, allergies, and recurrent respiratory infections are associated with either transient or persistent hypertrophy of lymphadenoid tissue in the upper airways of snoring children (6-8). Interestingly, all of these risk factors involve the generation of an inflammatory response, suggesting that the latter may promote the onset and maintenance of proliferative signals to lymphadenoid tissues. In the vast majority of children diagnosed with OSA, the initial treatment approach is the surgical removal of enlarged tonsils and adenoids. Although 50-115 of every 10,000 children undergo tonsillectomy and adenoidectomy (9), the overall efficacy of this procedure has not been firmly established, with recent data suggesting that the success rate is significantly less than previously reported (10). Furthermore, this procedure is associated with pain, other morbidities, and substantial healthcare costs. Therefore, identification of a set of genes orchestrating the proliferation of adenotonsillar tissue in the upper airway of children with OSA may allow for development of novel, cost-effective, nonsurgical therapeutic strategies.

In this study, we integrated genome-wide expression analysis with novel bioinformatics methods to identify putative functional networks involved in the proliferation of tonsillar tissue in the upper airway of children with OSA (Fig. 1). These analyses were conducted on tonsils from children with polysomnographically demonstrated OSA and from age-, gender-, and ethnicity-matched children without OSA who underwent tonsillectomy for recurrent tonsillar infections. We developed a computational framework to systematically narrow down differentially expressed genes to a restricted set derived from a network of proliferative pathways. To further validate this approach, the effect of antagonists directed against one of the network-associated candidate genes, phosphoserine phosphatase (PSPH), was assessed in a mixed tonsil cell culture system. We confirmed the pro-proliferative role played by PSPH in tonsillar tissue from children with OSA, but not in those derived from children with recurrent infections.

METHODS

Please see **online data supplement Methods** for more details.

Patients

The study was approved by the University of Louisville Human Research Committee, and informed consent was obtained from the legal caregiver of each participant, with assent being obtained from children older than 7 years of age. Consecutive prepubertal non-obese children diagnosed with OSA at the University of Louisville Pediatric Sleep Research Center in Louisville, KY, were invited to participate. Inclusion criteria were the presence of OSA according to polysomnographic criteria and age between 6 and 11 years. Exclusion criteria were any children with chronic medical condition, receiving medications, and any genetic or craniofacial syndromes.

Children with recurrent tonsillar infection (RI) were selected based on a history of at least five tonsillar infections requiring administration of antibiotic courses over a period of less than 6 months, as well as the absence of sleep-disordered breathing as assessed by an overnight sleep study. Subject characteristics are provided in Table 1.

Polysomnographic assessment

Overnight polysomnography was performed using standard measuring procedures. The diagnostic criteria for OSA included an obstructive apnea index >1 per hour of total sleep time and/or an obstructive apnea-hypopnea index >5 per hour of total sleep time with a nadir oxygen saturation value <92% (11).

Statistical analysis

Results are presented as mean ± SD unless stated otherwise. Comparisons according to group assignment were made with independent *t* tests or analysis of variance followed by *post hoc* comparisons, with *P*-values adjusted for unequal variances when appropriate (Levene test for equality of variances), or χ^2 analyses with Fisher's exact test (dichotomous outcomes). A two-tailed *P*-value < 0.05 was considered statistically significant.

Tonsillar tissue collection

Since tonsil cannot be obtained from normal children for obvious ethical reasons, consecutive children undergoing tonsillectomy at Kosair Children's Hospital for either OSA or RI were identified before surgery and recruited to the study. OSA and RI children were also required to have received their last dose of antibiotic therapy at least 6 wk before the day of the surgery. Children with OSA were excluded if they suffered from RI. Tonsils were removed by a pediatric ENT specialist, and a portion of each tonsil was stabilized in RNALater (Applied Biosytems, Woodward St. Austin, TX) and stored at –80°C.

RNA isolation and microarray hybridization

Total RNA from tonsils of 18 children with RI and 18 children with OSA (their clinical characteristics are shown in Table E1 in Supplement) was isolated using RNeasy Lipid Tissue Mini Kit with DNase treatment (Qiagen, Valencia, CA), and hybridized to Agilent human whole-genome arrays containing 44,000 transcripts as previously described (14).

Identification of enriched pathways in tonsillar tissue

Gene expression intensities of all 36 subjects (OSA and RI), whose clinical characteristics are shown in Table E1 in Supplement, were normalized and log-transformed. Enriched pathways in tonsillar tissue from children with OSA were identified using gene set enrichment analysis (15) at a false discovery rate cutoff ≤ 10%.

Gene interaction network analysis

Genes mapped to gene sets enriched in OSA subjects and involved in proliferative pathways were combined, and an interaction network was created using Ingenuity's knowledge base (16) and several publicly available gene product relationship databases (17-19). The topological characteristics of the interactome, i.e., the number of nodes and connectivity, were extracted.

Ranking of network-associated genes based on their Significance Score

A score based on the connectivity and the differential expression levels of the interactome's nodes was developed to identify the genes most likely to be important in tonsillar hypertrophy.

$$\text{Significance Score} = \text{Ln}[\text{Connectivity}] - \text{Ln}[P\text{-value}]$$

Where the *P*-value was based on a gene-by-gene inter-group comparison using the parametric *t*-test (two-sided, unequal variance). Statistical cutoff values were determined by performing a random permutation analysis on the subjects and obtaining a null frequency distribution. Genes with scores above the 95^{th} percentile of the null distribution were deemed significant.

Tonsil Immunohistochemistry

Tonsils were fixed overnight and sectioned on a freezing microtome. Coronal sections incubated with primary PSPH antisera (Abcam cta# ab58125; 1:1000) followed by exposure to biotinylated anti-rabbit antibody and incubation with streptavidin-horseradish peroxidase. Sections were prepared from 5 sets of tonsils and of adenoids from either OSA or RI groups, and visualized using a fluorescent microscope by an investigator blinded to the sample source.

Mixed adenotonsillar primary cell culture system

Surgically removed tonsils and adenoids were used for isolation of cells and establishment of a mixed cell culture as previously described (12). Cells were incubated to evaluate basal proliferation or treated with PSPH inhibitors such as okadaic acid, Calyculin A, and PPI2 at 10^{-6} to 10^{-9} M concentrations.

BrdU cell proliferation and apoptosis annexin V assays with flow cytometry

To detect global cell, T-cell and B-cell specific proliferation, we employed bromodeoxyuridine (BrdU) pulsed proliferation analysis using flow cytometry (13). A similar approach was undertaken using Annexin V mouse anti-human antibodies to quantify global, T-cell or B-cell specific apoptosis) (13). All data are expressed as the percentage of positive cell from the total cell population.

RESULTS

OSA activates distinct biological processes in tonsillar tissue of children

An overview of our approach is depicted in Fig. 1. We applied gene set enrichment analysis (15) to the gene expression profiles in tonsils of children with OSA (n = 18) and RI (n = 18). Out of approximately 1800 curated pathways sampled, 22 were enriched in the OSA phenotype at a false discovery rate ≤ 10%, whereas no gene sets were enriched in the RI group. The gene sets associated with OSA were functionally organized into five broad categories: proliferation, hypoxia, glutathione metabolism, cytochrome P450 activity, and fatty acid/steroid biosynthesis (supplementary Fig. E1). The proliferation module contained the largest number of member genes (n=564), and given its potential biological relevance to tonsillar hypertrophy, became the focus of subsequent analyses. Members of this module were linked together based on previously reported gene product interactions, resulting in a complex network comprised of 361 nodes and 2476 edges. Like many biological networks (20), the tonsillar hypertrophy "interactome" followed scale-free topological characteristics (supplementary Figs. E2, E3, and E4).

An integrative scoring strategy identifies putative candidate genes mediating tonsillar hypertrophy in OSA

Using the connectivity matrix of the proliferation interactome and the differential expression of its nodes, we developed a significance scoring metric that ranked the gene members (score range: 0.01–11.35). A random permutation analysis was then used to determine a 95% significance cut-off value of 4.87, resulting in the selection of 69 genes (supplementary Table E2, Fig. 2). Many of these gene candidates were involved in inflammation signaling (e.g., IL1B,

IL1A, IL1F6, IL6, CCL19) and regulation (e.g., JUNB, FOS), and tissue growth and remodeling (e.g., TGFB1, TGFB2, HBEGF, CTGF, FN1). We have recently demonstrated that adenotonsillar tissue from children with sleep apnea express pro-inflammatory cytokines and is in a proliferative state (21). Several members of the carcinoembryonic gene family were among the top-ranked hits in our analysis, including CEACAM1, CEACAM5, and CEACAM6. These molecules are critical mediators of cell-cell adhesion and determinants of tissue architecture, aberrant growth, and hypertrophy (22-24). Two protein phosphatases, dual specificity phosphatase 1 (DUSP1) and phosphoserine phosphatase (PSPH), were among the statistically significant network genes (score: 7.24, 5.92 respectively). Since little is known about the role of protein phosphatases in promoting tonsillar hypertrophy, we proceeded to investigate whether PSPH and DUSP1 represented novel therapeutic targets.

Selective inhibition of phosphoserine phosphatase is anti-proliferative

We initially determined that PSPH protein expression seemed to localize primarily to the germinal centers within the tonsil structure using immunohistochemistry, and was qualitatively more abundant in children with OSA than those with RI (Fig. 3). Next, we treated mixed cellular tonsil and adenoid cultures (12) with the phosphatase inhibitors okadaic acid, calyculin A, and protein phosphatase inhibitor 2 (PPI2) at increasing concentrations. Exposure to okadaic acid (which inhibits both PSPH and DUSP1) and calyculin A (a DUSP and protein phosphatase 1 and 2A inhibitor) reduced the proliferation of tonsillar/adenoid cell cultures harvested from children with OSA; however, this anti-proliferative effect was not seen with PPI2 (a preferential protein phosphatase 2B antagonist; n=6 for all experiments) (Fig. 4). Much milder and non-significant effects were seen in tonsillar/adenoid cell cultures from children with recurrent infections (n=6

for all experiments). Therefore, these findings confirm that phosphatases orchestrate proliferative signaling pathways in upper airway lymphadenoid tissues of children with obstructive sleep apnea. Our expression profiling-based strategy implies that important mechanisms regulating adenotonsillar proliferation occur at the transcriptional level, and targeted perturbation of gene expression can alter the production and activity of its corresponding protein, reversing the lymphadenoid hypertrophy associated with pediatric OSA.

Inhibition of phosphoserine phosphatase blocks proliferation and promotes programmed cell death

To confirm the anti-proliferative effects of calyculin and the potential induction of apoptosis by this phosphatase inhibitor in the mixed cell adenotonsillar cultures of OSA-derived tonsils, we performed flow cytometric analysis using BRDu and Annexin V staining respectively (13). Exposure of the cell cultures to 100 nM calyculin dramatically reduced cellular proliferation (Fig. 5A, control vs. calyculin A in total cells: 8.87±0.35% vs. 3.06±0.72%, $P < 0.001$; control vs. calyculin A in T-cells: 22.4±1.78% vs. 9.8±0.61%, $P < 0.001$; control vs. calyculin A in B-cells: 7.56±0.56% vs. 1.78±0.57%, $P < 0.001$) and resulted in a significantly higher rate of programmed cell death (Fig. 5B, control vs. calyculin A in total cells: 45.5±2.5% vs. 72.4±3.40%, $P < 0.001$). The selective difference in apoptosis was further characterized by differentiating T-cell (sorted by CD3+) and B-cell subtypes (sorted by CD19+). Treatment with 100 nM calyculin elicited cell death in 85.7 ±2.2% of T-cells compared to control conditions (38.4±2.2%; $P < 0.001$). Similarly, exposure to calyculin resulted in apoptosis among 71.2±4.4% of B-cell lymphocytes compared to 53.7±3.6% in untreated conditions ($P < 0.001$). These data imply that the anti-proliferative effect of targeting phosphoserine phosphatase is mediated, in

part, through induction of apoptosis in adenotonsillar cells. Furthermore, programmed cell death appears to be more prominent in T-cells compared to B-cells.

Quantitative RT-PCR validation of selected candidate genes

Up-regulation of PSPH, DUSP1, CEACAM5, in children with OSA was validated with qRT-PCR in the original cohort of 36 subjects (see online data supplement Table E3).

DISCUSSION

Pediatric sleep apnea is a common disorder primarily caused by enlarged tonsils and adenoids impinging upon the patency of the upper airway during sleep. Mechanisms leading to the proliferation and enlargement of the tonsils and adenoids in children who subsequently develop obstructive sleep apnea remain unknown. Furthermore, the usual treatment for pediatric sleep apnea, i.e., tonsillectomy and adenoidectomy, is costly, and fraught with measurable adverse consequences ranging from mild events such as pain to serious complications such as hemorrhage, infections, acute respiratory insufficiency, and potentially death (25). Therefore, effective non-surgical strategies for the treatment of pediatric sleep apnea are imperatively needed and can dramatically impact clinical practice.

In the present study, we performed genome-wide transcriptional profiling of tonsillar tissues obtained from children with and without OSA and outlined a computational framework to identify novel candidate targets regulating adenotonsillar hypertrophy. We validated our unbiased approach by performing follow-up *in vitro* proliferation assays to demonstrate that selective targeting of two up-regulated candidate genes, PSPH and DUSP1, profoundly reverses the proliferative state of adenotonsillar tissue in OSA. Thus, phosphatases play a critical role in pediatric tonsillar hypertrophy, and mediate the mechanisms leading to this proliferative state selectively in children with OSA, but not with RI.

Phosphoserine phosphatase belongs to a subfamily of phosphotransferases and catalyzes the rate-limiting step in serine biosynthesis by converting L-phosphoserine to L-serine (26). The only reported case of PSPH deficiency in a child described pre- and postnatal growth retardation, moderate psychomotor retardation, facial abnormalities and reduced PSPH activity in

lymphoblasts and fibroblasts (27). Treatment with oral serine led to normalization of serine levels and some improvement in head growth. In mice, PSPH is abundantly expressed in proliferating embryonic and hematopoietic stem cells, and in neural progenitors in the developing brain (28, 29). Furthermore, siRNA knockdown of PSPH expression inhibited neural stem cell proliferation (29) suggesting that this enzyme can be selectively targeted to affect cellular proliferation. To our knowledge, a role for PSPH in promoting tonsillar tissue hypertrophy has not been previously reported. We used several strategies to target PSPH expression and activity in adenotonsillar primary cell cultures, including pharmacological and siRNA inhibition (data not shown), resulting in significant anti-proliferative effects specifically in cell cultures derived from children with OSA. Furthermore, inhibition of PSPH appears to promote programmed cell death in tonsillar cell cultures. Together, these observations suggest that PSPH is a logical therapeutic target in reversing the adenotonsillar enlargement of pediatric OSA.

Dual specificity phosphatases have also been extensively investigated as critical modulators of lymphocyte function, proliferation, and apoptosis. Indeed, immune cell functions are intimately associated with the enzymatically catalyzed addition of phosphate to key tyrosine, threonine and serine residues in proteins (30-32). While the specific role of DUSP1 in tonsillar proliferation remains to be established in the context of pediatric OSA, our preliminary experiments using siRNA also supports a regulatory role for this group of phosphatases in the regulation of proliferation and apoptosis among critical lymphocyte populations within tonsillar tissues.

We have previously demonstrated the effectiveness of nonsurgical approaches for treatment of mild sleep disordered breathing in children, including the use of anti-inflammatory agents

such as oral leukotriene modifiers (33) and intranasal steroids (34). Furthermore, we recently utilized adenotonsillar cell culture assays to confirm that these pharmacologic interventions promote an anti-proliferative phenotype (13, 35). The current study provides a much broader framework for the selection of novel candidate pathways and critical mediators of adenotonsillar hypertrophy in children with OSA. Our computational network analysis identified several other putative targets with well-known roles in tissue remodeling and growth including members of the carcinoembryonic gene family (CEACAM1, CEACAM5, CEACAM6), insulin growth factor binding protein 2 (IGFBP2), lipocalin 2 (LCN2), and connective tissue growth factor (CTGF) as well as many pro-inflammatory mediators (IL1B, IL1A, IL1F6, IL6, CCL19, JUN, FOS). Future work on the molecular mechanisms by which these candidates potentially regulate tonsillar proliferation will help elucidate the pathophysiology of obstructive sleep apnea in children, and may result in the development of combinatorial therapies for this complex disorder.

There are several notable strengths in this work. First, since the data was generated from human tonsillar tissue samples, our findings are likely to be directly relevant to pediatric OSA. Second, in addition to the proliferation module, our unbiased approach identified several other biological modules selectively enriched in children with OSA, including those involved in hypoxia, fatty acid metabolism and glutathione biosynthesis. Previous reports strongly suggest that exposure to intermittent hypoxia promotes the activation of oxidative stress pathways and can result in perturbations in lipid synthesis and metabolism (36). Therefore, a similar integrative network analysis may yield additional biologically relevant targets for treatment of pediatric OSA. Finally, our general computational methodology for target selection is applicable to any transcriptional or proteomic-based data acquisition from human tissue samples.

Our study has a number of limitations. Since tonsils cannot be obtained from normal, asymptomatic children for obvious ethical reasons, we compared enlarged tonsils in children with OSA with tonsils of children without OSA who suffered from a history of recurrent tonsillitis (RI). Although tonsils were removed at least 6 weeks after the last dose of antibiotics in the RI group, significant differences in gene expression may persist between the tonsillar tissue of RI subjects and healthy children. Additionally, intra-operative conditions such as anesthesia and hypoxia may alter gene expression, but such differences would cancel out since both groups of children, namely OSA and RI, underwent similar surgical and anesthetic procedures. The bioinformatics analyses in the present work are limited by our current state of knowledge in biological pathways and gene product relationships. Therefore, future iterations of the same data can yield different results. Furthermore, since our analyses are based on transcriptional signatures, we are unable to assess post-transcriptional effects. Finally, it is important to note that we confirmed the role of only a few selected putative candidate genes in mediating adenotonsillar proliferation. Future work is necessary to establish the biological relevance of the other candidates.

Despite its prevalence and associated morbidity, little is known about the pathophysiologic mechanisms leading to sleep apnea in children. Since adenotonsillar enlargement is the primary cause of pediatric OSA, our human-based approach provides a paradigm for systematically identifying targets for pharmacological reversal of adenotonsillar proliferation and avoiding surgical extirpation. Applying global gene expression analysis, bioinformatics and network-based methods to clinically relevant phenotypes is a promising strategy for discovering novel therapeutic sites in complex human disorders.

REFERENCES

1. Lumeng JC, Chervin RD. Epidemiology of pediatric obstructive sleep apnea. *Proc Am Thorac Soc* 2008;5:242-252.
2. Capdevila OS, Kheirandish-Gozal L, Dayyat E, Gozal D. Pediatric obstructive sleep apnea: Complications, management, and long-term outcomes. *Proc Am Thorac Soc* 2008;5:274-282.
3. Muzumdar H, Arens R. Diagnostic issues in pediatric obstructive sleep apnea. *Proc Am Thorac Soc* 2008;5:263-273.
4. Arens R, McDonough JM, Corbin AM, Rubin NK, Carroll ME, Pack AI, Liu J, Udupa JK. Upper airway size analysis by magnetic resonance imaging of children with obstructive sleep apnea syndrome. *Am J Respir Crit Care Med* 2003;167:65-70.
5. Katz ES, D'Ambrosio CM. Pathophysiology of pediatric obstructive sleep apnea. *Proc Am Thorac Soc* 2008;5:253-262.
6. Kaditis AG, Finder J, Alexopoulos EI, Starantzis K, Tanou K, Gampeta S, Agorogiannis E, Christodoulou S, Pantazidou A, Gourgoulianis K, et al. Sleep-disordered breathing in 3,680 greek children. *Pediatr Pulmonol* 2004;37:499-509.
7. Teculescu DB, Caillier I, Perrin P, Rebstock E, Rauch A. Snoring in french preschool children. *Pediatr Pulmonol* 1992;13:239-244.
8. Ersu R, Arman AR, Save D, Karadag B, Karakoc F, Berkem M, Dagli E. Prevalence of snoring and symptoms of sleep-disordered breathing in primary school children in istanbul. *Chest* 2004;126:19-24.
9. Van Den Akker EH, Hoes AW, Burton MJ, Schilder AG. Large international differences in (adeno)tonsillectomy rates. *Clin Otolaryngol Allied Sci* 2004;29:161-164.
10. Tauman R, Gulliver TE, Krishna J, Montgomery-Downs HE, O'Brien LM, Ivanenko A, Gozal D. Persistence of obstructive sleep apnea syndrome in children after adenotonsillectomy. *J Pediatr* 2006;149:803-808.
11. Montgomery-Downs HE, O'Brien LM, Gulliver TE, Gozal D. Polysomnographic characteristics in normal preschool and early school-aged children. *Pediatrics* 2006;117:741-753.
12. Serpero LD, Kheirandish-Gozal L, Dayyat E, Goldman JL, Kim J, Gozal D. A mixed cell culture model for assessment of proliferation in tonsillar tissues from children with obstructive sleep apnea or recurrent tonsillitis. *Laryngoscope* 2009;119:1005-1010.
13. Kheirandish-Gozal L, Serpero LD, Dayyat E, Kim J, Goldman JL, Snow A, Bhattacharjee R, Gozal D. Corticosteroids suppress in vitro tonsillar proliferation in children with obstructive sleep apnoea. *Eur Respir J* 2009;33:1077-1084.
14. Khalyfa A, Capdevila OS, Buazza MO, Serpero LD, Kheirandish-Gozal L, Gozal D. Genome-wide gene expression profiling in children with non-obese obstructive sleep apnea. *Sleep Med* 2009;10:75-86.
15. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005;102:15545-15550.
16. Calvano SE, Xiao W, Richards DR, Felciano RM, Baker HV, Cho RJ, Chen RO, Brownstein BH, Cobb JP, Tschoeke SK, et al. A network-based analysis of systemic inflammation in humans. *Nature* 2005;437:1032-1037.
17. Alfarano C, Andrade CE, Anthony K, Bahroos N, Bajec M, Bantoft K, Betel D, Bobechko B, Boutilier K, Burgess E, et al. The biomolecular interaction network database and related tools 2005 update. *Nucleic Acids Res* 2005;33:D418-424.
18. Peri S, Navarro JD, Kristiansen TZ, Amanchy R, Surendranath V, Muthusamy B, Gandhi TK, Chandrika KN, Deshpande N, Suresh S, et al. Human protein reference database as a discovery resource for proteomics. *Nucleic Acids Res* 2004;32:D497-501.
19. Salwinski L, Miller CS, Smith AJ, Pettit FK, Bowie JU, Eisenberg D. The database of interacting proteins: 2004 update. *Nucleic Acids Res* 2004;32:D449-451.

20. Barabasi AL, Oltvai ZN. Network biology: Understanding the cell's functional organization. *Nat Rev Genet* 2004;5:101-113.
21. Kim J, Bhattacharjee R, Dayyat E, Snow AB, Kheirandish-Gozal L, Goldman JL, Li RC, Serpero LD, Clair HB, Gozal D. Increased cellular proliferation and inflammatory cytokines in tonsils derived from children with obstructive sleep apnea. *Pediatr Res* 2009.
22. Abou-Rjaily GA, Lee SJ, May D, Al-Share QY, Deangelis AM, Ruch RJ, Neumaier M, Kalthoff H, Lin SH, Najjar SM. Ceacam1 modulates epidermal growth factor receptor--mediated cell proliferation. *J Clin Invest* 2004;114:944-952.
23. Benchimol S, Fuks A, Jothy S, Beauchemin N, Shirota K, Stanners CP. Carcinoembryonic antigen, a human tumor marker, functions as an intercellular adhesion molecule. *Cell* 1989;57:327-334.
24. Kuespert K, Pils S, Hauck CR. Ceacams: Their role in physiology and pathophysiology. *Curr Opin Cell Biol* 2006;18:565-571.
25. Ramsden R. National prospectivetonsillectomy audit: Final report of an audit carried out in england and northern ireland between july 2003 and september 2004. 2005:1-52.
26. Cohen P. The structure and regulation of protein phosphatases. *Annu Rev Biochem* 1989;58:453-508.
27. Jaeken J, Detheux M, Fryns JP, Collet JF, Alliet P, Van Schaftingen E. Phosphoserine phosphatase deficiency in a patient with williams syndrome. *J Med Genet* 1997;34:594-596.
28. Geschwind DH, Ou J, Easterday MC, Dougherty JD, Jackson RL, Chen Z, Antoine H, Terskikh A, Weissman IL, Nelson SF, et al. A genetic analysis of neural progenitor differentiation. *Neuron* 2001;29:325-339.
29. Nakano I, Dougherty JD, Kim K, Klement I, Geschwind DH, Kornblum HI. Phosphoserine phosphatase is expressed in the neural stem cell niche and regulates neural stem and progenitor cell proliferation. *Stem Cells* 2007;25:1975-1984.
30. Liu JO. Calmodulin-dependent phosphatase, kinases, and transcriptional corepressors involved in t-cell activation. *Immunol Rev* 2009;228:184-198.
31. Tanzola MB, Kersh GJ. The dual specificity phosphatase transcriptome of the murine thymus. *Mol Immunol* 2006;43:754-762.
32. Won J, Lee GH. T-cell-targeted signaling inhibitors. *Int Rev Immunol* 2008;27:19-41.
33. Goldbart AD, Goldman JL, Veling MC, Gozal D. Leukotriene modifier therapy for mild sleep-disordered breathing in children. *Am J Respir Crit Care Med* 2005;172:364-370.
34. Kheirandish L, Goldbart AD, Gozal D. Intranasal steroids and oral leukotriene modifier therapy in residual sleep-disordered breathing after tonsillectomy and adenoidectomy in children. *Pediatrics* 2006;117:e61-66.
35. Dayyat E, Serpero LD, Kheirandish-Gozal L, Goldman JL, Snow A, Bhattacharjee R, Gozal D. Leukotriene pathways and in vitro adenotonsillar cell proliferation in children with obstructive sleep apnea. *Chest* 2009;135:1142-1149.
36. Gozal D, Capdevila OS, Kheirandish-Gozal L. Metabolic alterations and systemic inflammation in obstructive sleep apnea among nonobese and obese prepubertal children. *Am J Respir Crit Care Med* 2008;177:1142-1149.

FIGURE LEGENDS

Figure 1. Schematic overview of the experimental approach for identifying and confirming candidate targets in adenotonsillar hypertrophy of pediatric OSA.

Figure 2. Gene product interaction network in tonsillar proliferation of OSA. Candidate genes reaching significance based on our scoring algorithm (cutoff > 4.86) are highlighted (red: upregulated relative to RI, green: downregulated relative to RI). PSPH and DUSP1 have been labeled. See text for details.

Figure 3. Immunohistochemical staining for PSPH of palatine tonsils from a child with OSA (A panels) and a matched child with RI (B panels). In OSA children, PSPH preferentially localizes to germinal centers, where it is less abundant in children with RI.

Figure 4. Proliferative rates in tonsil cell cultures from children with OSA following administration of okadaic acid, calyculin A, and PPI2 at increasing concentrations. In RI-derived tonsil cell cultures, no significant differences emerged for any of the compounds.

Figure 5. A) Representative example of BrDU-based proliferation assay in tonsil cell cultures from a 6-year-old child with OSA treated with vehicle (left panel) or calyculin (right panel). Marked reductions in global cell proliferation, and in T-cell and B-cell proliferation are apparent. **B)** Representative example of Annexin V-based apoptosis assay in a 4-year old child with OSA treated with vehicle (left panel) or calyculin (right panel). Marked increases in global cellular apoptosis, and in T-cell and B-cell apoptosis are apparent.

Table 1. Demographic and polysomnographic characteristics of children with obstructive sleep apnea (OSA) and recurrent tonsillitis (RI).

	OSA (n=44)	**RI (n=44)**
Age (years)	6.0±1.6	5.5±2.3
Gender (F/M)	20/24	20/24
African American (n)	14	14
BMI z score	0.76±0.07	0.54±0.06
Total Sleep Time (TST; min)	487.5±9.4	482.9±8.3
SWS (%TST)	27.5±2.8	31.3±3.6
REM Sleep (%TST)	19.5±3.1	23.2±2.8
AHI (/hrTST)*	12.7±2.9	0.4±0.1
Nadir SaO2 (%)*	83.5±3.4	95.7±0.3
Arousal Index (/hrTST)*	17.4±3.7	7.7±0.5

All data are expressed as mean±SD; data for the 18 initial subjects in each group and those for subsequent gene validation and proliferation studies were compared and found to be similar, and were therefore merged. *$P < 0.001$

Figure 1.

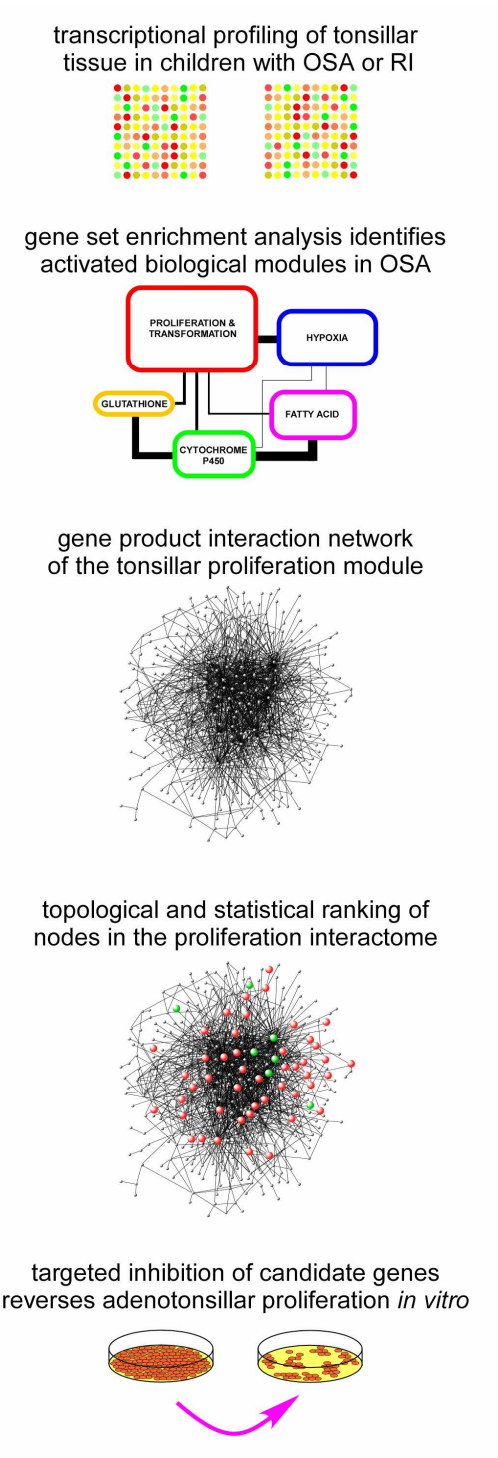

transcriptional profiling of tonsillar tissue in children with OSA or RI
gene set enrichment analysis identifies activated biological modules in OSA
PROLIFERATION & TRANSFORMATION
HYPOXIA
GLUTATHIONE
FATTY ACID
CYTOCHROME P450
gene product interaction network of the tonsillar proliferation module
topological and statistical ranking of nodes in the proliferation interactome
targeted inhibition of candidate genes reverses adenotonsillar proliferation *in vitro*

Figure 2.

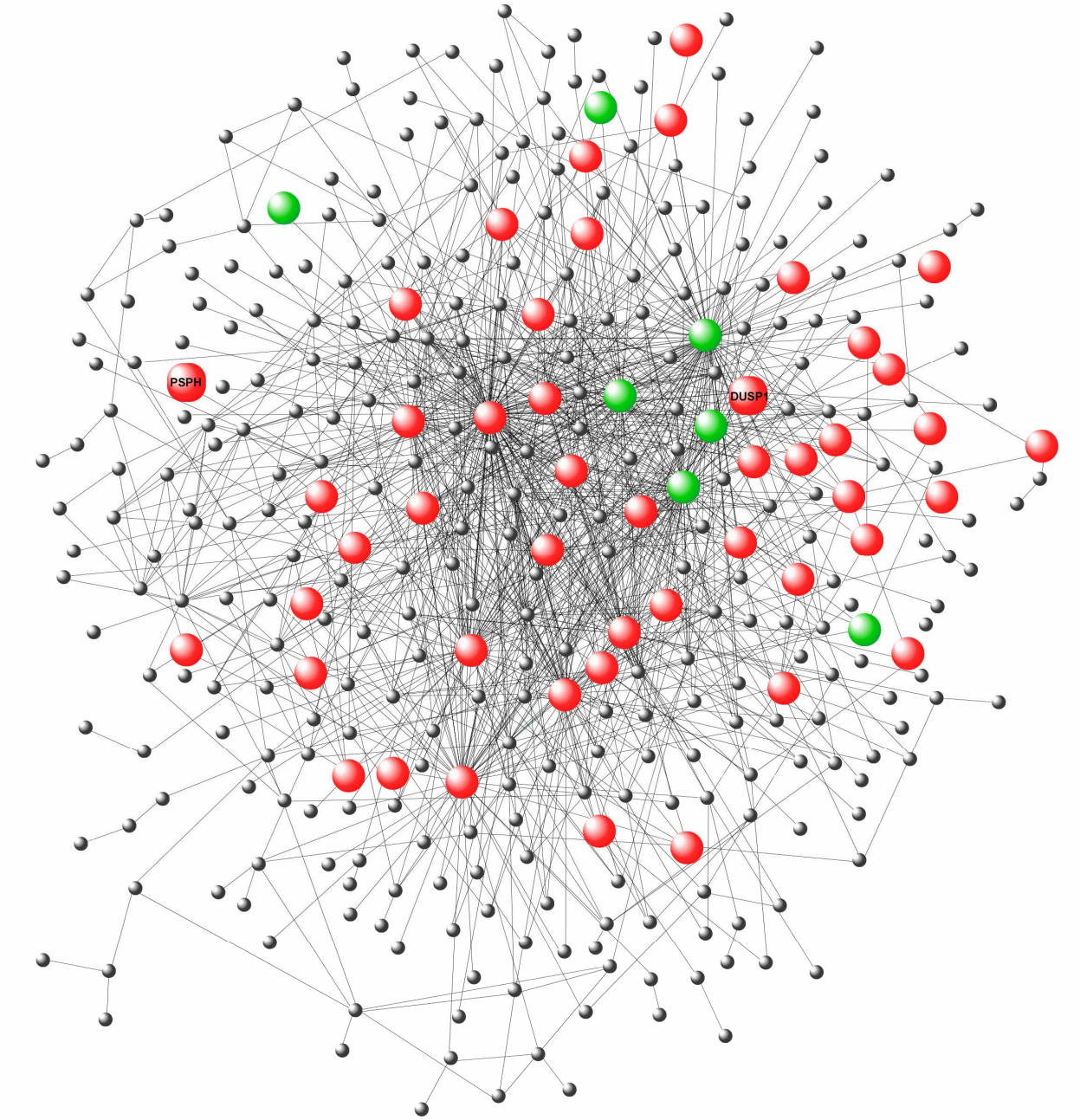


Figure 3.

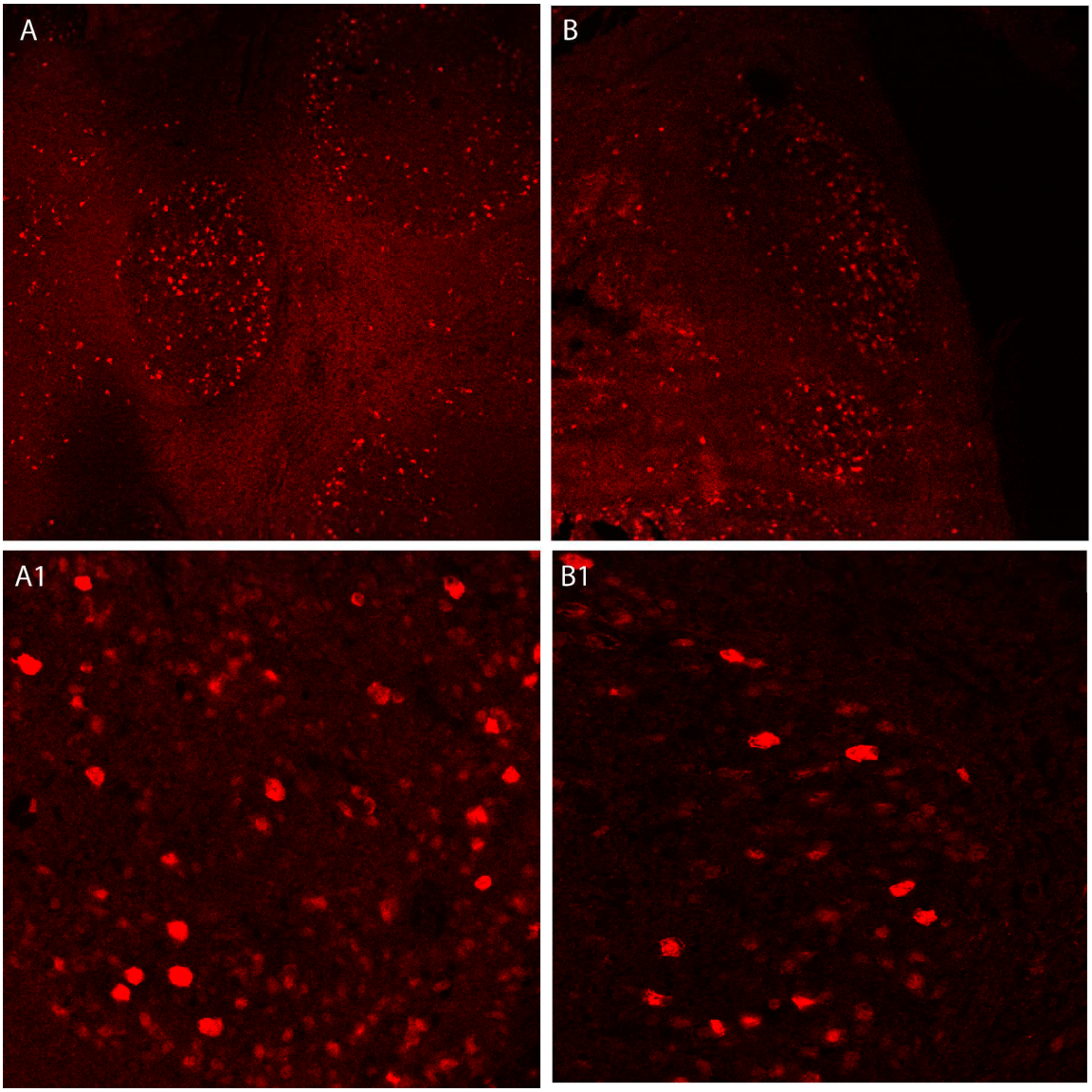
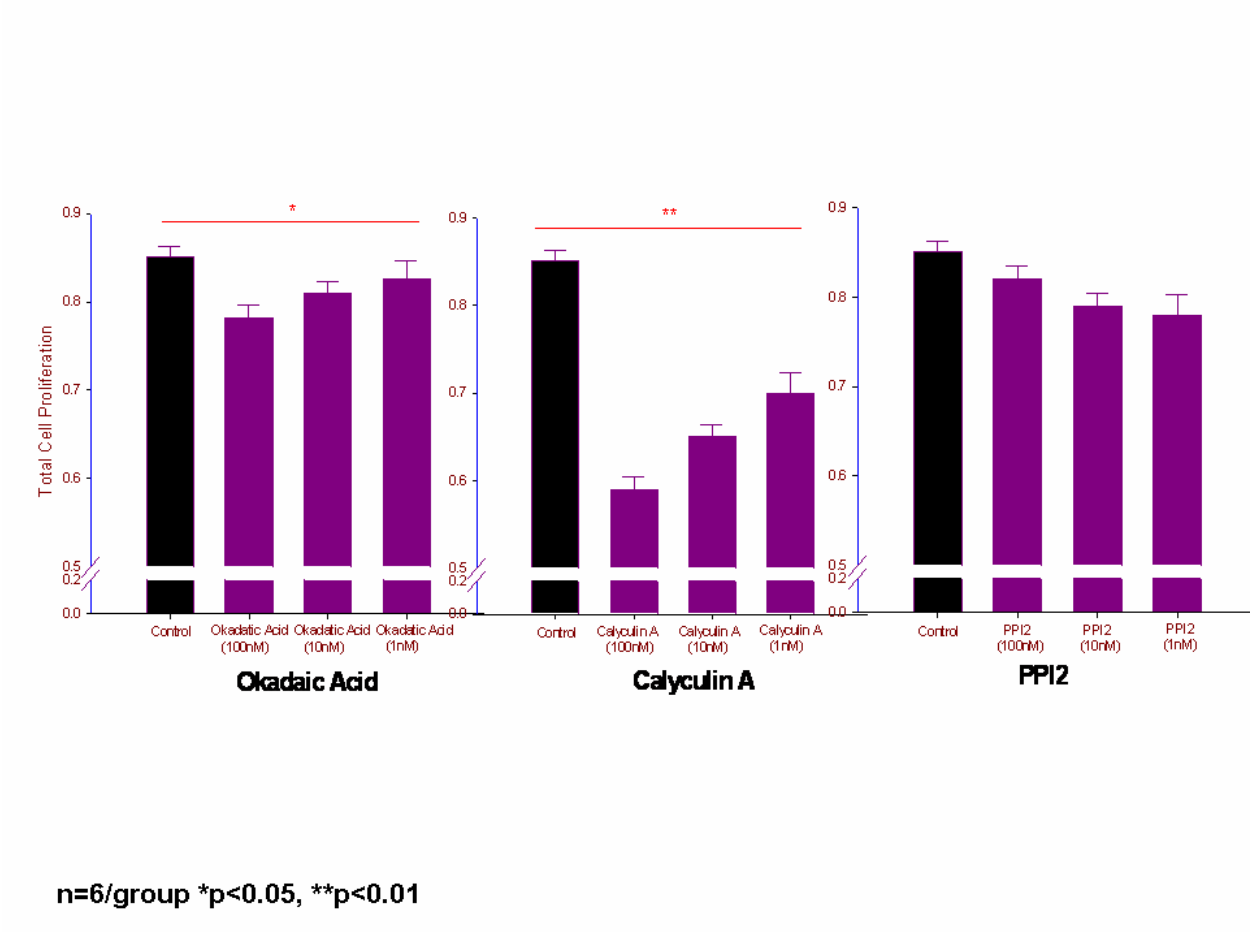
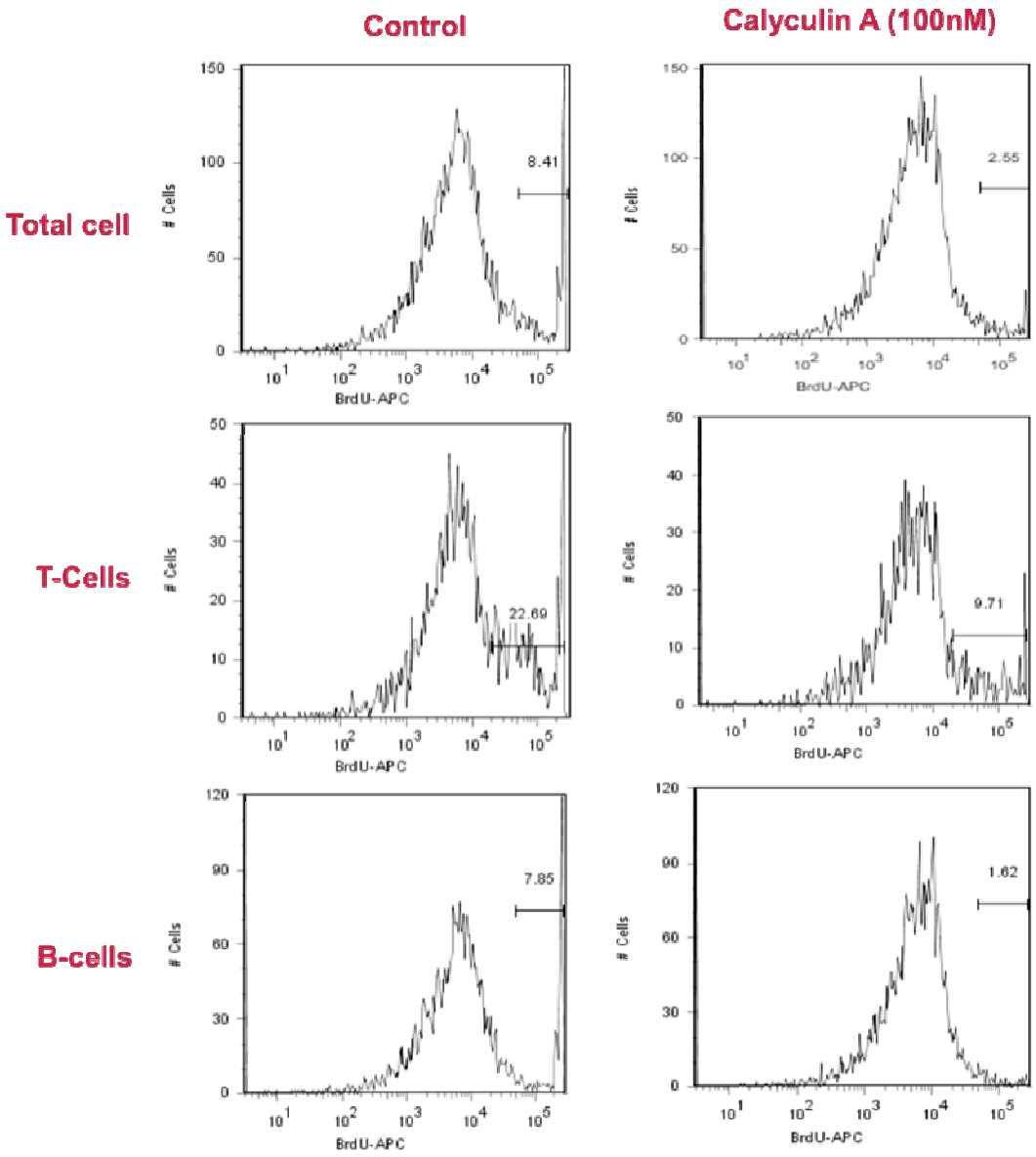


Figure 4.



n=6/group *p<0.05, **p<0.01

Figure 5A.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Total cell
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B-cells
Cells
BrdU-APC
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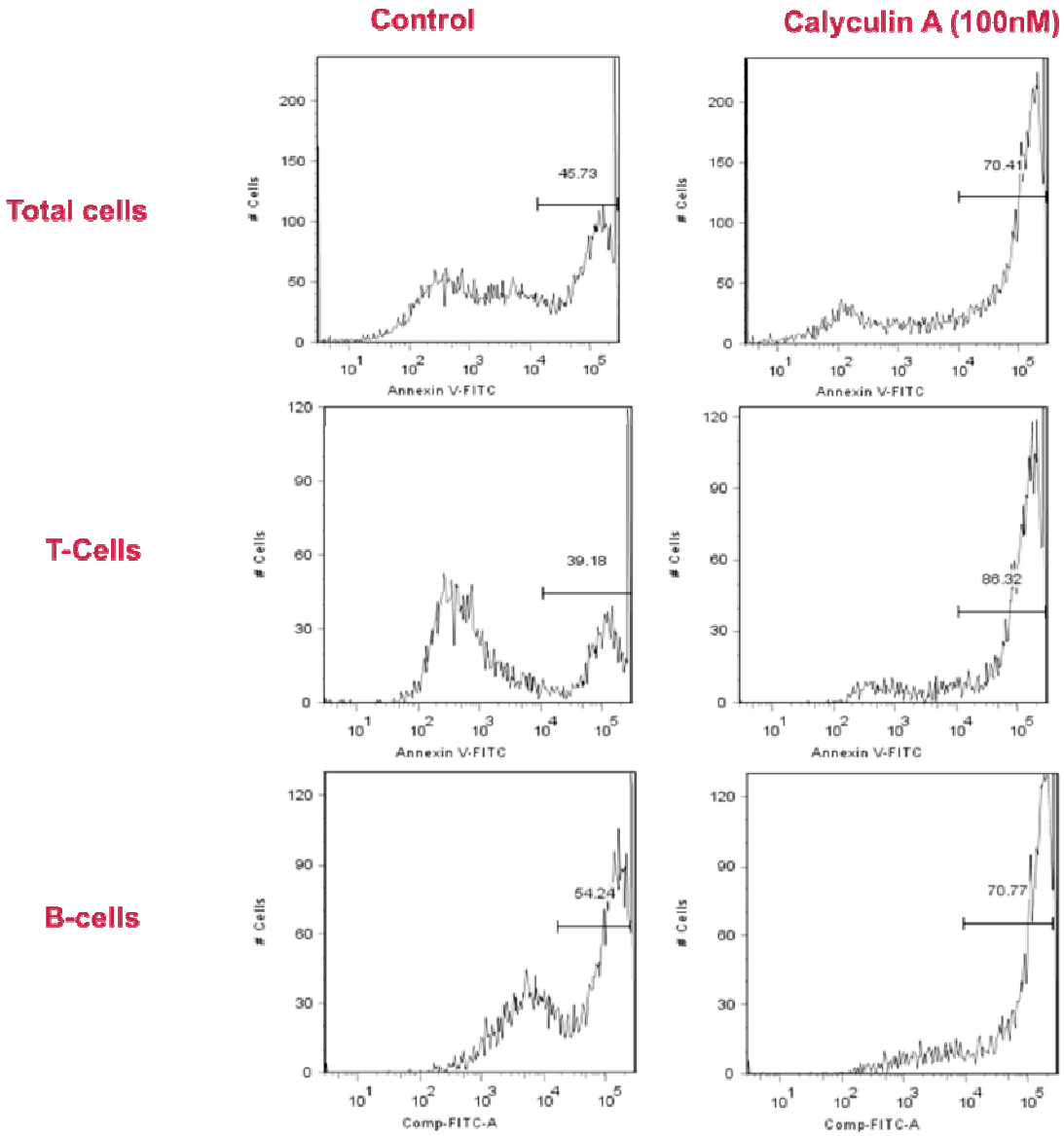
2.55
22.69
9.71
7.85
1.62

Figure 5B.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B-cells
45.73
70.41
39.18
86.32
54.24
70.77
Cells
Annexin V-FITC
Comp-FITC-A

Transcriptomic Analysis Identifies Phosphatases as Novel Targets for Adenotonsillar Hypertrophy of Pediatric OSA

Abdelnaby Khalyfa, Sina A. Gharib, Jinkwan Kim, Ehab Dayyat, Ayelet B. Snow, Rakesh Bhattacharjee, Leila Kheirandish-Gozal, Julie L. Goldman, David Gozal

Online Data Supplement

SUPPLEMENTARY METHODS

Patients

The study was approved by the University of Louisville Human Research Committee, and informed consent was obtained from the legal caregiver of each participant, with assent being obtained from children older than 7 years of age. Consecutive prepubertal non-obese children diagnosed with OSA at the University of Louisville Pediatric Sleep Research Center in Louisville, KY, invited to participate. Inclusion criteria were the presence of OSA according to polysomnographic criteria and age between 6 and 11 years. Exclusion criteria were any children with chronic medical condition, receiving medications, and any genetic or craniofacial syndromes. Age, gender, and ethnicity; use of medications, antihistamines, and presence of co-morbidities were gathered from each participant.

Children with recurrent tonsillar infection (RI) were selected based on a history of at least five tonsillar infections requiring administration of antibiotic courses over a period of less than 6 months, as well as the absence of any symptoms suggestive of OSA using a previously validated questionnaire (1). Patients referred for RI were selected based on a history of at least five tonsillar infections in < 6 months and the absence of any symptoms suggestive of OSA using a previously validated questionnaire (2). However, to further ascertain the absence of sleep-disordered breathing they were evaluated by an overnight sleep study.

Polysomnographic assessment

Children were studied for up to 12 hours in a quiet, darkened room with an ambient temperature of 24°C in the company of one of their parents. No drugs were used to induce sleep. The following parameters were measured during the overnight sleep recordings: chest and abdominal wall movement by respiratory impedance or inductance plethysmography; heart rate by ECG; and air flow, which was triply monitored with a side-stream end-tidal capnograph that also provided breath-by-breath assessment of end-tidal carbon dioxide levels (PET_{CO_2}; BCI SC-300, Menomonee Falls, Wis), a nasal pressure cannula, and an oronasal thermistor. Arterial oxygen saturation (Sp_{O_2}) was assessed by pulse oximetry (Nellcor N 100; Nellcor Inc, Hayward, CA), with simultaneous recording of the pulse waveform. The bilateral electro-oculogram, 8 channels of electroencephalogram, chin and anterior tibial electromyograms, and analog output from a body-position sensor (Braebon Medical Corp, Ogdensburg, NY) were also monitored. All measures were digitized with a commercially available polysomnography system (Rembrandt, MedCare Diagnostics, Amsterdam, Netherlands). Tracheal sound was monitored with a microphone sensor (Sleepmate, Midlothian, VA), and a digital time-synchronized video recording was performed.

The proportion of time spent in each sleep stage was expressed as percentage of total sleep time. Obstructive apnea was defined as the absence of airflow with continued chest wall and abdominal movement for duration of at least 2 breaths (2, 3). Hypopneas were defined as a decrease in oronasal flow of ≥50% with a corresponding decrease in Sp_{O_2} of ≥4% and/or arousal (2). The obstructive apnea/hypopnea index was defined as the number of apneas and hypopneas per hour of total sleep time. The obstructive apnea index was defined as the number of apneas per hour of total sleep time. The diagnostic criteria for OSA included an obstructive apnea index >1 per hour of total sleep time and/or an obstructive apnea-hypopnea index >5 per hour of total sleep time with a nadir oxygen saturation value <92% (2). The diagnosis of OSA was established by

overnight polysomnography in the sleep laboratory and required the presence of an apnea-hypopnea index more than five events per hour of sleep (4).

Body mass index

Height and weight were obtained using standard techniques from each child. BMI was then calculated (body mass/height^2) and was expressed as BMI z-score using an online BMI z score calculator (http://www.cdc.gov/epiinfo/). Children with BMI z-score values exceeding 1.20 were classified as fulfilling the criteria for overweight/obesity (5), and were excluded from this study.

Statistical analysis

Results are presented as mean ± SD unless stated otherwise. All analyses were conducted using statistical software (version 11.5; SPPS; Chicago, IL). Comparisons according to group assignment were made with independent *t* tests or analysis of variance followed by *post hoc* comparisons, with p values adjusted for unequal variances when appropriate (Levene test for equality of variances), or χ^2 analyses with Fisher's exact test (dichotomous outcomes). A two-tailed *P*-value < 0.05 was considered statistically significant.

Tonsillar tissue collection

Since tonsil cannot be obtained from normal children for obvious ethical reasons, consecutive children undergoing tonsillectomy at Kosair Children's Hospital for either OSA or RI were identified before surgery and recruited to the study. OSA and RI children were also required to have received their last dose of antibiotic therapy at least 6 wk before the day of the surgery. Children with OSA were excluded if they suffered from RI (based on aforementioned criteria). Children with known asthma, allergic rhinitis, history of allergies, and/or having received corticosteroid or leukotriene modifier therapy within 12 months from surgery were excluded (for both groups). Tonsils were removed by a pediatric ENT specialist, and a portion of each tonsil was stored in RNALater (Applied Biosytems/Ambion Woodward St. Austin, TX) as recommended by the manufacturer protocol, and stored at -80°C.

Tonsil Immunohistochemistry

Tonsils were placed overnight in a fixative containing 1% paraformaldehyde in PBS and 30% sucrose at 4^{o}C. Post-fixed tissues were sectioned on a freezing microtome. Coronal sections (30 microns) of tonsils were initially incubated in 0.3% H_2O_2 for 30 minutes, washed several times in PBS, and blocked with a PBS/0.4% Triton X-100/0.5%TSA (Tyramide Signal Amplification, Perkin Elmer Life Sciences, Boston, MA) blocking reagent/10% normal goat serum (Vector Laboratories, Burlingame CA) for 1 hour. Sections were then incubated with primary PSPH antisera (Abcam cta# ab58125; 1:1000) at 4 ^{o}C for 24 hours, and then washed in PBS 6 times for 5 minutes each wash. Sections were then incubated at room temperature for 1 hour in biotinylated anti-rabbit antibody (Vectastain Elite ABC kit, Burlingame CA; 1:600) in a PBS/0.5% TSA blocking reagent /10% goat serum solution. After 3 5-min washes, sections were incubated at room temperature with streptavidin-horseradish peroxidase diluted 1:100 in PBS/0.5% TSA blocking reagent. Subsequently, the sections were incubated with tetramethyl rhodamine tyramide (red) diluted 1:50 in amplification diluent (Perkin Elmer Life Sciences , Boston, MA) for 2 minutes. Sections were then washed in PBS, and mounted onto glass slides. Negative controls were prepared by either omitting the primary or the secondary antibody. Sections were prepared from 5 sets of tonsils and of adenoids from either OSA or RI groups, and

were visualized using a fluorescent microscope by an investigator who was blinded to the sample source.

Mixed adenotonsillar primary cell culture system

Surgically removed tonsils and adenoids were placed in ice cold phosphate buffered saline (PBS) plus antibiotics and processing was started within 30 minutes after surgical excision under aseptic conditions. Briefly, tonsils were washed thoroughly with PBS, manually dissected into Petri dishes, and gently grounded with a syringe plunger through a 70 μm mesh screen to obtain a mixed cell suspension through mechanical dissociation. Red blood cells were removed by lysis buffer. Cells viability of all specimens was determined by trypan blue exclusion. Specimens with a viability of less than 70% were discarded. Cells cultures were established in standard medium RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) plus antibiotics, which included streptomycin, fungisone, gentamycin, and penicillin to prevent bacterial and fungal contamination. Mixed cell suspensions were transferred onto 24-well or 96-round bottom-well plates at a concentration of $1x10^6$ cells/well. Cells were cultured in a 5 % CO_2 incubator at 37°C for 48 hours. Cells were incubated to evaluate basal proliferation or treated with PSPH inhibitors such as okadaic acid, Calyculin A, and PPI2 at 10^{-6}M to a10^{-9} concentrations.

BrdU cell proliferation and apoptosis annexin V assays with flow cytometry

To detect global cell, T-cell and B-cell specific proliferation, we employed bromodeoxyuridine (BrdU) pulsed proliferation analysis using flow cytometry. All procedures were measured using the APC BrdU flow kit (BD Biosciences, San Diego, CA) as previously described [13, 18] and as recommended by the manufacturer. In brief, at the end of 48 hours of cell culture in 24-well plates, cells were pulse-labeled with 1 mM BrdU for 4 hours. The cells were then washed with PBS, and BrdU labeled cells were stained with a 3-color antibody combination consisting of mouse anti-human CD45/PerCP Cy7, CD3/PE, and CD19/APC-Cy7 antibodies (BD Biosciences, San Diego, CA) in 50 μl staining buffer for 15 min on ice. Following binding, the cell-surface antibodies, cells were fixed and permeabilized with cytofix/cytoperm buffer. After this procedure, cells were suspended with DNase (300 μg/ml) for 1 hour at 37°C. The anti-BrdU APC antibody was added in perm/wash buffer and incubated for 20 min at room temperature. Isotype controls relevant for each of the antibodies were used to establish background fluorescence. Negative control was used as a sample that was untreated with BrdU and was not stained with specific fluorescence antibodies. Data were acquired on a FACS Aria flow cytometer using the FACS Diva 5.5 software (BD Biosciences, San Diego, CA). After gating of lymphocytes based on CD45+ cells, T-cell and B-cell numbers were calculated as CD3+/CD19- and CD3-/CD19+ cell populations, respectively. Proliferation of T-cells and B-cells was identified by counting CD3+/BrdU+ and CD19+/BrdU+ cell populations. A similar approach was undertaken using Annexin V mouse anti-human antibodies to quantify global, T-cell or B-cell specific apoptosis. The results were displayed as two color dot-plots and analyzed by FlowJo software (Tree Star, San Carlos, CA). All data are expressed as the percentage of positive cell from the total cell population.

RNA isolation and microarray hybridization

Total RNA from tonsils of 18 children with RI and 18 children with OSA was isolated using RNeasy Lipid Tissue Mini Kit with DNase treatment (Qiagen, Valencia, CA). Tissues were homogenized in 1 ml of QIAzol Lysis Reagent (Qiagen, Valencia, CA) using a PolyTron homogenizer. RNA integrity was assessed for each sample using the Agilent 2100 Bioanalyzer

(Agilent Technologies, Palo Alto, CA), cRNA was generated, fluorescently labeled with Cyanine 3-dCTP (Perkin Elmer, Boston, MA), and hybridized to the Agilent human whole-genome arrays containing 44,000 transcripts. Microarrays were scanned (SureScan, Agilent Technologies) followed by image processing and filtering using Agilent Feature Extraction software.

Quantitative RT-PCR

qRT-PCR analysis was performed using ABI PRISM 7500 System (Applied Biosystems, Foster City, CA). The same total RNA was used for both microarray and RT-PCR experiments. cDNA synthesis was performed using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Ribosomal 18S rRNA was used as a reference gene to normalize the expression ratios for the gene of interest. The primer sequences of the genes of interest were designed to be within the same region of the microarray sequence probes. One microgram of total RNA from OSA and RI samples was used to generate cDNA templates and TaqMan® Master Mix Reagent Kit (Applied Biosystems, Foster City, CA) was used to amplify and quantify each transcript of interest in 25 μl reactions. Triplicate PCR reactions were performed in 96-well plates for each gene in parallel with the 18S rRNA. The steps involved in the reaction program included: the initial step of 2 minutes at 50^{o}C; denaturation at 95^{o}C for 10 min, followed by 45 thermal cycles of denaturation (15 seconds at 95^{o}C) and elongation (1 min at 60^{o}C). The expression values will be obtained from the cycle number (Ct value) using the Biosystems analysis software. All the genes of interest and 18S rRNA will be performed in triplicates to determine the Ct-diff. These Ct values will be averaged and the difference between the 18S Ct (Avg) and the gene of interest Ct (Avg) will be calculated (Ct-diff). The relative expression of the gene of interest will be analyzed using the $2^{-\Delta\Delta CT}$ method. Quantitative results were expressed as the mean ±standard deviation (SD). Statistical significance was evaluated by the Student's t-test

Identification of enriched pathways in tonsillar tissue

Initially, the filtered gene expression intensities of all 36 subjects (OSA and RI) were log-transformed and normalized using the quantile method. Intensities of multiple probes mapping to the same gene were averaged, resulting in ~31,000 unique gene expression values. Enriched pathways in tonsillar tissue from children with OSA were identified using gene set enrichment analysis (6) (GSEA). Approximately 1800 gene sets were computationally sampled and a random permutation analysis of the subjects (n = 1000) was performed to determine enrichment of gene sets in each group at a false discovery rate cutoff ≤ 10%.

Gene interaction network analysis

Genes mapped to gene sets enriched in OSA subjects and involved in proliferative pathways were combined, and an interaction network was created using Ingenuity's knowledge base (7) and several publicly available gene product relationship databases (8-10). The interaction network, or interactome, was built around genes with the highest connectivity using an iterative algorithm that systematically connects additional nodes to the initial seed. The topological characteristics of the interactome, i.e., the number of nodes and connectivity, were extracted.

Ranking of network-associated genes based on their Significance Score

A score based on the topologic properties of the interactome and the differential expression levels of its nodes was developed to identify the genes most likely to be important in tonsillar

hypertrophy. There is mounting evidence that the functional integrity of genetic networks is highly dependent on hubs of high connectivity (11, 12). Using the interactome's topology, we rank-ordered its members based on their connectivity. Since this method does not provide information on the relative differential expression of a given gene in the tonsillar tissue of children with OSA compared to RI, an integrated Significance Score was defined as:

$$\text{Significance Score} = \text{Ln}[\text{Connectivity}] - \text{Ln}[P\text{-value}]$$

Where the *P*-value was based on a gene-by-gene inter-group comparison using the parametric *t*-test (two-sided, unequal variance) on log-transformed gene expression values. In general, the greater a gene's connectivity and the more significant its differential expression, the higher its score. However, genes with highly significant expression, even if sparsely connected will receive relatively high scores, as will genes with a modest degree of differential expression but high connectivity within the network. Statistical cutoff values were determined by performing a random permutation analysis on the subjects (n = 10,000) and obtaining a null frequency distribution for the Significance Score. Genes with scores above the 95^{th} percentile of the null distribution were deemed significant (Supplementary Fig. E3).

References

E1. Montgomery-Downs HE, O'Brien LM, Holbrook CR, Gozal D. Snoring and sleep-disordered breathing in young children: Subjective and objective correlates. *Sleep* 2004;27:87-94.

E2. Montgomery-Downs HE, O'Brien LM, Gulliver TE, Gozal D. Polysomnographic characteristics in normal preschool and early school-aged children. *Pediatrics* 2006;117:741-753.

E3. Standards and indications for cardiopulmonary sleep studies in children. American thoracic society. *Am J Respir Crit Care Med* 1996;153:866-878.

E4. Goodwin JL, Kaemingk KL, Fregosi RF, Rosen GM, Morgan WJ, Sherrill DL, Quan SF. Clinical outcomes associated with sleep-disordered breathing in caucasian and hispanic children--the tucson children's assessment of sleep apnea study (tucasa). *Sleep* 2003;26:587-591.

E5. Kuczmarski RJ, Ogden CL, Grummer-Strawn LM, Flegal KM, Guo SS, Wei R, Mei Z, Curtin LR, Roche AF, Johnson CL. Cdc growth charts: United states. *Advance data* 2000:1-27.

E6. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005;102:15545-15550.

E7. Calvano SE, Xiao W, Richards DR, Felciano RM, Baker HV, Cho RJ, Chen RO, Brownstein BH, Cobb JP, Tschoeke SK, et al. A network-based analysis of systemic inflammation in humans. *Nature* 2005;437:1032-1037.

E8. Alfarano C, Andrade CE, Anthony K, Bahroos N, Bajec M, Bantoft K, Betel D, Bobechko B, Boutilier K, Burgess E, et al. The biomolecular interaction network database and related tools 2005 update. *Nucleic Acids Res* 2005;33:D418-424.

E9. Peri S, Navarro JD, Kristiansen TZ, Amanchy R, Surendranath V, Muthusamy B, Gandhi TK, Chandrika KN, Deshpande N, Suresh S, et al. Human protein reference database as a discovery resource for proteomics. *Nucleic Acids Res* 2004;32:D497-501.

E10. Salwinski L, Miller CS, Smith AJ, Pettit FK, Bowie JU, Eisenberg D. The database of interacting proteins: 2004 update. *Nucleic Acids Res* 2004;32:D449-451.

E11. Gharib SA, Liles WC, Klaff LS, Altemeier WA. Noninjurious mechanical ventilation activates a proinflammatory transcriptional program in the lung. *Physiol Genomics* 2009;37:239-248.

E12. Luscombe NM, Babu MM, Yu H, Snyder M, Teichmann SA, Gerstein M. Genomic analysis of regulatory network dynamics reveals large topological changes. *Nature* 2004;431:308-312.

Supplementary Table E1. Demographic and polysomnographic characteristics of children with obstructive sleep apnea (OSA) and recurrent tonsillitis (RI) undergoing T&A who were analyzed using gene arrays.

	OSA (n=18)	**RI (n=18)**
Age (years)	5.9±1.8	5.7±1.9
Gender (F/M)	9/9	9/9
African American (n)	6	6
BMI z score	0.80±0.12	0.74±0.14
Total Sleep Time (TST; min)	491.5±14.7	487.9±16.8
SWS (%TST)	28.1±5.7	30.1±6.2
REM Sleep (%TST)	18.7±6.0	22.9±5.7
AHI (/hrTST)*	13.9±4.1	0.5±0.2
Nadir SaO2 (%)*	82.9±5.1	96.2±0.6
Arousal Index (/hrTST)*	18.1±5.3	7.9±0.9

All data are expressed as mean±SD; *$P < 0.001$

Supplementary Table E2. List of tonsillar proliferation network candidate genes (significance score > 4.86). See main text and supplementary methods for details.

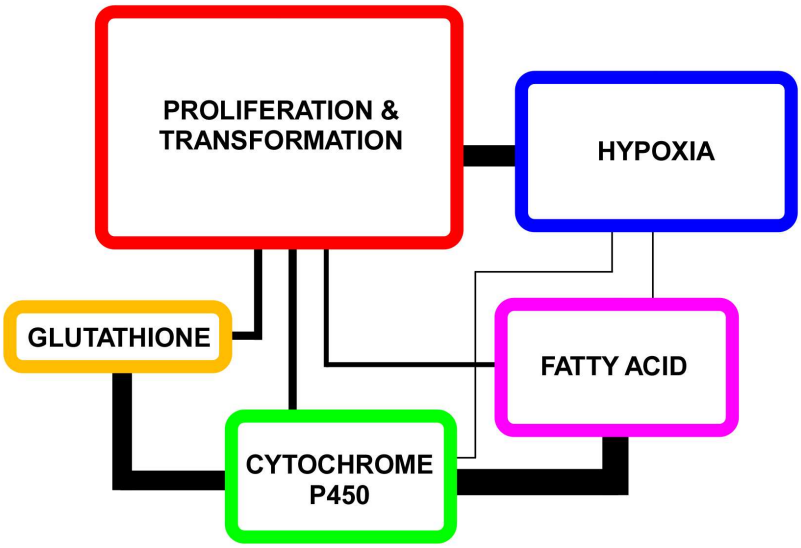
Gene Symbol	Connectivity	P-value	Significance Score
CEACAM5	5	5.89E-05	11.35
CEACAM6	3	1.68E-04	9.79
SERPINB5	12	7.80E-04	9.64
CEACAM1	5	3.28E-04	9.63
LDLR	7	1.51E-03	8.44
IVL	8	1.91E-03	8.34
CD9	7	3.03E-03	7.75
CDKN1A	45	1.99E-02	7.72
IGFBP2	6	3.14E-03	7.55
LCN2	3	1.63E-03	7.52
HBEGF	13	7.33E-03	7.48
DUSP1	**14**	**1.00E-02**	**7.24**
ANXA9	1	7.67E-04	7.17
CCL19	2	1.86E-03	6.98
CTGF	10	9.43E-03	6.97
GJB2	2	2.10E-03	6.86
ITGA2	9	1.03E-02	6.77
ANXA2	15	1.75E-02	6.75
PRSS3	5	6.55E-03	6.64
KLKB1	4	5.60E-03	6.57
CITED2	4	5.76E-03	6.54
ATF3	12	1.80E-02	6.50
PLAT	19	2.91E-02	6.48
PRSS2	3	4.93E-03	6.41
SPRR1A	2	3.77E-03	6.27
SDC1	13	2.73E-02	6.17
PI3	3	6.56E-03	6.13
TGFB1	111	2.48E-01	6.10
LGALS7	3	6.93E-03	6.07
SERPINB1	1	2.38E-03	6.04
SPRR1B	3	7.79E-03	5.95
IL1F6	3	7.86E-03	5.94
CTSB	18	4.74E-02	5.94
PSPH	**1**	**2.68E-03**	**5.92**
DEFB4	4	1.13E-02	5.87
IL1B	100	2.96E-01	5.82
ADM	6	1.85E-02	5.78
KLF5	6	1.94E-02	5.74
KLK7	3	1.03E-02	5.67
KRT18	10	3.77E-02	5.58
ITGA7	1	3.83E-03	5.57
JUNB	26	1.03E-01	5.53
SLPI	10	4.08E-02	5.50
PTGS2	32	1.32E-01	5.49
CCK	4	1.67E-02	5.48
ELF3	6	2.69E-02	5.41
SKP2	9	4.03E-02	5.41
NR4A2	4	1.98E-02	5.31
SPRR2G	1	5.05E-03	5.29
CCND1	47	2.44E-01	5.26
FOS	56	3.03E-01	5.22
TGFB2	10	5.82E-02	5.15
CDCP1	9	5.29E-02	5.14
BCL2	44	2.66E-01	5.11
ATP1B1	6	3.64E-02	5.10
CSTB	5	3.09E-02	5.09
S100A9	12	7.56E-02	5.07
IER2	3	1.96E-02	5.03
HPSE	5	3.35E-02	5.01
IL6	70	4.75E-01	4.99
MXD1	3	2.06E-02	4.98
NDRG1	10	7.09E-02	4.95
SGK	6	4.26E-02	4.95
CD55	2	1.43E-02	4.94
FN1	47	3.37E-01	4.94
S100A10	5	3.61E-02	4.93
HRAS	52	3.78E-01	4.92
IL1A	32	2.35E-01	4.92
PTHLH	12	9.17E-02	4.87

Supplementary Table E3. Microarray genes validated by qRT-PCR.

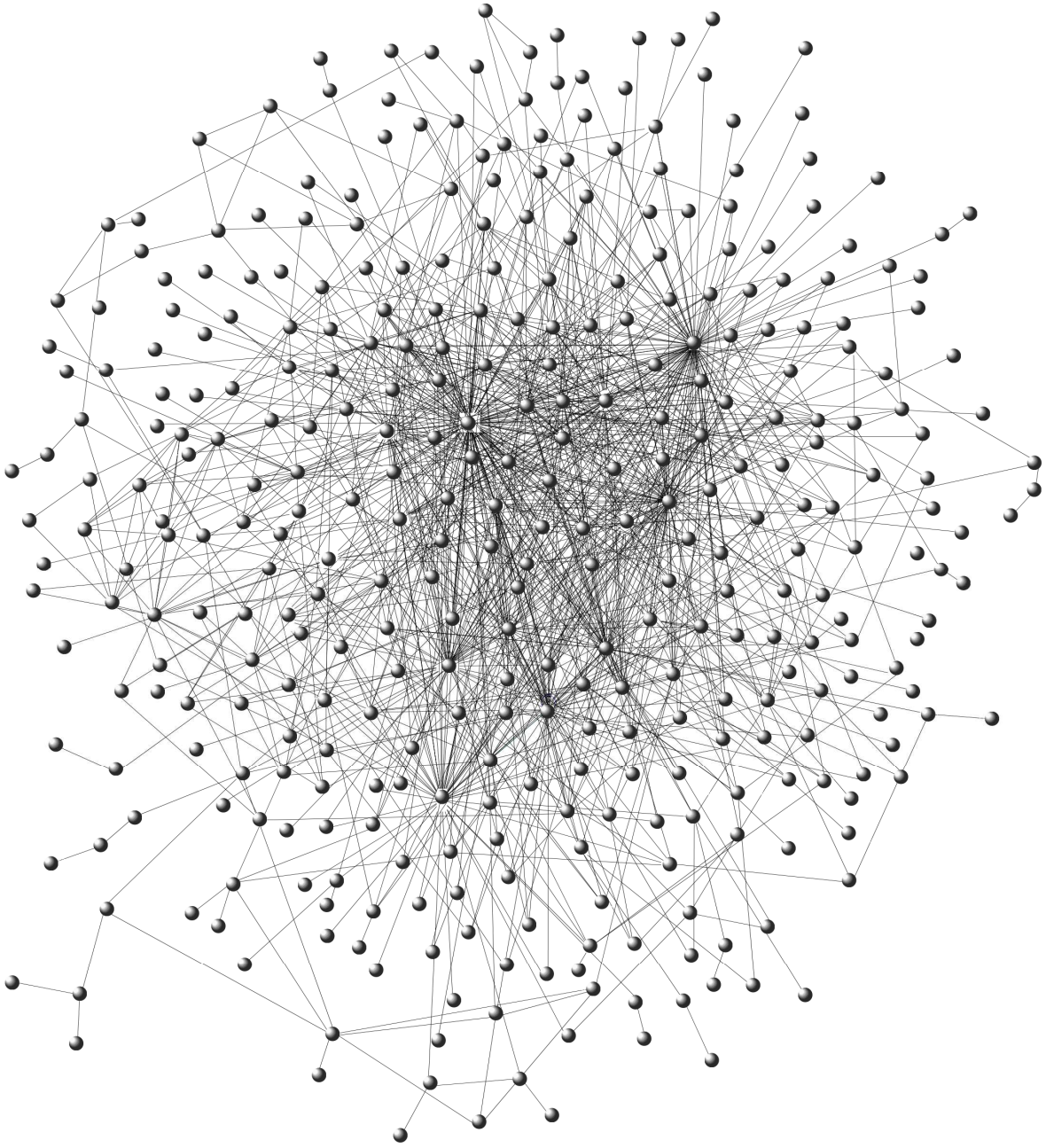
Gene Symbol	Microarray		qRT-PCR	
	Fold change	P-value	Fold change	P-value
CEACAM5	1.58	0.004	2.89	0.006
PSPH	3.36	0.0027	1.53	0.04
DUSP1	2.20	0.01	1.80	0.02

Fold change: OSA/RI; n=18/group.

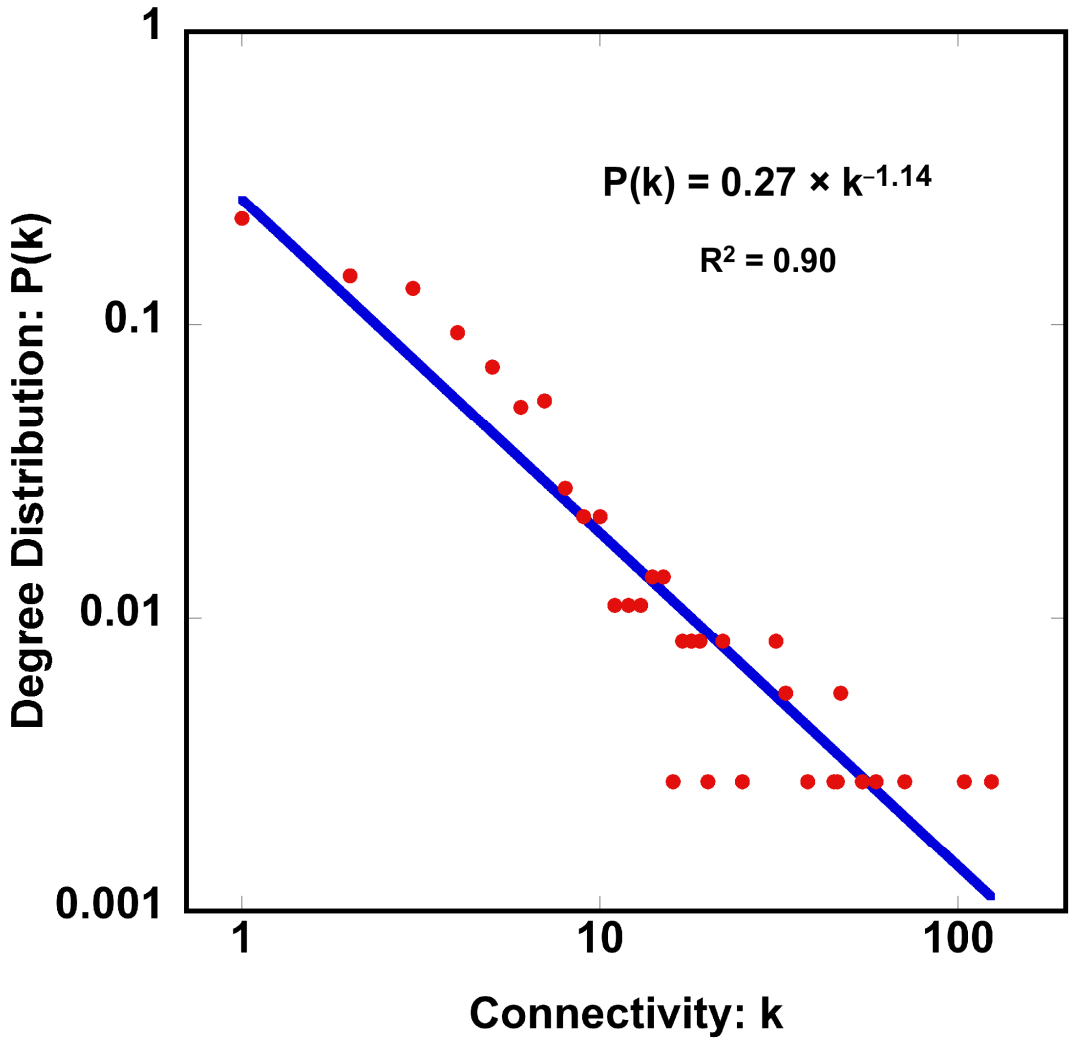
Supplementary Figure E1. Functional categorization of differentially expressed genes in tonsils of children with OSA. Significantly enriched biological modules are displayed using a wiring diagram. The size of each module is proportional to the number of genes members (e.g., n = 564 for proliferation/transformation). The intermodular connections reflect the fact that some genes map to multiple modules, with the thickness of the lines being proportional to the number of shared genes.



Supplementary Figure E2. Gene product interaction network in tonsillar proliferation of OSA. This interactome is comprised of 361 nodes and 2476 edges.



Supplementary Figure E3. A log-log diagram of the tonsillar proliferation interactome's fractional degree distribution, P_k, versus connectivity, k, demonstrates a linear relationship consistent with power-law distribution.



Supplementary Figure E4. The null frequency distribution of the Significance Score applied to the 361 interactome genes and generated from 10,000 random permutations. The 95th percentile cutoff is shaded and corresponds to a significance score of 4.86.