

Caveolar transport through nasal epithelium of birch pollen allergen Bet v 1 in allergic patients

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Background: Previous work in type I pollen allergies has focused on aberrant immunoresponses.

Objective: Our systems-level analyses explore the role of epithelium in early pathogenesis of type I allergic reactions.

Methods: We began top-down analyses of differences in human nasal epithelial cells and biopsy specimens obtained from patients with birch allergy and healthy control subjects in the resting state and after intranasal *in vivo* birch pollen challenges. Immunohistochemistry, immunotransmission electron microscopy, mass spectrometry, transcriptomics, and integration of data to a pathway were conducted.

Results: Bet v 1 allergen bound to epithelium immediately after *in vivo* birch pollen challenge during winter only in allergic individuals. It also travelled through epithelium with caveolae to mast cells. Sixteen unique proteins were found to bind to the Bet v 1 column only in lysates from allergic epithelial cells; 6 of these were caveolar and 6 were cytoskeletal proteins. The nasal epithelial transcriptome analysis from allergic and healthy subjects differed during the winter season, and these subjects also responded differentially to birch pollen challenge. Within this pollen-induced response, the gene ontology categories of cytoskeleton and actin cytoskeleton were decreased in allergic patients, whereas the actin-binding category was enriched in healthy subjects. Integration of microscopic, mass spectrometric, and transcriptomic data to a common protein-protein binding network showed how these were connected to each other.

Conclusion: We propose a hypothesis of caveolae-dependent uptake and transport of birch pollen allergen in the epithelium

of allergic patients only. Application of discovery-driven methodologies can provide new hypotheses worth further analysis of complex multifactorial diseases, such as type I allergy. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Pollen, allergy, epithelium, caveola, lipid raft, cytoskeleton, systems biology, pathway, integration

Type I hypersensitivity causes acute allergic reactions to allergens by cross-linking with specific anti-IgE molecules on mast cells in immunologically sensitized patients. A large body of previous work in type I pollen allergies has focused on aberrant immunoresponses.¹⁻⁶ Although the role of epithelium as the first line of defense against allergens has recently been emphasized,⁷⁻⁹ its true role is far from clear. We have already demonstrated the early *in vivo* leukocyte response after Bet v 1 pollen allergen challenge in human subjects.¹⁰⁻¹² The aim of our systems-level analyses is to begin to explore the role of epithelial cells in the early pathogenesis of type I allergic reactions.

We focused on type I allergy because it is very common. In Europe up to 15% of the population has pollen allergies, which are associated with significant morbidity and indirect loss of productivity in society.^{3,9,13} This disease was also selected because it is acute, and some of its causative agents have been identified, such as the birch pollen allergen Bet v 1.^{3,5,14} The way nasal mucosa interacts with airborne allergens is not completely understood. In the intestine epithelial cells rapidly uptake specific allergens and transport them across the epithelium, resulting in allergic sensitization. This sensitization induces the expression of CD23 (the low-affinity IgE receptor or FcεRII) on intestinal epithelial cells, which enhances IgE-dependent transepithelial antigen uptake.¹⁵⁻¹⁷

Here we analyze the role of respiratory epithelium in antigen uptake. We collected nasal epithelial specimens from both patients with birch pollen allergy and healthy control subjects. Specimens were collected during the nonsymptomatic winter season, both at baseline and after nasal pollen challenge, and during the natural spring pollen season, when the allergic patients experienced symptoms. To minimize *in vitro* artefacts, we collected and analyzed only freshly isolated nasal epithelial cells and biopsy specimens and not samples cultured *in vitro*. We used electron microscopy (EM) to locate Bet v 1 within the epithelium, mass spectrometry to identify proteins associated with Bet v 1, and transcriptomics to identify genes uniquely expressed in samples from allergic patients. Based on these data, we propose a hypothesis of caveolae-dependent uptake and transport of the birch pollen allergen, a mechanism present only in epithelium from allergic patients.

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Abbreviations used

EM: Electron microscopy

GO: Gene ontology

Immuno-EM: Immunotransmission electron microscopy

NCBI: National Center for Biotechnology Information

METHODS**Subjects**

The study was performed according to the Declaration of Helsinki, and the study protocol was reviewed and approved by the Committee on Ethics of Helsinki University Eye and Ear Hospital and Tampere University Hospital. Informed consent was obtained from all subjects.

The diagnosis of birch-induced allergic rhinitis was based on a history of spring seasonal allergic rhinitis, clinical examination, and skin prick test results. Inclusion criteria for these patients were as follows: monosensitization to the birch pollen allergen based on skin prick test results and symptoms of early-phase allergic response after *in vivo* nasal challenge with the birch pollen extract. The healthy control subjects had negative allergy skin test results and had no history of seasonal allergies. All subjects included in this study were nonsmokers and were taking any medication when study procedures were performed.

Microscopy and mass spectrometry were performed with 6 allergic subjects (3 female and 3 male subjects; mean age, 27.4 years; age range, 22–33 years) and 6 healthy control subjects (3 female and 3 male subjects; mean age, 28.6 years; age range, 24–35 years). Transcriptomics was carried out in 3 allergic and 3 healthy control subjects.

Nasal epithelial specimens

Biopsy specimens from nasal epithelium and nasal epithelial cell swabs were obtained from both healthy subjects and allergic patients (see the [Methods](#) section in this article's Online Repository at www.jacionline.org). Lidocaine (1%) plus epinephrine (adrenalin, 250 µL; 1:1000) was used for local anesthesia of the inferior turbinate 20 minutes before biopsies. Fokken forceps were used to take the specimens from the anterior surface of the inferior turbinate, and the biopsy specimens were immediately frozen in liquid nitrogen to prevent mRNA degradation and other *in vitro* artefacts. The cell swabs were taken without any anesthesia with a cotton wool applicator. The nasal cell swabs were used immediately for analysis (see below). Morphologic data indicated that 95% of the cells in a cytocentrifuge preparation represented epithelial cells, whereas the rest were mostly leukocytes (data not shown). Specimens were collected during winter, at baseline (off season baseline), and after the *in vivo* challenge and during spring (in-season sample), when a fulminant birch pollen season causes symptoms in allergic patients.

Immunohistochemistry

Bet v 1 location within epithelial tissue was studied with polyclonal rabbit anti-Bet v 1 antibodies detected with secondary fluorescent anti-rabbit antibody (Alexa 488, Molecular Probes, Eugene, Ore). Likewise, we stained for IgG, IgM, IgA, IgE, caveolin 1, caveolin 2, caveolin 3, flotillin 2, and CD1a (see the [Methods](#) section in this article's Online Repository).

Immunotransmission EM

To locate Bet v 1 in epithelium, we used polyclonal anti-Bet v 1 antibody, followed by incubation with rat anti-rabbit IgG and protein A–gold complex. Double staining used 5- and 10-nm gold particles to label Bet v 1 and caveolin 2, respectively (see the [Methods](#) section in this article's Online Repository).¹⁸ Specimens from 3 allergic and 3 healthy subjects were analyzed.

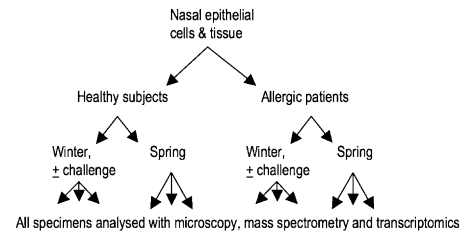


FIG 1. Nasal epithelial cells and tissues were sampled from patients with birch pollen allergy and healthy subjects during the symptom-free winter period, before the intranasal pollen challenge, and 10 minutes after perturbation. The same subjects were sampled during the spring birch pollen season, when allergic individuals had symptoms. Three datasets were generated: Bet v 1 location and quantity (microscopy), Bet v 1-associated epithelial proteins (mass spectrometry), and epithelial transcriptomics.

Mass spectrometry

Recombinant Bet v 1 with either N- or C-terminal TAP-tag was incubated with nasal epithelial cells *in vitro*, followed by hypotonic cell lysis, and Bet v 1 with associated proteins was isolated by using affinity column purification, digested with trypsin, and analyzed with the LC-MS (see the [Methods](#) section in this article's Online Repository).^{19–21}

Transcriptomics

Nasal epithelial cells from 3 healthy and 3 allergic subjects were collected during winter and spring. RNA was isolated, and gene expression was analyzed with the Affymetrix HG-U133-PLUS-2 array. The [Methods](#) section in this article's Online Repository describes sample processing and data analysis in detail.²² The microarray data were submitted to ArrayExpress (accession no. E-MTAB-17).

Medicel reference objects and reference pathway

The nonredundant objects in the reference pathway model depict data for genes, their transcripts, and corresponding proteins integrated from various biologic databases to a data warehouse (the Integrator; Medicel Ltd, Helsinki, Finland). GeneG and transcript data are integrated from Ensembl and National Center for Biotechnology Information (NCBI)/RefSeq, and protein data are integrated from UniProt, Ensembl, and NCBI/RefSeq. In addition to the previously mentioned databases, the Medicel reference pathway takes advantage of the interactions of the Intact and KEGG databases. Protein sets (SwissProt) resulting from microarray analyses, as well as from the 2 other data domains, were converted to Medicel reference objects and visualized on the reference pathway.

RESULTS

The study design is summarized in [Fig 1](#). Nasal epithelial specimens from patients with birch pollen allergy and healthy control subjects were collected off season (winter) and in season (spring, natural allergen exposure) and in addition at 1 and 10 minutes after nasal pollen challenge performed off season. Copy analyses showed that 1 minute after the *in vivo* nasal challenge with birch pollen allergen, healthy subjects did not show any binding of allergen to the epithelium ([Fig 2, A](#), top panel pair), whereas allergic patients had Bet v 1 bound to the epithelium in a patchy manner. Specimens from all 6 allergic patients showed this binding, whereas those of the healthy subjects remained negative ([Fig 2, A](#), blue staining, lower panel pair). During the spring season, Bet v 1 was found in the nasal epithelium only in allergic patients and not in healthy control subjects (data not shown).

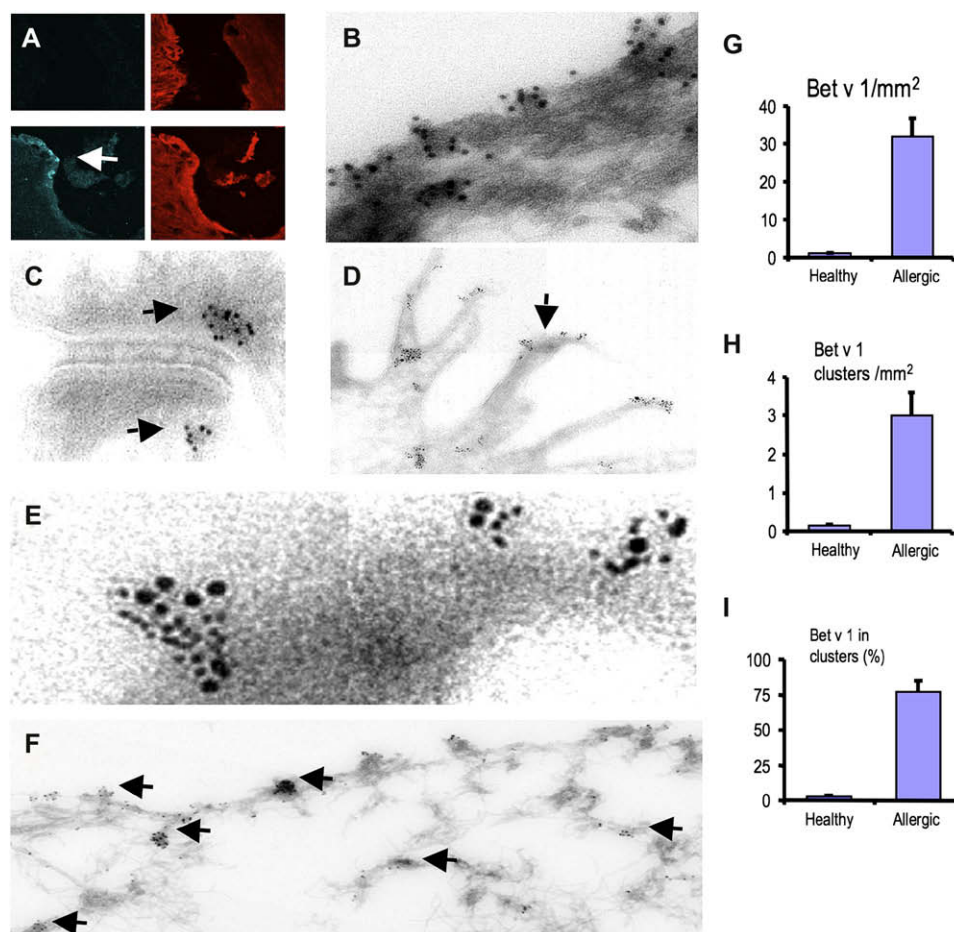


FIG 2. **A**, Nasal epithelium from healthy subjects shows no Bet v 1 binding to the epithelium, whereas concanavalin A staining clearly indicated the epithelial lining in red (upper pair). One minute after the *in vivo* birch pollen challenge, there was a strong and patchy Bet v 1 clustering to the epithelium of allergic patients (lower pair, blue staining, white arrow; original magnification $\times 400$). **B**, After 1 minute of nasal birch pollen challenge, most of the gold label in immuno-EM was on the epithelial surface (original magnification $\times 27,000$). **C**, Often the clusters of Bet v 1 were located in the vicinity of desmosomes (original magnification $\times 18,000$). **D**, Double immuno-EM showed coclustering of caveolin 2 (10 nm of gold) and Bet v 1 (5 nm of gold; arrows; original magnification $\times 10,000$). **E**, Higher magnification of the area of the arrow in Fig 2, D (original magnification $\times 100,000$). **F**, The clusters of Bet v 1 allergen were colocalized on the top of the cytoskeletal structures (arrows). **G-I**, Quantification of Bet v 1/mm² (Fig 2, G) and Bet v 1 clusters/mm² (Fig 2, H) and percentage of anti-Bet v 1 gold particles present in clusters within the nasal epithelium (Fig 2, I).

Immunotransmission EM (immuno-EM) analyses indicated that Bet v 1 clustered at the surface of the epithelium and travelled across the intact epithelium in allergic patients (Fig 2, B-I). Specimens from all 6 allergic subjects showed this clustering, whereas those of the healthy subjects did not. In addition, Bet v 1 accumulated often in the vicinity of desmosomes (Fig 2, C). Double-labeled immuno-EM analyses with lipid raft and caveolar markers, such as anti-caveolin 2 (Fig 2, D and E) and flotillin 2 (data not shown) suggested that Bet v 1 was transported within lipid rafts and caveolae. In Fig 2, F, several Bet v 1 clusters can be seen colocalizing to cytoskeletal structures, which are known to participate in caveolar movements.^{23,24} Interestingly, all specimens from allergic patients showed the same Bet v 1 uptake, whereas this was not the case for specimens from healthy control subjects. Immuno-EM of nasal mucosal biopsy specimens also showed that Bet v 1 reached mast cells located close to or under the basal lamina within minutes after the *in vivo* nasal challenge only in allergic patients (Fig 3). A biopsy specimen was

photographed from the upper apical epithelial cell surface all the way down to the mast cells lying under the basal lamina with a series of overlapping frames (>500 individual photographs at magnification $\times 27,000$) zoomed down and collated to show a cross-section of the epithelium. A few high-magnification snapshots showing the anti-Bet v 1 gold particles with arrows linking to the original positions within the tissue are shown to verify that Bet v 1 indeed was transported within a minute through all epithelial layers to the underlying mast cells.

Caveolae and lipid rafts are known to transport viruses, bacteria, and signaling molecules.²⁵⁻³⁰ Because our data indicated that lipid rafts and caveolae can also transport allergens through nasal epithelial cells, our next aim was to identify the Bet v 1-associated proteins acting as receptors, participating in the intracellular transport, or both. We performed affinity chromatography with the covalently matrix-bound TAP-tagged Bet v 1 and nasal epithelial cell lysates from both healthy and allergic subjects during the winter season. After the eluting procedure, the Bet v 1-

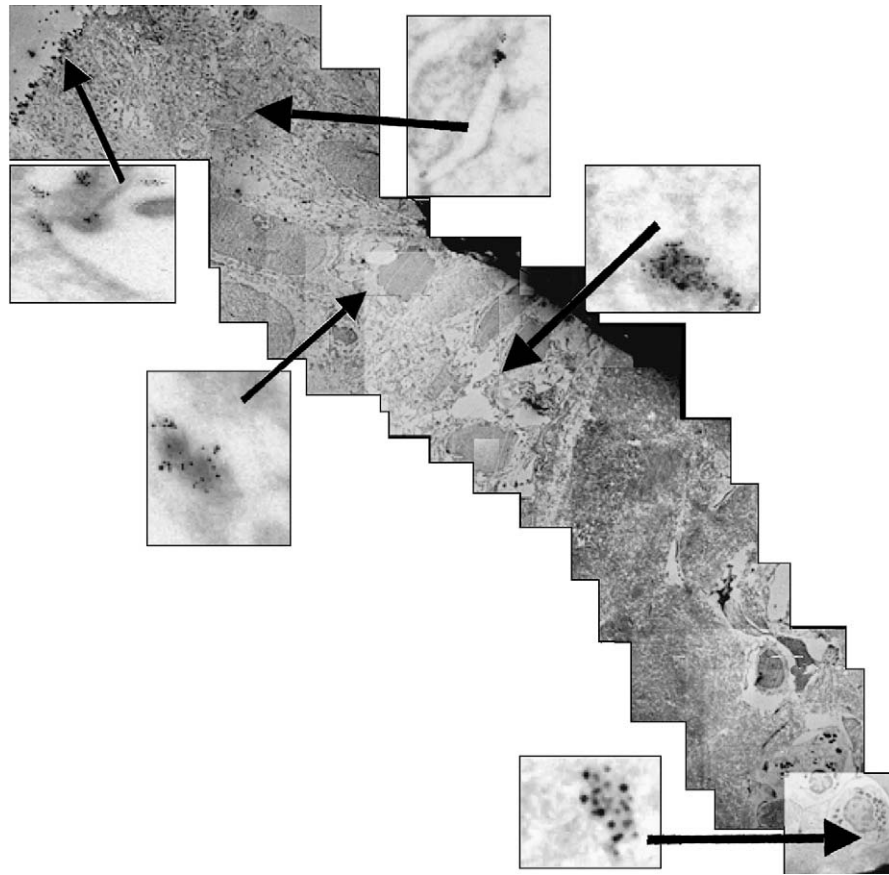


FIG 3. More than 500 individual photograph frames were taken from a nasal biopsy specimen from a challenged allergic patient (original magnification $\times 27,000$), magnified, and collated to show the cross-section of the entire pseudostratified epithelium with underlying mast cells. A few high-magnification frames showing the anti-Bet v 1 gold particles and with arrows linking to the original positions within the tissue are shown.

associated proteins were subjected to 2-dimensional HPLC and mass spectrometry MS/MS stop-flow analyses. The peptides were identified with database-related and *de novo* identifications, and a short list of Bet v 1-associated proteins was generated with very stringent criteria.¹⁹ Among the 16 proteins found to associate with Bet v 1 in epithelial cells from allergic, but not healthy, subjects, there were 6 caveolar proteins: ACTG, PLEC1, STML2, KCNA5, CALM, and ANXA2 (see definitions in Table I). The proportion of caveolar proteins within the Bet v 1-associated proteins in allergic specimens was greater than 37% and thus strongly enriched because known caveolar proteins represent less than 1% of the whole human proteome. At the same time, no caveolar proteins were found to associate with Bet v 1 in the epithelial cell lysates from healthy control subjects. This observation strongly favored the hypothesis that Bet v 1 allergen could travel through the epithelial tissues within caveolae.

The actin cytoskeleton participates in caveolar dynamics, internalization, and traffic, whereas microtubules are required for the long-range caveolar movements.^{23,24} Another subset of 6 proteins from the allergic-specific list of Bet v 1-associated proteins (ie, ACTG, K1C18, TBA8, EPIPL, PLEC1, and CROCC; Table I) belonged or linked to the cytoskeleton. Other Bet v 1-associated proteins were involved in intracellular vesicle transport and antibacterial defense (Table I). In contrast, no cytoskeletal proteins were found to associate with Bet v 1 in the epithelial

cell lysates from healthy control subjects. Taken together, the mass spectrometry observations provided further evidence that caveolar transport along the various components of the cytoskeleton could be involved in the uptake and transcellular migration of Bet v 1.

Transcriptomics revealed the third set of data comparing epithelial cells from allergic and healthy subjects. We first compared nasal samples from these 2 groups obtained during the symptom-free winter season and analyzed them with the Affymetrix HG-U133-PLUS-2 arrays (Affymetrix, Santa Clara, Calif). The raw CEL file data were extracted together with the project information (ie, metadata) to the Integrator (Medicel Ltd) for further analyses. We found 95 transcripts differentially expressed between epithelial specimens from allergic and healthy subjects. Similarly, comparison of samples obtained during the spring identified 86 transcripts differentially expressed between the 2 groups, with only 1 overlapping gene in this pairwise comparison (TFPI2_HUMAN [tissue factor pathway inhibitor 2], which is known to regulate matrix remodeling; Fig 4, A, and Table I). Interestingly, when comparing healthy subjects during winter and spring, 605 differentially expressed transcripts and corresponding proteins were found, whereas only 331 transcripts/proteins differed significantly in the allergic subjects between winter and spring (Fig 4, B, and see Table E1 in this article's Online Repository at www.jacionline.org). Thus the nasal epithelium from

Table I. Bet v 1–associated proteins found only in the nasal epithelial cells from allergic patients, but not healthy control subjects, during winter

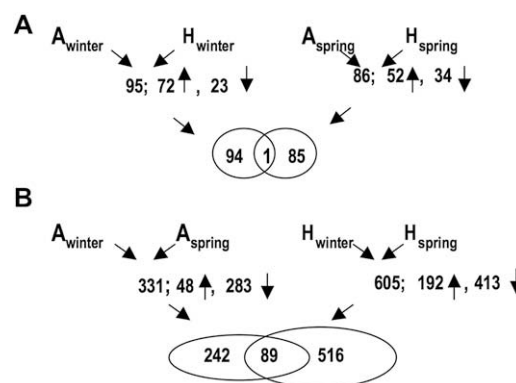
*ACTG_HUMAN	Actin, γ actin, a subunit of microfilaments, one of the 3 major components of the cytoskeleton
K1C18_HUMAN	Cytokeratin 18, a subunit of intermediate filament, one of the 3 major components of the cytoskeleton
TBA8_HUMAN	α -Tubulin, the major constituent of microtubules, one of the 3 major components of the cytoskeleton
EPIPL_HUMAN	Epiplakin, a cytoskeletal linker protein, with interactions between epiplakin and intermediate filaments
*PLEC1_HUMAN	Plectin intermediate filament binding protein
CROCC_HUMAN	Ciliary rootlet, a cytoskeletal-like structure in ciliated cells
*STML2_HUMAN	Stomatin-like protein 2, colocalized at intercellular junctions and regulated gap junctions and lipid domain organization
GNDS_HUMAN	Ral/RalBP1, role in endocytosis and vesicle sorting and migration
ECHB_HUMAN	Participates in lipid metabolism
*KCNA5_HUMAN	Voltage-dependent potassium ion permeability, role in regulating the secretion of insulin in normal pancreatic islets
S100P_HUMAN	Protein S100-P, overexpressed in epithelium in psoriasis, wound healing, skin cancer, inflammation, calcium binding
*CALM_HUMAN	Calmodulin, calcium-binding proteins, regulates nuclear transport
*ANXA2_HUMAN	Annexin 2, lipocortin belongs to the calcium-binding proteins, suppresses phospholipase A ₂ , and thus inhibits inflammation
MYCB2_HUMAN	MYCB2 mediates ubiquitination and subsequent proteasomal degradation of target proteins
DCD_HUMAN	Dermcidin displays antimicrobial activity, thus limiting infection by potential pathogens, such as <i>Escherichia coli</i> and <i>Staphylococcus aureus</i>
MARCO_HUMAN	Scavenger receptor MARCO, a major mediator of nonopsonized <i>E coli</i> phagocytosis clearance of bacteria <i>in vivo</i>

Only those proteins that were identified in at least 2 different individuals or at least 2 different spectra within 1 individual are included.

*Proteins found in lipid rafts and caveolae.

allergic and healthy subjects differed at the transcriptomics level during the winter season. Both healthy and allergic subjects responded to the birch pollen challenge, with allergic subjects displaying a much less pronounced response.

The gene ontology (GO) categories provide a controlled vocabulary to describe the gene and gene product attributes of any organism. Further analyses of the mRNA expression profiles against GO categories indicated that the nasal epithelium of allergic and healthy subjects differed during spring in lipid metabolism and responses to virus (Table II and see Table E2 in this article's Online Repository at www.jacionline.org). GO enrichment analysis with allergic specimens showed that the category of protein transport was upregulated, whereas the cytoskeleton and actin cytoskeleton categories were decreased during spring. Concomitantly, the immune response and actin binding were enriched in GO categories during the spring, with

**FIG 4.** Nasal epithelial transcriptome analyses between healthy and allergic subjects. **A**, When specimens were compared, 94 and 85 transcripts were differentially expressed between the 2 groups during winter and spring, respectively. GO categories were enriched in pairwise combinations. **B**, Comparison between the nonchallenged (winter) and challenged (spring) specimens revealed great differences. There were many more transcripts with altered expression in healthy compared with allergic specimens.

upregulated response in specimens from healthy subjects (Table II). These observations also support the role of caveolar transport in Bet v 1 entry to the nasal epithelium in allergic patients.

Finally, we combined the 3 data sets (microscopy, mass spectrometry, and transcriptomics). We first integrated a unique set of data from Swiss-Prot, Ensembl, NCBI RefSeq, KEGG, and Intact using a common information model and integrated it to generate cellular networks from genes-transcripts-proteins, from protein-protein interactions, and from enzymatic reactions into a data warehouse (the Integrator). This approach allowed analysis of combined data from these 3 technologies, namely microscopy showing lipid raft and caveolar raft proteins colocalized with Bet v 1, mass spectrometry showing additional caveolar proteins associated with Bet v 1, and microarray transcriptomes of nasal epithelium. As a result, this analysis suggested protein-protein interactions involved in Bet v 1 epithelial transport as follows. Lipid rafts and the caveolar proteins caveolin 2 and flotillin 2 (CAV2 and FLT2 from microscopy data) are connected to the Bet v 1–associated proteins GNDS, a Ras-related GTPase, and MYCBP2, a E3 ubiquitin-protein ligase (from mass spectrometric data), and to PTN4, one of the cytoskeleton binding proteins (from transcriptomics; see definitions in Table I). PTN4 protein binds directly to LIPL, a lipase that hydrolyzes triglycerides, circulating chylomicrons, and very low density lipoproteins. Such lipid metabolism could be central in the regulation of lipid rafts and caveolar transport. No dataset from a single technologic platform alone could have provided such a connected network needed to understand the new systems level of diseases.

DISCUSSION

The most significant effect of such a top-down explorative study using human specimens is that it can generate new hypotheses. Before the present work, few would have argued that the nasal epithelium was so clearly different between healthy and allergic subjects during the nonsymptomatic winter season. An unexpected finding was that allergic epithelium displayed a markedly reduced gene expression response after allergen challenge compared with that seen in healthy epithelium. Together these observations suggest that there are major complex

Table II. The number of differently expressed transcripts (converted to corresponding proteins) was enriched to GO categories, and the most interesting observations are displayed

	GO category	In category	Found	Expected	P value
A _{winter} vs H _{winter}	Metabolism	404	7	3.1	.038
A _{spring} vs H _{spring}	Lipid metabolism	181	4	1.1	.031
	Response to virus	70	3.0	0.4	.010
A _{spring} vs A _{winter}					
Upregulated	Protein transport	250	4	1	.017
Downregulated	Cytoskeleton	232	11	5.3	.023
	Actin cytoskeleton	64	6	1.4	.003
H _{spring} vs H _{winter}					
Upregulated	Immune response	303	13	4.3	.000
	Actin binding	170	6	2.4	.035
Downregulated	RNA binding	385	28	12.9	.000
	Transcript fact binding	66	9	2.2	.000

P values were calculated with the 2-sided Fisher exact probability test. The full lists of all significant enrichments are available in Table E2.

A, Allergic subjects; H, healthy control subjects.

alterations in the epithelial cells in allergic patients compared with healthy control subjects. Previous studies have already demonstrated several differences in the epithelium of allergic patients,^{9,31} but the result of our analysis is that allergic subjects actively and rapidly transport pollen allergen through the epithelium,^{9,31} a process that does not occur in healthy subjects.

We show that Bet v 1, used here as an example of pollen allergens, could be taken up through lipid rafts and caveolae into epithelial cells in allergic patients. Although we can clearly show functional differences between the lipid raft/caveolar transport of allergens in allergic versus healthy subjects, the underlying mechanism for this remains unclear. Neither the expression of transcripts categorized to lipid rafts and caveolae nor the quantitative immuno-EM analysis of caveolar protein numbers differed between the 2 populations (data not shown). The actual distribution of lipid rafts and caveolar proteins on the cell surface (ie, clustering or capping) and their posttranslational modifications, such as phosphorylation or glycosylation, could be the underlying cause of the observed phenomenon. One known mechanism of antigen uptake in the gut is mediated through the uptake of IgE or receptors for IgE.^{16,17} However, we did not detect IgE with immunohistology in any nasal biopsy specimens, nor did we detect IgE or Fc receptors for IgE in the mass spectrometric analysis of proteins from allergic subjects. Although the receptor for Bet v 1 on the nasal epithelium of allergic subjects is unknown, our data indicate that the receptor is likely associated with lipid rafts and caveolae, which then transport it across the epithelium toward the basement membrane.

Because conventional human epithelial cell lines do not specifically represent the allergic state space, the next step would be to generate cell lines from both allergic and healthy nasal epithelium and recapitulate parts of the Bet v 1 transport data. However, one must realize that such an *ex vivo* cell-line system approach might not reproduce *in vivo* tissue-level observations of allergen transport through epithelial tissue to the basal lamina mast cells.

Antigen-presenting cells, such as dendritic cells, exist in nasal epithelium and might be involved in the uptake of Bet v 1 allergens. Our results indicated that the majority of the uptake documented here was not mediated by dendritic cells because immunohistology never showed any colocalization of CD1a (a marker for dendritic cells) with the Bet v 1 allergen. Likewise, the large EM panels (Fig 3 and see Fig E1 in this article's Online

Repository at www.jacionline.org), representing more than 500 individual photograph frames collated together and displaying the Bet v 1 uptake on morphologically pseudostratified epithelium, did not reveal any dendritic cells.

As first-line evidence that this phenomenon might be more general and not restricted to nasal epithelial tissues, we have also shown that the same transport of Bet v 1 allergens takes place through morphologically intact stratified conjunctival epithelium in allergic, but not healthy, subjects.³² Based on our results, the caveolae-dependent mechanisms of the Bet v 1 pollen allergen binding to and transporting through the epithelium can be further explored and analyzed in the context of pathogenetic mechanisms leading to allergy.

Before this study, the role of lipid rafts and caveolae in pollen transport was unknown. It was known that caveolar transport can facilitate entry and transepithelial transport of certain viruses, toxin proteins, and even whole bacteria, as well as parasites.^{27,33-41} The caveolae/lipid rafts are especially rich in lipids, such as cholesterol, gangliosides, and sphingomyelins.^{42,43} It is possible that lipid interactions within caveolae could also play a role in the binding of Bet v 1.^{44,45}

Our gene expression data relied on gene chip data without PCR-based verification. Our observations are not based on any single or even several transcripts but rather on groups of transcripts and their enriched presentations (GO enrichment analysis). Thus our approach indicates that certain cellular processes and functions could be involved in a few hundred differentially expressed transcripts. With this network/pathway analysis method, we are able to point out that the transcriptomics analysis corroborated microscopic and mass spectrometric data, which also indicated participation of the lipid raft and caveolae-dependent transport mechanisms in Bet v 1 transepithelial transport.

This study is our first attempt to apply systems biology, integrating data from different technology platforms to allergy research. The individual data points gathered from such experiments are often very large, and hence we and others have developed computational approaches to analyze them further.⁴⁶⁻⁴⁸ This study is one of the first to generate a small network that could have relevance in the pathogenesis of allergen mucosal transport. This network is built by integrating the pre-existing public databases with our own observations into one data warehouse (see Figs E2-E4 in this article's Online Repository at

www.jacionline.org). This is not a hypothetical model but an integrated network displaying data from several databases combined with our own new observations from this study, which might be relevant to a better understanding of the molecular pathogenesis of allergy.

The ability to discover new, causatively relevant disease mechanisms is the major motivation for unbiased explorative approaches. It is of crucial importance that several data from different technologies all point toward the same pathogenetic mechanism. In our case microscopy, mass spectrometry, and transcriptomics all implicated lipid rafts and caveolar and cytoskeletal proteins in Bet v 1 transport across the nasal epithelial cell. The drawback of such top-down experiments is that they are very expensive to conduct and produce much more raw data than hypothesis-derived approaches, thus generating an overflow of data. In addition, they are not definitive on their own, and therefore they still need to be followed up with hypothesis-driven experiments to confirm that lipid rafts and caveolar and cytoskeletal proteins are essential for Bet v 1 transport across the respiratory epithelium.

We have shown here with a systems-level integrative analysis that there is a vast difference in the nasal epithelium of patients with pollen allergy and healthy subjects, which allows the active transport of pollen through the epithelium to mast cells within minutes in allergic patients. This transport and its prevention might provide a new site of intervention in allergic subjects. Taken together, the systems-level understanding of complex, multifactorial pathophysiologic phenomena will require all the current laboratory techniques available, as well as substantial computer support, where the biology will be transformed into an integrated, computer-readable format.

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Clinical implications: Birch pollen allergen travels through nasal epithelium to mast cells in caveolae only in allergic patients (not in healthy control subjects).

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METHODS

Immunohistochemistry

Bet v 1 location within epithelial tissue was studied with polyclonal anti-Bet v 1 antibodies (first obtained from R. Valenta, University of Vienna, Austria, and later polyclonal rabbit anti-Bet v 1 antibody toward recombinant Bet v 1 was generated by our group). Rabbit IgG and the depletion of the primary antibody were used as negative controls. Secondary fluorescent anti-rabbit antibody (Alexa 488, Molecular Probes) was used to detect the signal, and concanavalin A lectin was coupled with Alexa 633 (Molecular Probes) to detect epithelial tissue. Other antibodies used for immunohistochemistry were anti-IgG, anti-IgM, anti-IgA, anti-IgE, caveolin 1, caveolin 2, caveolin 3, flotillin 2, and CD1a (all from Immunodiagnostic Oy, Hämeenlinna, Finland).

Immuno-EM

Immuno-EM was performed to analyze the presence of the Bet v 1 allergen within the nasal epithelial tissue. Tissue biopsy specimens were fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 1 hour, postfixated with OsO₄ in 0.1 mol/L phosphate buffer (pH 7.4) for 1 hour, dehydrated in acetone, and embedded in Epon LX 112. Ultrathin sections were cut with a Reichert Ultracut E-ultramicrotome and, after cutting the sections, were incubated in 5% BSA and 0.1% gelatin in NaCl/Pi. Antibodies and gold conjugates were diluted in 0.1% BSA-C (Aurion, Wageningen, The Netherlands) in NaCl/Pi, and all washing steps were performed in 0.1% BSA-C in NaCl/Pi. For the double-labeling experiments, blocking was done as described above. The sections were exposed to the first primary antibody (polyclonal rabbit anti-Bet v 1) for 60 minutes, followed by incubation with rat anti-rabbit IgG for 30 minutes and protein A–gold complex (5 nm) for 30 minutes. After washing, 1% glutaraldehyde in 0.1 mol/L phosphate buffer was used to block free binding sites on protein A. The sections were then incubated with the second antibody (murine anti-human caveolin 2 antibody) for 60 minutes, followed by anti-mouse IgG for 30 minutes and the protein A–gold complex (10 nm) for 30 minutes, as described above. The control specimens were prepared by carrying out the labeling procedure without the primary antibody. The sections were embedded in methylcellulose and examined with a Philips CM100 transmission electron microscope. Images were captured with a CCD camera equipped with tcl-em-menu, version 3, from Tietz Video and Image Processing Systems GmbH (Gauting, Germany).^{E1}

The quantification of immuno-EM images was carried out with dedicated *in silico* tools and algorithms linked directly into a computer to generate a workflow (Medicel Ltd). The cell surface and the apical epithelial layers were included in the calculations. Between 50 and 100 visual fields from each image were chosen. Specimens from 3 allergic and 3 healthy subjects were analyzed. The application of workflows allowed automated counting, as well as traceable integration of the metadata with calculated results and combination with other data domains, such as mass spectrometry and transcriptomics. All microscopic analyses were done blindly, so that the person doing the analysis did not know the origin of the specimens.

Mass spectrometry

Recombinant Bet v 1 with either N- or C-terminal TAP-tag was cloned, produced, and purified in *Escherichia coli* fermentation and coupled to a cyanogen bromide-activated Sepharose column. Nasal epithelial cells from both healthy and allergic subjects were collected during winter without pollen challenge. Nasal epithelial cells were immediately suspended in 2 × 500 μL of ice-cold PBS, placed in pellets (at 3000 rpm for 5 minutes at +4°C), and washed twice with 1 mL of ice-cold PBS. Hypotonic lysis was carried out with 20 mmol/L TRIS-HCl, 1 mmol/L EDTA, 5 mmol/L MgCl₂, 1 mmol/L dithiothreitol, and 1% octylglucoside. The debris was flushed with a 30-gauge needle 10 times and spun down (at 8000g and +4°C for 30 minutes). The supernatant was ready for affinity column dialysis to 50 mmol/L TRIS-HCl (pH 7.5), 150 mmol/L NaCl, and 0.1% octylglucoside. The pellet contained the membranes and was further suspended in the lysis buffer (50 mmol/L TRIS-HCl [pH 7.5], 150 mmol/L NaCl, and 1% octylglucoside) with proteinase inhibitors by using the 30-gauge needle flushing, as previously described, and incubated on ice for 30 minutes. The membrane lysates were centrifuged at 8000g for 30 minutes, and the supernatant from this centrifugation was ready for Bet v 1 affinity column

purification. The column-bound proteins were released, TCA/acetone precipitated, digested with trypsin overnight, and analyzed with the LC-MS.^{E2-E4}

The peptide LC-MS2 spectra were acquired with a Q-TOF Ultima Global mass spectrometer (Micromass Ltd, Manchester, United Kingdom) by using flow nano HPLC with Waters CapLC system (Waters, Corp, Milford, Mass). The analytic column was a 25-cm-long PepMapC18 with a 75-μm diameter (LC Packings, Amsterdam, The Netherlands). The electrospray ionization (ESI) spectra were acquired in the positive mode during a 600-minute gradient from 5% B (95% acetonitrile and 0.1% formic acid) to 30% B (buffer A was 5% acetonitrile and 0.1% formic acid), and the acquisition range was set to 800 to 1600 m/z in MS Survey and 100 to 4000 m/z in MS2 mode. Spectra were collected in data-dependent acquisition fashion, including charge states +2 to +6.

The LC-MS2 spectra were extracted and deconvoluted with Mascot Distiller (Matrix Science, Inc, London, United Kingdom). The protein identification was done with database-related X!Tandem and *de novo* LuteFisk integrated to the Integrator (Medicel Ltd). The database search was done against SwissProt human sequences, with 2 potential miscleavages in the trypsin cleavage.

A short list of proteins, which appeared in specimens from the allergic subjects only, was taken for further analyses. Those that were found in 2 different subjects or with 2 different peptides in 1 subject (of higher significance than 1 peptide only) were interpreted in our analyses as being Bet v 1-associated proteins in allergic specimens.

Transcriptomics

Nasal epithelial cells from 3 healthy and 3 allergic subjects were collected during winter and spring. RNA isolation was carried out according to Qiagen's Rneasy Mini Handbook, the concentration was measured with the RNA 6000 Nano kit for Agilent Bioanalyser, and the specimens were hybridized with Affymetrix chip platform on HG-U133-PLUS-2 and conducted in Finnish DNA Microarray Centre (Turku Centre for Biotechnology, Turku, Finland).

During microarray analyses, the following integrated databases were used for probe annotations and conversions between genes, transcripts, and proteins: HG-U133-PLUS-2 (2006-11-15-a), NCBI/RefSeq/RNA (2007/05/24), Ensembl (45-a), SwissProt (53.2-a), TrEMBL (36.2), GeneOntology (2007-07-30), KeGG (2006/12/15-1), and Intact (2007-07-27).

Data analysis was done with the Integrator (Medicel Ltd). The data preprocessed with the GCRMA method were normalized with quantile normalization. Only those probes known to represent 1 transcript were selected for analysis. Statistically differing expressions between different groups were calculated with a workflow by using ANOVA. The GO categories provide a controlled vocabulary to describe gene and gene product attributes in any organism and here in human subjects. The enrichments/depletions of GO categories were essentially computed as previously described.^{E5} The microarray data were submitted to ArrayExpress, accession E-MTAB-17.

Challenge protocol

First, nasal endoscopy was performed to state the status of the nasal mucosa and to make sure that the patient did not have nasal polyposis or moderate-to-severe septal deviation. A cotton wool applicator soaked in 1% lidocaine with epinephrine was placed under the inferior turbinate on both sides. An additional cotton wool applicator with the same local anesthesia solution was placed on the anterior insertion of both inferior turbinates. After 20 minutes, the first biopsy specimen from the mucosa 1 cm posterior to the front edge of the right inferior turbinate was taken with the Fokken forceps with a cup diameter of 2.5 mm. Intranasal challenge was performed blind either by dropping 250 μL of Alutard SQ *Betula verrucosa* (birch pollen) allergen extract 100 SQ-U/mL (ALK-Abelló, Hørsholm, Denmark) or 250 μL of ALK diluent 122820 (placebo; ALK-Abelló) on the anterior part of the left inferior turbinate. After 1 minute of incubation of the allergen or placebo, the second biopsy was performed from the left inferior turbinate with the same method and from the same place as the first biopsy. Epistaxis was resolved by pinching the nasal alae together for 10 minutes and in some cases by covering the operated area with a gelatin sponge (Spongostan, Ferrosan, Denmark). The symptoms of the early-phase allergic response (eg, sneezing, rhinorrhea, and itching) were documented by using a questionnaire taken before and 30 minutes after the challenge.

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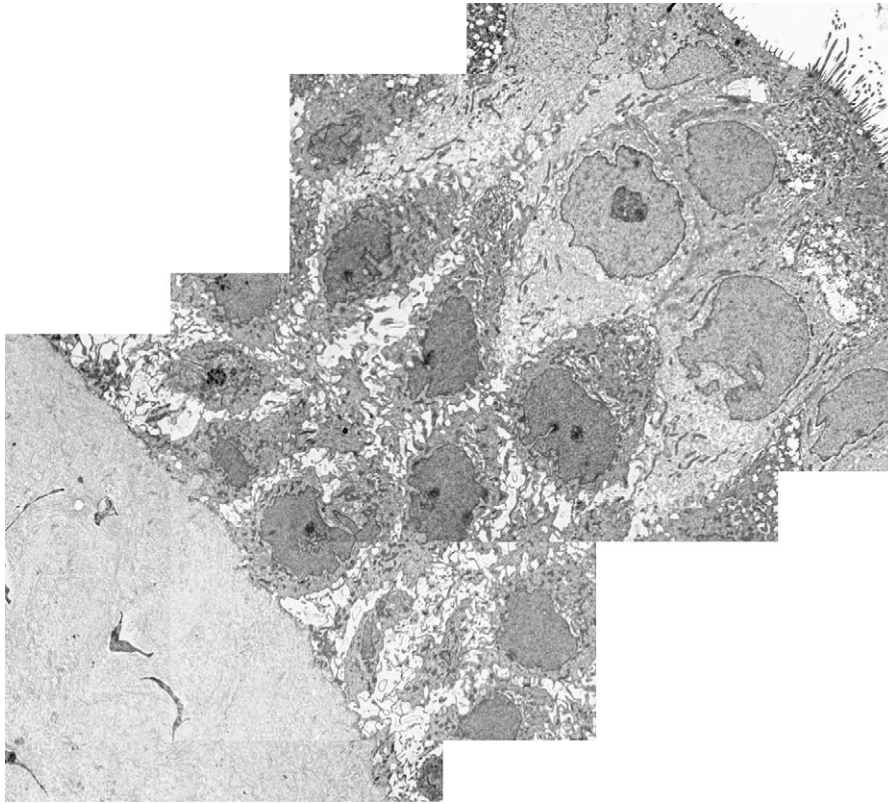
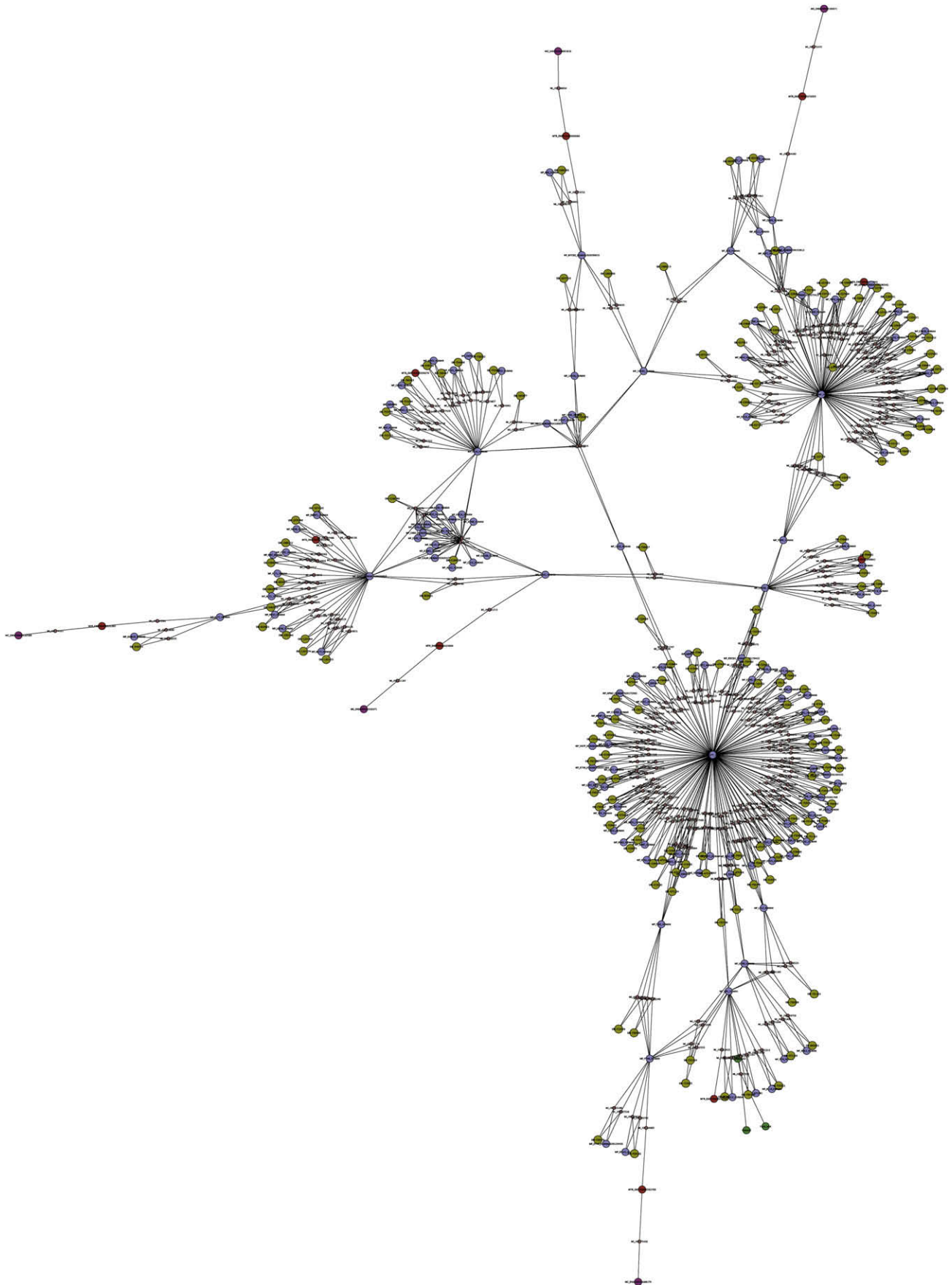


FIG E1. A tiled EM micrograph of the normal pseudostratified epithelium for orientation when viewing Fig 3.



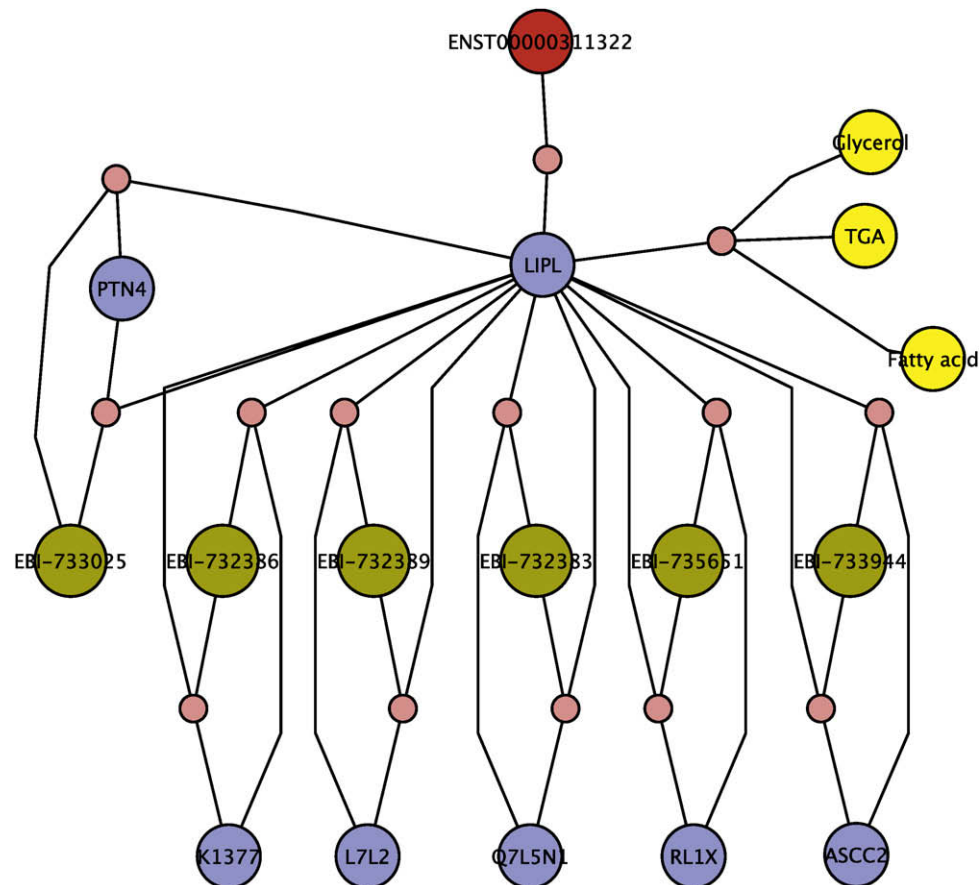


FIG E3. The neighborhood of PTN4 and LIPL on the Medical Integrator reference pathway displayed with Cytoscape. *Red circle*, Transcript; *blue circles*, protein; *green circles*, multimolecular complex from EBI database; *small pink circles*, interaction; *yellow circle* = metabolite, here glycerol, triacylglycerol (TGA) and fatty acid.

FIG E2. A comprehensive file of the pathway connecting 3 lists of data we had generated in this study from microscopy, mass spectrometry, and transcriptomics. Because this file contains 690 nodes and 113 edges, it is viewable only on a computer screen. Here 2 caveolar/lipid raft proteins, caveolin 2 and flotillin 2 (CAV2 and FLT2 from microscopy data), are connected to the Bet v 1-associated proteins GNDS, a Ras-related GTPase, and MYCBP2, an E3 ubiquitin-protein ligase, which carry out the proteasomal degradation of target proteins (both mass spectrometric data) and are linked to PTN4, a member of the cytoskeleton-binding proteins (found in transcriptome data).

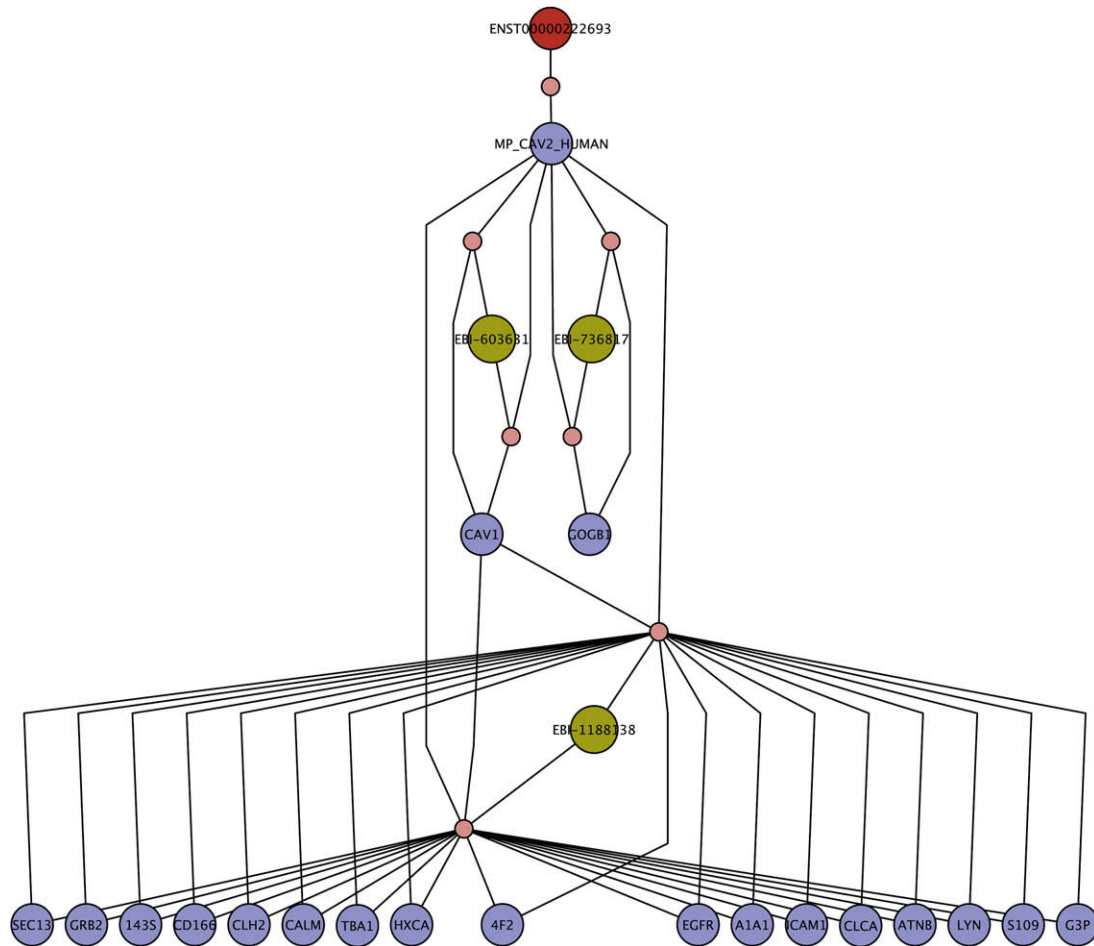


FIG E4. The neighborhood of 2 caveolar/lipid raft proteins, caveolin 1 and 2, on the Medical Integrator reference pathway displayed with Cytoscape. *Red circle*, Transcript; *blue circles*, protein; *green circle*, multimolecular complex from the EBI database; *small pink circles*, interaction.

Table E1. Transcriptomic results converted to corresponding protein names

A_{winter} vs H_{winter}
Upregulated in A
4ET_HUMAN
ASB1_HUMAN
CD034_HUMAN
CD166_HUMAN
CG027_HUMAN
CG044_HUMAN
CI167_HUMAN
CLM1_HUMAN
CMAH_HUMAN
CNO2_HUMAN
CPEB4_HUMAN
CTC9_HUMAN
DEOC_HUMAN
ENSP00000244815
ENSP00000343452
FAH2A_HUMAN
FKBP1_HUMAN
FUT1_HUMAN
FX16_HUMAN
FX33_HUMAN
GMDS_HUMAN
GNAI1_HUMAN
GOA1_HUMAN
GORS2_HUMAN
GR65_HUMAN
HC58_HUMAN
HOMZ_HUMAN
ID4_HUMAN
IKKE_HUMAN
IRE2_HUMAN
K2C7_HUMAN
KLF3_HUMAN
LBH2_HUMAN
MRP3_HUMAN
N2L2_HUMAN
NFRKB_HUMAN
PASK_HUMAN
PC20_HUMAN
PDA5_HUMAN
PDD2L_HUMAN
PDPT_HUMAN
PGDH_HUMAN
PHR2_HUMAN
PI51B_HUMAN
PIN4_HUMAN
PMGE_HUMAN2
PPBT_HUMAN
PTD4_HUMAN
PTN4_HUMAN
Q6NT76
Q6NZY7
Q6PL18
Q8TBZ8
Q8WTS1
Q8WVP7
Q9NXL6
Q9UH65
RABE1_HUMAN
RL36L_HUMAN
RPP14_HUMAN

(Continued)

TABLE E1. (Continued)

A_{winter} vs H_{winter}
SNW1_HUMAN
SP3_HUMAN
SRP72_HUMAN
STL4_HUMAN
STX8_HUMAN
SUCB2_HUMAN
SXB6_HUMAN
T4S9_HUMAN
TFP2_HUMAN
UBL3_HUMAN
UBP53_HUMAN
UPK1B_HUMAN
Downregulated in A
A1AT_HUMAN
BAL_HUMAN
CD53_HUMAN
CKRB_HUMAN
COMD7_HUMAN
CP39A_HUMAN
CYH4_HUMAN
ELOB_HUMAN
ENSP00000244815
ENSP00000261333
IFT5_HUMAN
LRC50_HUMAN
Q719H9
Q9NPF7
Q9Y661
RABP2_HUMAN
SAM9L_HUMAN
SI7B_HUMAN
THA6_HUMAN
TT21A_HUMAN
UCRP_HUMAN
ZDHL_HUMAN
ZN683_HUMAN
A_{spring} vs H_{spring}
Upregulated in A
ACH9_HUMAN
AL3B2_HUMAN
APC_HUMAN
ARBK1_HUMAN
BMP3_HUMAN
CAH4_HUMAN
CBPD_HUMAN
CD032_HUMAN
CDN3_HUMAN
CFAI_HUMAN
CHD2_HUMAN
CO1A1_HUMAN
CPT2_HUMAN
CT038_HUMAN
CTNB_HUMAN
ENSP00000358606
FRM4B_HUMAN
GP37_HUMAN
HLAE_HUMAN
INAR1_HUMAN
INGR_HUMAN
IRAK2_HUMAN

(Continued)

TABLE E1. (Continued)

A_{spring} vs H_{spring}

JHD2C_HUMAN
 KLK4_HUMAN
 KPCL_HUMAN
 LARG2_HUMAN
 LKAP_HUMAN
 MFSD4_HUMAN
 NCO4_HUMAN
 NK25_HUMAN
 ORP8_HUMAN
 P2BA_HUMAN
 PNR1_HUMAN
 PPR3C_HUMAN
 Q6TFL4
 Q8IZI9
 Q8N357
 Q8NC42
 Q96B02
 Q96DR8
 Q9BQ75
 RA21_HUMAN
 RIF1_HUMAN
 S10A7_HUMAN
 SLAP1_HUMAN
 SLFN5_HUMAN
 SNN_HUMAN
 SPRE2_HUMAN
 TM11E_HUMAN
 TMTC2_HUMAN
 TRAB_HUMAN
 ZN642_HUMAN

Downregulated in A

AAAD_HUMAN
 AKBA_HUMAN
 AKIP_HUMAN
 BIK_HUMAN
 BTB14_HUMAN
 CJ086_HUMAN
 CT77_HUMAN
 CYFP1_HUMAN
 DOC6_HUMAN
 DPY1_HUMAN
 ENSP00000365643885
 FYCO1_HUMAN
 H10_HUMAN
 HNRDL_HUMAN
 K513_HUMAN
 KCTD6_HUMAN
 MFSD5_HUMAN
 MOT14_HUMAN
 MT1F_HUMAN
 MT1L_HUMAN
 PPME1_HUMAN
 Q6PCB7
 Q9Y421
 RFNG_HUMAN
 RL5_HUMAN
 SFR3_HUMAN
 SPUF_HUMAN
 TFP2_HUMAN
 TGFB2_HUMAN
 TMM53_HUMAN
 TR2A_HUMAN

(Continued)

TABLE E1. (Continued)

A_{spring} vs H_{spring}

WDR6_HUMAN
 WSDU1_HUMAN
 Z335_HUMAN

A_{spring} vs A_{winter}**Upregulated in A_{spring}**

AOAH_HUMAN
 ARF3_HUMAN
 BCAT_HUMAN
 BTG2_HUMAN
 C79A_HUMAN
 CA108_HUMAN
 CALU_HUMAN
 CF62_HUMAN
 COPA_HUMAN
 CYTB_HUMAN
 CYTT_HUMAN
 DPY1_HUMAN
 ENSP00000373198
 ENSP00000373614
 ER53_HUMAN
 H2B1C_HUMAN
 H2B1D_HUMAN
 H2B2E_HUMAN
 H33_HUMAN
 IFT5_HUMAN
 IMA1_HUMAN
 JIP2_HUMAN
 K513_HUMAN
 KDD5_HUMAN
 MCM4_HUMAN
 NXT2_HUMAN
 O75477
 O95297
 PBEF_HUMAN
 PP1B_HUMAN
 PSB4_HUMAN
 PXDC1_HUMAN
 Q15555
 Q86UU1
 Q9BTE1
 Q9BV40
 Q9H204
 Q9HC07
 Q9NPE3
 Q9UKI2
 RABP2_HUMAN
 RB5A_HUMAN
 RFX5_HUMAN
 ROA0_HUMAN
 SFR3_HUMAN
 SM31_HUMAN
 SPUF_HUMAN
 TRLT_HUMAN

Downregulated in A_{spring}

2A5E_HUMAN
 A32B_HUMAN
 AFG32_HUMAN
 AMYC_HUMAN
 ANR50_HUMAN
 ARHC_HUMAN
 ARHGA_HUMAN

(Continued)

TABLE E1. (Continued)

A_{spring} vs A_{winter}

ARID2_HUMAN
 AS250_HUMAN
 ASH1L_HUMAN
 ATF6A_HUMAN
 ATX7_HUMAN
 ATY3_HUMAN
 AXN2_HUMAN
 BCAS1_HUMAN
 BGLR_HUMAN
 BIG3_HUMAN
 BTG1_HUMAN
 C122_HUMAN
 CAN13_HUMAN
 CATB_HUMAN
 CBL_HUMAN
 CD166_HUMAN
 CENPL_HUMAN
 CG027_HUMAN
 CIRP_HUMAN
 CLF4_HUMAN
 CLN8_HUMAN
 CLR2_HUMAN
 CMC1_HUMAN
 CNA1_HUMAN
 CO017_HUMAN
 CO2A_HUMAN
 COA2_HUMAN
 CPEB4_HUMAN
 CRCM_HUMAN
 CROP_HUMAN
 CS022_HUMAN
 CUE1_HUMAN
 CXCR7_HUMAN
 D2HDD_HUMAN
 DAK1_HUMAN
 DEOC_HUMAN
 DICE_HUMAN
 DNJCD_HUMAN
 DOCK5_HUMAN
 DR36_HUMAN
 DSG2_HUMAN
 DTX2_HUMAN
 DYNA_HUMAN
 E2AK4_HUMAN
 EDD_HUMAN
 EF2K_HUMAN
 EFC4A_HUMAN
 ELL2_HUMAN
 EML4_HUMAN
 ENSP00000244815
 ENSP00000253251
 ENSP00000339123
 ENSP00000343288
 ENSP00000345275
 EP15_HUMAN
 EPLI_HUMAN
 EPN4_HUMAN
 ETV6_HUMAN
 EVI1_HUMAN
 F119A_HUMAN
 FA3D_HUMAN
 FA98B_HUMAN

(Continued)

TABLE E1. (Continued)

A_{spring} vs A_{winter}

FARP2_HUMAN
 FCMD_HUMAN
 FGD4_HUMAN
 FLNB_HUMAN
 FMO2_HUMAN
 FREM2_HUMAN
 FUBP1_HUMAN
 FX21_HUMAN
 GBQ_HUMAN
 GOA1_HUMAN
 GOGB1_HUMAN
 GOLP4_HUMAN
 GORS2_HUMAN
 HCR_HUMAN
 HEAT2_HUMAN
 HERP2_HUMAN
 HINT1_HUMAN
 HMCS_HUMAN
 HNRPO_HUMAN
 HOK1_HUMAN
 I2C2_HUMAN
 I4G3_HUMAN
 IF3A_HUMAN
 IMB1_HUMAN
 IPP_HUMAN
 ISL1_HUMAN
 ITF2_HUMAN
 JHD2C_HUMAN
 JHD3C_HUMAN
 K1718_HUMAN
 K1815_HUMAN
 K1967_HUMAN
 K2022_HUMAN
 K2026_HUMAN
 KLH12_HUMAN
 L7L2_HUMAN
 LAS1L_HUMAN
 LAT2_HUMAN
 LKAP_HUMAN
 LMB2_HUMAN
 LMG1_HUMAN
 LONP2_HUMAN
 LPP3_HUMAN
 MAML3_HUMAN
 MAST2_HUMAN
 MDM4_HUMAN
 MECP2_HUMAN
 MEI1_HUMAN
 MKLN1_HUMAN
 MOT2_HUMAN
 MRCKB_HUMAN
 MRP3_HUMAN
 MTAP_HUMAN
 MTMRA_HUMAN
 MYCB2_HUMAN
 MYLIP_HUMAN
 MYOP_HUMAN
 MYST4_HUMAN
 NCR1_HUMAN
 NCR2_HUMAN
 NFIB_HUMAN
 NFRKB_HUMAN

(Continued)

TABLE E1. (Continued)

A_{spring} vs A_{winter}

NIAM_HUMAN
 NIGM_HUMAN
 NLFA_HUMAN
 NOTC2_HUMAN
 NRD2_HUMAN
 NU43_HUMAN
 NUFP1_HUMAN
 NUKS_HUMAN
 O95199
 P2Y5_HUMAN
 P2YR_HUMAN
 PAC3_HUMAN
 PAS1_HUMAN
 PASK_HUMAN
 PC20_HUMAN
 PCYXL_HUMAN
 PDCD4_HUMAN
 PDPT_HUMAN
 PDXK_HUMAN
 PER1_HUMAN
 PFTB_HUMAN
 PGT1_HUMAN
 PI51B_HUMAN
 PLCG_HUMAN
 PPCE_HUMAN
 PPCT_HUMAN
 PPRB_HUMAN
 PTN4_HUMAN
 Q15208
 Q6P2H3
 Q6PEV8
 Q6PGP7
 Q6PL18
 Q6T4R5
 Q6TFL4
 Q6UW14
 Q6VVX0
 Q71F56
 Q7Z5K2
 Q86SQ7
 Q86WG5
 Q86XF0
 Q86XS8
 Q86YS7
 Q8IX01
 Q8IZL2
 Q8N3Z6
 Q8N573
 Q8TDY4
 Q8TF01
 Q8WTS1
 Q8WVP7
 Q8WXS4
 Q8WYH8
 Q8WYQ9
 Q96AE7
 Q96IQ7
 Q96IZ0
 Q9BWU1
 Q9H204
 Q9H2U1
 Q9H3M7

(Continued)

TABLE E1. (Continued)

A_{spring} vs A_{winter}

Q9H7S9
 Q9H7T0
 Q9NRF8
 Q9NVF7
 Q9NVV4
 Q9NZC4
 Q9NZI6
 Q9UBF9
 Q9UH65
 Q9UIK5
 Q9UKG1
 RAB14_HUMAN
 RABE1_HUMAN
 RB35A_HUMAN
 RB56_HUMAN
 RBMF_HUMAN
 RBP1_HUMAN
 RCQ1_HUMAN
 RH26_HUMAN
 RHBL2_HUMAN
 RIF1_HUMAN
 RNF10_HUMAN
 RPOM_HUMAN
 RSAD1_HUMAN
 RUFY2_HUMAN
 RUN1_HUMAN
 S2536_HUMAN
 S3A3_HUMAN
 SA1_HUMAN
 SCAB_HUMAN
 SDS3_HUMAN
 SEB_HUMAN
 SETD5_HUMAN
 SF45_HUMAN
 SFPQ_HUMAN
 SFR8_HUMAN
 SFRB_HUMAN
 SJ2B_HUMAN
 SL9A1_HUMAN
 SMA1_HUMAN
 SMA3_HUMAN
 SMA5_HUMAN
 SMRC1_HUMAN
 SP1_HUMAN
 SRRM1_HUMAN
 SSRA_HUMAN
 STL4_HUMAN
 SUCB2_HUMAN
 SUV41_HUMAN
 SYNJ2_HUMAN
 SYQ_HUMAN
 TF_HUMAN
 TFH3_HUMAN
 TM164_HUMAN
 TM168_HUMAN
 TM41A_HUMAN
 TMF1_HUMAN
 TMM80_HUMAN
 TMTC2_HUMAN
 TNP1_HUMAN
 TR2A_HUMAN
 TRAB_HUMAN

(Continued)

TABLE E1. (Continued)

A_{spring} vs A_{winter}

TTC13_HUMAN
TTC19_HUMAN
TTC3_HUMAN
TUS4_HUMAN
TXLNA_HUMAN
UAP56_HUMAN
UBP53_HUMAN
UBP8_HUMAN
UE3A_HUMAN
UGNT_HUMAN
VAN2_HUMAN
VGLL4_HUMAN
VILL_HUMAN
VIPR_HUMAN
VPS8_HUMAN
WD36_HUMAN
XAB2_HUMAN
Y153_HUMAN
YPL2_HUMAN
Z291_HUMAN
Z292_HUMAN
Z337_HUMAN
ZN318_HUMAN
ZN395_HUMAN
ZN529_HUMAN
ZN638_HUMAN
ZRAB3_HUMAN
ZZZ3_HUMAN

H_{spring} vs H_{winter}**Upregulated in H_{spring}**

143Z_HUMAN
AAAS_HUMAN
ABI3_HUMAN
AL3B2_HUMAN
ANXA1_HUMAN
APG5_HUMAN
APP2_HUMAN
ARI1_HUMAN
ASF1A_HUMAN
ATF2_HUMAN
B3GN5_HUMAN
BC13_HUMAN
BCL2_HUMAN
BM02_HUMAN
BMP3_HUMAN
BTG2_HUMAN
BTG4_HUMAN
C561_HUMAN
CAR10_HUMAN
CATS_HUMAN
CBPD_HUMAN
CCRL2_HUMAN
CD032_HUMAN
CDK8_HUMAN
CDN3_HUMAN
CF62_HUMAN
CFAI_HUMAN
CFLA_HUMAN
CHD2_HUMAN
CHP1_HUMAN
CI167_HUMAN

(Continued)

TABLE E1. (Continued)

H_{spring} vs H_{winter}

CN032_HUMAN
CNC9_HUMAN
CO1A1_HUMAN
CP51_HUMAN
CRAD_HUMAN
CXAR_HUMAN
CXCR4_HUMAN
DHRS8_HUMAN
DHS7_HUMAN
DIAC_HUMAN
DNL4_HUMAN
ELP3_HUMAN
ENSP00000257897
ENSP00000271679
ENSP00000283376
ENSP00000305243
ER53_HUMAN
EV2B_HUMAN
EZRI_HUMAN
FA26F_HUMAN
FA62B_HUMAN
FGL2_HUMAN
FLAP_HUMAN
FMNL_HUMAN
FRIH_HUMAN
FRIL_HUMAN
G3P_HUMAN
GB13_HUMAN
GB14_HUMAN
GBB1_HUMAN
GLT5_HUMAN
GMFB_HUMAN
GNAI3_HUMAN
GO45_HUMAN
GS27_HUMAN
GTO1_HUMAN
H2B1C_HUMAN
HAX1_HUMAN
HB21_HUMAN
HB24_HUMAN
HBP1_HUMA
HC58_HUMAN
HECD1_HUMAN
HLAE_HUMAN
IEFS_HUMAN
IL8_HUMAN
INGR_HUMAN
JAK1_HUMAN
KBT2_HUMAN
KLF3_HUMAN
KLF6_HUMAN
KLK4_HUMAN
KPCL_HUMAN
KS6B1_HUMAN
LAL1_HUMAN
LAM5_HUMAN
LCB2_HUMAN
LCP2_HUMAN
LFA3_HUMAN
LSS6_HUMAN
M3K2_HUMAN
MARE1_HUMAN
MARH7_HUMAN

(Continued)

TABLE E1. (Continued)

H _{spring} vs H _{winter}
MGLL_HUMAN
MK01_HUMAN
MSRX_HUMAN
MU13_HUMAN
NAT13_HUMAN
NFIL3_HUMAN
NFKB2_HUMAN
NK25_HUMAN
NUDT4_HUMAN
NXT2_HUMAN
O14792
O95297
OAZ1_HUMAN
OAZ2_HUMAN
ODO1_HUMAN
OTM1_HUMAN
P66B_HUMAN
PAFR_HUMAN
PAG1_HUMAN
PBEF_HUMAN
PDI_HUMAN
PDK1_HUMAN
PGSG_HUMAN
PHR2_HUMAN
PIP2_HUMAN
PM14_HUMAN
PNPH_HUMAN
PNR1_HUMAN
PP1B_HUMAN
PPR3C_HUMAN
PSPC_HUMAN
PTEN_HUMAN
PTN11_HUMAN
PTNC_HUMAN
Q6P3W7
Q7L1Q6
Q7Z5R6
Q7Z739
Q86Y79
Q8IZI9
Q8TBM7
Q8WTS1
Q8WVD5
Q8WYR1
Q96B02
Q96BD6
Q96BX8
Q96K12
Q9BQ75
Q9BUB7
Q9BUP3
Q9H7X3
Q9H8T0
Q9HC07
Q9NP73
Q9NQ25
Q9NT62
Q9NYV6
Q9NZC3
Q9NZC4
Q9UHA3
RA21_HUMAN

(Continued)

TABLE E1. (Continued)

H _{spring} vs H _{winter}
RAC2_HUMAN
RAMP4_HUMAN
RASN_HUMAN
RB31_HUMAN
RB4B_HUMAN
RHEB_HUMAN
RHOA_HUMAN
RPCX_HUMAN
S401_HUMAN
S6A6_HUMAN
SAA4_HUMAN
SEL1L_HUMAN
SGCB_HUMAN
SHB1_HUMAN
SKT_HUMAN
SM31_HUMAN
SM3C_HUMAN
SN15_HUMAN
SNXA_HUMAN
SY04_HUMAN
TANK_HUMAN
TIG1_HUMAN
TM140_HUMAN
TMTC2_HUMAN
TP4A1_HUMAN
TPM4_HUMAN
TSN2_HUMAN
TUSC2_HUMAN
UB2G1_HUMAN
UBL3_HUMAN
UCHL5_HUMAN
UCP3_HUMAN
WASIP_HUMAN
XRN2_HUMAN
YPL5_HUMAN
ZNF79_HUMAN
Downregulated in H_{spring}
2ABA_HUMAN
5NTC_HUMAN
A10B_HUMAN
A2A2_HUMAN
A8B1_HUMAN
AAC4_HUMAN
ACIN_HUMAN
ACOX3_HUMAN
AD28_HUMAN
ADNP_HUMAN
AF4_HUMAN
AGRIN_HUMAN
AKA10_HUMAN
AKIP_HUMAN
AMP2_HUMAN
ANC5_HUMAN
ANR12_HUMAN
ANRX_HUMAN
ANXA1_HUMAN
ANXB_HUMAN
APIG1_HUMAN
API5_HUMAN
ARI1_HUMAN
AS250_HUMAN
ASP1_HUMAN

(Continued)

TABLE E1. (Continued)

H _{spring} vs H _{winter}
ENSP00000262547
ENSP00000268989
ENSP00000284290
ENSP00000295952
ENSP00000297979
ENSP00000327961
ENSP00000342480
ENSP00000343288
ENSP00000345275
ENSP00000348906
ENSP00000354588
ENSP00000354785
ENSP00000359027
ENSP00000360207
ENSP00000365643
ENSP00000366160
ENSP00000369752
ENSP00000373637
ENSP00000374213
ER22_HUMAN
ETV6_HUMAN
EVII_HUMAN
EXT1_HUMAN
F111A_HUMAN
F131_HUMAN
FA62B_HUMAN
FA76B_HUMAN
FAK1_HUMAN
FARP2_HUMAN
FATSO_HUMAN
FBX11_HUMAN
FGD4_HUMAN
FLNB_HUMAN
FNB2_HUMAN
FNBP1_HUMAN
FUBP1_HUMAN
FXC1_HUMAN
FXL3_HUMAN
FYCO1_HUMAN
GATB_HUMAN
GCN1L_HUMAN
GNT5_HUMAN
GO67_HUMAN
GOA4_HUMAN
GOGB1_HUMAN
GPT2_HUMAN
GSP1_HUMAN
GTA4_HUMAN
HBAP1_HUMAN
HECD1_HUMAN
HES1_HUMAN
HNRDL_HUMAN
HRX_HUMAN
I2C2_HUMAN
I4G3_HUMAN
IF44_HUMAN
IHA_HUMAN
INGR_HUMAN
ITAV_HUMAN
ITF2_HUMAN
JHD2C_HUMAN
K0401_HUMAN

(Continued)

TABLE E1. (Continued)

H _{spring} vs H _{winter}
ATX2_HUMAN
B4G5_HUMAN
BA2B_HUMAN
BIR4_HUMAN
BIR6_HUMAN
BMRB_HUMAN
BRD1_HUMAN
BRD2_HUMAN
CANB1_HUMAN
CB04_HUMAN
CBLB_HUMAN
CBP_HUMAN
CBX3_HUMAN
CD166_HUMAN
CDR2_HUMAN
CENPL_HUMAN
CEPT1_HUMAN
CFLA_HUMAN
CHD2_HUMAN
CHD4_HUMAN
CJ038_HUMAN
CJ086_HUMAN
CKRB_HUMAN
CLD1_HUMAN
CLF4_HUMAN
CLK4_HUMAN
CLR2_HUMAN
CN032_HUMAN
CO017_HUMAN
COMD7_HUMAN
CP042_HUMAN
CP35_HUMAN
CPB6_HUMAN
CPSA_HUMAN
CROP_HUMAN
CT121_HUMAN
CTLF1_HUMAN
CTNB_HUMAN
CU63_HUMAN
CUE1_HUMAN
CUL3_HUMAN
CX045_HUMAN
CYA9_HUMAN
DAF_HUMAN
DCXR_HUMAN
DGKD_HUMAN
DGR8_HUMAN
DHRS3_HUMAN
DHX8_HUMAN
DICE_HUMAN
DIP2_HUMAN
DOC6_HUMAN
DX56_HUMAN
DYHC_HUMAN
EDD_HUMAN
EFA5_HUMAN
EFB2_HUMAN
EFER_HUMAN
ELL2_HUMAN
ENSP00000244815
ENSP00000260506
ENSP00000261333

(Continued)

TABLE E1. (Continued)

H _{spring} vs H _{winter}
K0999_HUMAN
K1324_HUMAN
K1718_HUMAN
K2026_HUMAN
K685_HUMAN
KAP2_HUMAN
KC19_HUMAN
KC1E_HUMAN
KPCI_HUMAN
KR18_HUMAN
KSR1_HUMAN
KTN1_HUMAN
LAMA5_HUMAN
LAT2_HUMAN
LMO4_HUMAN
LONP2_HUMAN
LRBA_HUMAN
LU_HUMAN
LUZP1_HUMAN
M3K1_HUMAN
MA1B1_HUMAN
MAML3_HUMAN
MBD6_HUMAN
MCR_HUMAN
MEFA_HUMAN
MEI1_HUMAN
MINT_HUMAN
MKLN1_HUMAN
MLL2_HUMAN
MOT2_HUMAN
MOT5_HUMAN
MRCKB_HUMAN
MRP3_HUMAN
MTA1_HUMAN
MTF2_HUMAN
MY1C_HUMAN
MYCB2_HUMAN
MYG_HUMAN
NAH8_HUMAN
NAT13_HUMAN
NCO2_HUMAN
NCO5_HUMAN
NCR2_HUMAN
NDUS5_HUMAN
NEUL_HUMAN
NFIB_HUMAN
NOM1_HUMAN
NSF_HUMAN
NSUN6_HUMAN
NUMA1_HUMAN
O14964
O60502
O60671
ODB2_HUMAN
P2YR_HUMAN
P85B_HUMAN
PAB1_HUMAN
PABP2_HUMAN
PAR14_HUMAN
PARF_HUMAN
PAS1_HUMAN
PBX1_HUMAN
PCD6_HUMAN

(Continued)

TABLE E1. (Continued)

H _{spring} vs H _{winter}
PCGF3_HUMAN
PCGF5_HUMAN
PCM1_HUMAN
PCTL_HUMAN
PD XK_HUMAN
PECR_HUMAN
PGAP1_HUMAN
PGBM_HUMAN
PIA1_HUMAN
PIPNB_HUMAN
PKD1_HUMAN
PKL2_HUMAN
PLA5_HUMAN
PLS1_HUMAN
PO21_HUMAN
PO23_HUMAN
POLS_HUMAN
PPL2_HUMAN
PPME1_HUMAN
PR38B_HUMAN
PR4B_HUMAN
PRD15_HUMAN
PRP31_HUMAN
PUR_HUMAN
Q6ISB3
Q6NT76
Q6PCB7
Q6PH85
Q6T4R5
Q70CQ2
Q71F56
Q7Z3B3
Q86SQ7
Q86UV5
Q86VN1
Q8IX01
Q8IZL2
Q8N157
Q8N1G2
Q8N3Z6
Q8N823
Q8TF01
Q8WXI7
Q96AE7
Q96DE5
Q96FZ2
Q96HE9
Q96HH9
Q96IP4
Q96S59
Q96SQ5
Q96SW2
Q99590
Q9BWQ6
Q9GZS3
Q9H5J8
Q9H7T0
Q9HC07
Q9NRD9
Q9NXL6
Q9NZC4
Q9NZI6
Q9NZV1

(Continued)

TABLE E1. (Continued)

H _{spring} vs H _{winter}
Q9UHB7
R152_HUMAN
R35A_HUMAN
RAP2_HUMAN
RAYL_HUMAN
RB18_HUMAN
RB56_HUMAN
RBCC1_HUMAN
RBM25_HUMAN
RBM6_HUMAN
RBMF_HUMAN
RDH13_HUMAN
RERE_HUMAN
RFNG_HUMAN
RH26_HUMAN
RIOK3_HUMAN
RMP_HUMAN
RNF12_HUMAN
RNF19_HUMAN
ROA3_HUMAN
RPA2_HUMAN
RPB1_HUMAN
RSG2_HUMAN
RT29_HUMAN
RU2A_HUMAN
RUFY2_HUMAN
RUN1_HUMAN
RUND1_HUMAN
S3A3_HUMAN
SA1_HUMAN
SAB1_HUMAN
SAFB1_HUMAN
SAM4B_HUMAN
SCFD1_HUMAN
SDS3_HUMAN
SEC6_HUMAN
SETD5_HUMAN
SF45_HUMAN
SFB2_HUMAN
SFPQ_HUMAN
SFR15_HUMAN
SFR16_HUMAN
SFR4_HUMAN
SFR5_HUMAN
SFR7_HUMAN
SFR9_HUMAN
SFRC_HUMAN
SG223_HUMAN
SGA1_HUMAN
SH3R2_HUMAN
SI1L1_HUMAN
SKT_HUMAN
SL53_HUMAN
SLE1_HUMAN
SM3C_HUMAN
SM3F_HUMAN
SMA3_HUMAN
SMC5_HUMAN
SMCA4_HUMAN
SMG1_HUMAN
SMRC1_HUMAN
SMU1_HUMAN

(Continued)

TABLE E1. (Continued)

H _{spring} vs H _{winter}
SNB2_HUMAN
SORL_HUMAN
SP1_HUMAN
SP10_HUMAN
SP18_HUMAN
SPAG8_HUMAN
SPHM_HUMAN
SPTCS_HUMAN
SRRM1_HUMAN
SSRD_HUMAN
STRBP_HUMAN
STXG_HUMAN
SUV41_HUMAN
T172_HUMAN
T240_HUMAN
T2RE_HUMAN
TE2I_HUMAN
TEP1_HUMAN
TERA_HUMAN
TFB1M_HUMAN
TFCP2_HUMAN
TFH1_HUMAN
TGFB2_HUMAN
THB1_HUMAN
THI2_HUMAN
THO2_HUMAN
THO3_HUMAN
THU2_HUMAN
TIZ2_HUMAN
TMF1_HUMAN
TNP1_HUMAN
TOB2_HUMAN
TR2A_HUMAN
TRIB_HUMAN
TRPS_HUMAN
TSH2_HUMAN
TTC18_HUMAN
U520_HUMAN
UB21_HUMAN
UB6C_HUMAN
UBE3C_HUMAN
UBP36_HUMAN
UBXD7_HUMAN
UDG2_HUMAN
VAN2_HUMAN
VDP_HUMAN
VGLL4_HUMAN
VKGC_HUMAN
VP35_HUMAN
VP37B_HUMAN
WD11_HUMAN
WDR6_HUMAN
YAF2_HUMAN
YES_HUMAN
YTH1_HUMAN
Z141_HUMAN
Z251_HUMAN
Z292_HUMAN
Z335_HUMAN
Z339_HUMAN
ZBT5_HUMAN
ZDHC7_HUMAN

(Continued)

TABLE E1. *(Continued)***H_{spring} vs H_{winter}**

ZDHD_HUMAN
ZDHL_HUMAN
ZFAN6_HUMAN
ZIN_HUMAN
ZN294_HUMAN
ZN395_HUMAN
ZN44_HUMAN
ZN503_HUMAN
ZN638_HUMAN
ZO3_HUMAN
ZRAB3_HUMAN
ZSW6_HUMAN
ZUBR1_HUMAN
ZY16_HUMAN

Only probes yielding 1 protein sequence are included.

Table E2. GO enrichment of the differentially expressed transcripts converted to corresponding SwissProt proteins

A_{Winter} vs H_{Winter}	P value	All	Interest	Expected
Upregulated in A _{Winter}				
signal_recognition_particle	.001	11	2	0.066
Downregulated in A _{Winter}				
2-5-oligoadenylate_synthetase_activity	.003	2	1	0.003
ISG15-protein_conjugation	.003	2	1	0.003
All regulated transcripts				
metabolism	.038	404	7	3.137
binding	.039	241	5	1.877
lyase_activity	.022	77	3	0.597
isomerase_activity	.032	90	3	0.698
signal_recognition_particle	.002	11	2	0.085
A_{Spring} vs H_{Spring}	P value	All	Interest	Expected
Upregulated in A _{Spring}				
transport	.007	433	5	1.246
neurogenesis	.025	222	3	0.639
cadmium_ion_binding	.001	6	2	0.017
cell_body	.001	21	2	0.060
copper_ion_binding	.008	48	2	0.138
regulation_of_apoptosis	.015	67	2	0.192
heme_binding	.029	93	2	0.267
growth_factor_activity	.036	104	2	0.299
Downregulated in A _{Spring}				
cell_cycle	.030	322	4	1.185
proteolysis_and_peptidolysis	.032	329	4	1.212
heart_development	.001	59	3	0.217
response_to_virus	.002	70	3	0.257
serine-type_endopeptidase_activity	.002	75	3	0.276
Wnt_receptor_signaling_pathway_through_β-catenin	.001	2	2	0.007
β-catenin_destruction_complex	.001	2	2	0.007
lateral_plasma_membrane	.001	5	2	0.018
dephosphorylation	.003	7	2	0.025
androgen_receptor_binding	.001	14	2	0.051
androgen_receptor_signaling_pathway	.003	23	2	0.084
G1/S_transition_of_mitotic_cell_cycle	.007	25	2	0.092
dorsal/ventral_pattern_formation	.007	25	2	0.092
response_to_DNA_damage_stimulus	.003	25	2	0.092
innate_immune_response	.009	41	2	0.150
response_to_stress	.015	52	2	0.191
cell_cycle_arrest	.012	58	2	0.213
transcription_factor_complex	.019	59	2	0.217
calcium_ion_transport	.025	60	2	0.224
Wnt_receptor_signaling_pathway	.021	61	2	0.224
epidermis_development	.021	61	2	0.224
protein_kinase_activity	.021	61	2	0.224
chromatin_binding	.021	64	2	0.235
chromosome_organization_and_biogenesis	.031	75	2	0.276
skeletal_development	.046	93	2	0.342
cytokine_activity	.047	95	2	0.349
negat_regulation_of_transcription_from_Pol_II_promoter	.047	95	2	0.349
All regulated transcripts				
transport	.002	433	9	2.840
cell_differentiation	.022	263	5	1.725
heart_development	.006	59	4	0.387
lipid_metabolism	.031	181	4	1.187
response_to_virus	.010	70	3	0.459
chromosome_organization_and_biogenesis	.013	75	3	0.492
serine-type_endopeptidase_activity	.013	75	3	0.492
skeletal_development	.023	93	3	0.610
cytokine_activity	.024	95	3	0.623
growth_factor_activity	.030	104	3	0.682
Wnt_receptor_signaling_pathway_through_β-catenin	.004	2	2	0.013
β-catenin_destruction_complex	.004	2	2	0.013

(Continued)

Table E2. (Continued)

A _{Spring} vs H _{Spring}	P value	All	Interest	Expected
lateral_plasma_membrane	.004	5	2	0.032
heart_development	.006	59	4	0.387
cadmium_ion_binding	.006	6	2	0.039
dephosphorylation	.005	7	2	0.047
aldehyde_metabolism	.001	9	2	0.059
epithelial_to_mesenchymal_transition	.001	9	2	0.059
transport	.002	433	9	0.029
androgen_receptor_binding	.003	14	2	0.091
A _{Spring} vs A _{winter}	P value	All	Interest	Expected
Upregulated in A _{Spring}				
protein_transport	.017	250	4	1.040
ER_to_Golgi_transport	.001	60	3	0.240
defense_response_to_bacteria	.002	63	3	0.253
Golgi_apparatus	.022	151	3	0.606
GTPase_activity	.024	155	3	0.622
Structural_molecule_activity	.048	203	3	0.815
Downregulated in A _{Spring}				
nucleic_acid_binding	.013	426	18	9.866
ligase_activity	.004	190	11	4.400
cytoskeleton	.023	232	11	5.373
mRNA_processing	.006	146	9	3.381
integral_to_plasma_membrane	.020	802	9	18.57
posit_regulation_of_transcription_from_Pol_II_promoter	.002	99	8	2.292
transcriptional_activator_activity	.006	91	7	2.107
RNA_polymerase_II_transcription_factor_activity	.010	104	7	2.408
actin_cytoskeleton	.003	64	6	1.482
guanyl-nucleotide_exchange_factor_activity	.009	79	6	1.829
ubiquitin-protein_ligase_activity	.018	90	6	2.084
transcription_corepressor_activity	.019	92	6	2.130
negat_regulation_of_transcription_from_Pol_II_promoter	.022	95	6	2.200
ubiquitin-dependent_protein_catabolism	.024	96	6	2.223
spliceosome_complex	.025	97	6	2.246
GTPase_activator_activity	.038	107	6	2.478
positive_regulation_of_transcription_DNA-dependent	.012	60	5	1.389
negative_regulation_of_transcription	.019	67	5	1.551
cytoskeletal_protein_binding	.002	24	4	0.555
regulation_of_translational_initiation	.003	27	4	0.625
Notch_signaling_pathway	.006	32	4	0.741
Rho_guanyl-nucleotide_exchange_factor_activity	.019	45	4	1.042
regulation_of_Rho_protein_signal_transduction	.021	46	4	1.065
thyroid_hormone_receptor_binding	.004	15	3	0.347
Double-stranded_DNA_binding	.009	19	3	0.440
phospholipid_metabolism	.009	19	3	0.440
ER-Golgi_intermediate_compartment	.017	24	3	0.555
transcription_cofactor_activity	.017	24	3	0.555
protein_catabolism	.019	25	3	0.579
nuclear_mRNA_splicing_via_spliceosome	.023	27	3	0.625
regulation_of_GTPase_activity	.031	30	3	0.694
extracellular	.004	521	3	12.06
extracellular_space	.039	380	3	8.801
All regulated transcripts				
RNA_binding	.001	385	24	10.437
Nucleic_acid_binding	.015	426	20	11.548
RNA_splicing	.009	157	14	4.256
cytoskeleton	.036	232	12	6.289
mRNA_processing	.002	146	11	3.958
ligase_activity	.019	190	11	5.150
integral_to_plasma_membrane	.006	2	10	21.742
Golgi_apparatus	.021	15	9	4.093
chromatin_modification	.002	87	8	2.358

(Continued)

Table E2. (Continued)

H _{Spring} vs H _{winter}	P value	All	Interest	Expected
posit_regulation_of_transcription_from_Pol_II_promoter	.005	99	8	2.683
chromosome	.019	124	8	3.361
actin_cytoskeleton	.001	64	7	1.735
transcriptional_activator_activity	.011	91	7	2.467
ubiquitin-dependent_protein_catabolism	.015	96	7	2.602
RNA_polymerase_II_transcription_factor_activity	.022	104	7	2.819
nucleosome_1	.019	78	6	2.114
guanyl-nucleotide_exchange_factor_activity	.020	79	6	2.141
nucleosome_assembly	.033	89	6	2.412
ubiquitin-protein_ligase_activity	.035	90	6	2.439
transcription_corepressor_activity	.038	92	6	2.494
negat_regulation_of_transcript_from_Pol_II_promoter	.044	95	6	2.575
spliceosome_complex	.048	97	6	2.629
positive_regulation_of_transcription_DNA-dependent	.023	60	5	1.626
negative_regulation_of_transcription	.035	67	5	1.816
early_endosome	.002	20	4	0.542
ER-Golgi_intermediate_compartment	.003	24	4	0.650
cytoskeletal_protein_binding	.003	24	4	0.650
regulation_of_translational_initiation	.005	27	4	0.731
Notch_signaling_pathway	.010	32	4	0.867
Rho_guanyl-nucleotide_exchange_factor_activity	.033	45	4	1.219
regulation_of_Rho_protein_signal_transduction	.035	46	4	1.247
blood_coagulation	.049	51	4	1.382
extracellular_space	.037	380	4	10.30
nuclear_localization_sequence_binding	.001	8	3	0.216
thyroid_hormone_receptor_binding	.007	15	3	0.406
double-stranded_DNA_binding	.013	19	3	0.515
phospholipid_metabolism	.013	19	3	0.515
transcription_cofactor_activity	.026	24	3	0.650
protein_catabolism	.029	25	3	0.677
response_to_DNA_damage_stimulus	.029	25	3	0.677
nuclear_mRNA_splicing_via_spliceosome	.035	27	3	0.731
regulation_of_GTPase_activity	.046	30	3	0.813
extracellular	.002	521	3	14.12
ion_transport	.038	333	3	9.021
H _{Spring} vs H _{winter}	P value	All	Interest	Expected
Upregulated in H _{Spring}				
immune_response	.003	303	13	4.340
cell_cycle	.002	322	12	4.612
GTPase_activity	.006	155	10	2.220
oxidoreductase_activity	.032	341	10	4.884
small_GTPase_mediated_signal_transduction	.006	166	9	2.377
intracellular_signaling_cascade	.009	247	9	3.538
ubiquitin_cycle	.014	265	9	3.796
GTP_binding	.019	280	9	4.010
binding	.022	241	8	3.452
protein_transport	.027	250	8	3.581
cell_motility	.005	97	7	1.389
chemotaxis	.005	99	7	1.418
negative_regulation_of_cell_proliferation	.003	138	7	1.976
protein_modification	.013	175	7	2.506
inflammatory_response	.014	179	7	2.565
lipid_metabolism	.015	181	7	2.592
signal_transducer_activity	.031	210	7	3.008
cell_proliferation	.040	221	7	3.165
anti-apoptosis	.005	113	6	1.611
actin_binding	.035	170	6	2.435
regulation_of_apoptosis	.002	67	5	0.959
cell_division	.048	138	5	1.976
receptor_activity	.046	808	5	11.57
protein_tyrosine_phosphatase_activity	.004	48	4	0.687

(Continued)

Table E2. (Continued)

H _{Spring} vs H _{winter}	P value	All	Interest	Expected
cell_cycle_arrest	.009	58	4	0.830
ER_to_Golgi_transport	.010	60	4	0.859
protein_kinase_activity	.011	61	4	0.873
response_to_virus	.017	70	4	1.027
protein_amino_acid_dephosphorylation	.028	81	4	1.160
induction_of_apoptosis	.034	86	4	1.231
regulation_of_cell_migration	.001	17	3	0.243
Rho_protein_signal_transduction	.005	25	3	0.358
nuclear_membrane	.009	31	3	0.444
transmembr_receptor_prot_tyrosi_kinase_signal_pathway	.048	57	3	0.816
Downregulated in H _{Spring}				
RNA_binding	.000	385	28	12.91
metal_ion_binding	.000	1638	82	54.93
zinc_ion_binding	.000	1615	80	54.12
transcription_factor_binding	.000	66	9	2.213
ubiquitin_cycle	.001	265	21	8.887
nucleotide_binding	.002	1214	62	40.71
positive_regulation_of_transcription_DNA-dependent	.0021	60	8	2.012
nuclear_mRNA_splicing_via_spliceosome	.001	27	5	0.90
protein_ubiquitination	.002	28	5	0.93
intracellular	.002	407	68	47.18
transcription_coactivator_activity	.002	120	11	4.024
DNA_binding	.002	947	49	31.75
eye_morphogenesis	.002	9	3	0.301
transcription_factor_activity	.002	672	37	22.53
basal_lamina	.003	3	2	0.100
synapse_organization_and_biogenesis	.003	3	2	0.100
protein_binding	.003	578	148	119.9
transcription_from_Pol_II_promoter	.003	145	12	4.866
transcription_corepressor_activity	.003	92	9	3.085
RNA_splicing_factor_activity_transesterific_mechanism	.003	20	4	0.670
regul_of_small_GTPase_mediated_signal_transduction	.003	32	5	1.073
blood_vessel_remodeling	.006	4	2	0.134
clathrin_coat_of_trans-Golgi_network_vesicle	.006	4	2	0.134
NLS-bearing_substrate-nucleus_import	.006	12	3	0.402
negative_regulation_of_transcription	.007	67	7	2.246
response_to_hypoxia	.010	14	3	0.469
chromatin_assembly/disassembly	.010	26	4	0.871
hemopoiesis	.010	26	4	0.871
apoptotic_mitochondrial_changes	.010	5	2	0.167
lateral_plasma_membrane	.010	5	2	0.167
mitotic_anaphase	.010	5	2	0.167
translation_factor_activity_nucleic_acid_binding	.010	5	2	0.167
thyroid_hormone_receptor_binding	.012	15	3	0.503
vesicle_docking_during_exocytosis	.012	15	3	0.503
nucleic_acid_binding	.013	426	24	14.28
ligase_activity	.013	190	13	6.371
DNA-directed_RNA_polymerase_II_core_complex	.015	16	3	0.536
RNA_pol_II_transcription_factor_acti_enhan_binding	.015	16	3	0.536
Ubiquitin-dependent_protein_catabolism	.015	96	8	3.219
Ubiquitin_ligase_complex	.015	29	4	0.972
ATP-dependent_proteolysis	.015	6	2	0.201
embryonic_hindlimb_morphogenesis	.015	6	2	0.201
mRNA_splice_site_selection	.015	6	2	0.201
poly(A)_binding	.015	6	2	0.201
protein_modification	.017	175	12	5.868
posit_regulation_of_transcription_from_Pol_II_promoter	.018	99	8	3.320
ATP-dependent_RNA_helicase_activity	.021	18	3	0.603
induction_of_apoptosis_by_extracellular_signals	.021	18	3	0.603
ATP-dependent_peptidase_activity	.021	7	2	0.234
ephrin_receptor_binding	.021	7	2	0.234
Notch_signaling_pathway	.021	32	4	1.073

(Continued)

Table E2. (Continued)

A _{Spring} vs A _{winter}	P value	All	Interest	Expected
single-stranded_DNA_binding	.021	32	4	1.003
All regulated transcripts				
DNA_binding	.011	947	61	44.50
transcription_factor_activity	.006	672	47	31.57
RNA_binding	.013	385	29	18.09
ubiquitin_cycle	.004	265	28	12.45
RNA_splicing	.000	157	25	7.377
receptor_activity	.007	808	23	37.96
mRNA_processing	.001	146	22	6.860
protein_modification	.001	175	18	8.223
ligase_activity	.013	190	17	8.928
mitochondrion	.018	610	17	28.66
ubiquitin-protein_ligase_activity	.007	90	14	4.224
transcription_coactivator_activity	.001	120	14	5.634
transcription_from_Pol_II_promoter	.009	145	14	6.817
spliceosome_complex	.007	97	13	4.554
extracellular	.005	521	12	24.44
transcription_factor_binding	.007	66	10	3.101
transcription_corepressor_activity	.011	92	10	4.323
ubiquitin-dependent_protein_catabolism	.014	96	10	4.511
regulation_of_apoptosis	.003	67	9	3.148
lipid_binding	.019	86	9	4.041
cell_motility	.047	97	9	4.558
positive_regulation_of_transcription_DNA-dependent	.006	60	8	2.819
double-stranded_DNA_binding	.001	19	7	0.892
ER_to_Golgi_transport	.021	60	7	2.819
negative_regulation_of_transcription	.037	67	7	3.148
response_to_virus	.045	70	7	3.289
protein_ubiquitination	.001	28	6	1.315
regul_of_small_GTPase_mediated_signal_transduction	.003	32	6	1.503
diacylglycerol_binding	.016	44	6	2.067
regulation_of_cell_migration	.001	17	5	0.798
chromatin_assembly/disassembly	.006	26	5	1.221
nuclear_mRNA_splicing_via_spliceosome	.007	27	5	1.268
Notch_signaling_pathway	.015	32	5	1.503
Golgi_membrane	.020	34	5	1.597
regulation_of_cell_proliferation	.020	34	5	1.597
endoplasmic_reticulum_membrane	.045	42	5	1.973
DNA-directed_RNA_polymerase_II_core_complex	.005	16	4	0.751
RNA_splicing_factor_act_transesterifi_mechanism	.012	20	4	0.939
hemopoiesis	.031	26	4	1.221
ubiquitin_ligase_complex	.045	29	4	1.362
rhodopsin-like_receptor_activity	.036	219	4	10.29
double-strand_break_repair	.004	8	3	0.375
eye_morphogenesis	.007	9	3	0.422
histone_acetyltransferase_activity	.012	11	3	0.516
microvillus	.012	11	3	0.516
NLS-bearing_substrate-nucleus_import	.016	12	3	0.563
patterning_of_blood_vessels	.025	14	3	0.657
response_to_hypoxia	.025	14	3	0.657
C-C_chemokine_receptor_activity	.030	15	3	0.704
calcium-mediated_signaling	.030	15	3	0.704
negative_regulation_of_cell_differentiation	.030	15	3	0.704
thyroid_hormone_receptor_binding	.030	15	3	0.704
vesicle_docking_during_exocytosis	.030	15	3	0.704
RNA_pol_II_transcription_factor_act_enhancer_binding	.036	16	3	0.751
ubiquitin-like-protein_ligase_activity	.036	16	3	0.751
ATP-dependent_RNA_helicase_activity	.049	18	3	0.845
I-κB_kinase/NF-κB_cascade	.049	18	3	0.845
chromosome_segregation	.049	18	3	0.845
induction_of_apoptosis_by_extracellular_signals	.049	18	3	0.845

P values were determined by using the 2-sided Fisher exact probability test.

All, Number of all proteins within the GO category; Interest, number of observed proteins; Expected, number of proteins in a category by chance; GTPase, guanosine triphosphatase; GTP, guanosine triphosphate; ATP, adenosine triphosphate.