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ORIGINAL PAPER

Targeted disruption of the galectin-3 gene results in decreased susceptibility to NNK-induced lung tumorigenesis: an oligonucleotide microarray study

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Abstract
Purpose Galectin-3, a β-galactoside-binding animal lectin is a multifunctional protein, which regulates cell growth, cell adhesion, cell proliferation, angiogenesis, and apoptosis, and in turn contributes to tumorigenesis and metastasis. The aim of this study was to clarify the role or related mechanisms of galectin-3 in lung carcinogenesis.
Methods We administrated 4-(methylnitrosamino)-1-(3-pyridyle)-1-butanone (NNK), a powerful chemical carcinogen into galectin-3 wild-type (gal3+/+) and galectin-3 knock-out (gal3−/−) CD1 mice by intraperitoneal injection, examined the expression status of 22,690 mouse genes of the NNK-induced tumors using Affymetrix GeneChip mouse expression 430 A arrays, and then analyzed functional network and gene ontology by Ingenuity Pathway Analysis. Real-time PCR was also employed to partially confirm the genechip data.
Results Compared with the gal3+/+ mice, the incidence of lung tumors was significantly low in gal3−/− mice after 32 weeks (28.6 vs 52.1%, $P < 0.05$). Pathway analysis indicated that galectin-3 up-regulated carcinogenesis-related genes (e.g. B-cell receptor, ERK/MAPK, and PPAR signalings) in normal condition, and lung cancer and NNK-induced gene expression associated with cellular growth (e.g. Wnt/β-catenin signaling) or immunological disease (e.g. EGF and PDGF signalings) in lung carcinogenesis with or without the galectin-3 control, respectively.
Conclusion Disrupted galectin-3 may attenuate the lung carcinogenesis due to its regulatory role in the B-cell receptor, ERK/MAPK, and PPAR signal pathways.

Keywords Galectin-3 · NNK · Lung tumor · Oligonucleotide microarrays · IPA

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Introduction

Galectins are a family of animal β-galactoside-binding lectins characterized by conserved-sequence elements in their carbohydrate-binding sites (CRD) (Liu 2000). Galectin-3, a protein (~30 kDa) which is expressed in the monocyte/macrophage and a variety of epithelial cells (Barondes et al. 1994) has demonstrated to involve in several biological processes, such as cell–cell/matrix interactions, cell cycle and cell growth regulation, nuclear splicing of pre-mRNA, and angiogenesis (Liu et al. 2002; Perillo et al. 1998; Danguy et al. 2002). Galectin-3 expression has been found in a number of neoplastic cell types with possible roles in

tumorigenesis and metastasis (Hughes 2001; Hittelet et al. 2003). It has therefore been explored as a potential prognostic factor for colorectal (Castronovo et al. 1992; Nakamura et al. 1999; Bresalier et al. 1998; Lotz et al. 1993), head and neck (Piantelli et al. 2002; Honjo et al. 2000), pancreatic (Shimamura et al. 2002), and gastric cancers (Baldus et al. 2000). In lung cancers, differential expression of galectin-3 between histological types has suggested its important influences on tumor cell adhesion, apoptosis, and responses to chemotherapy (Buttery et al. 2004). In addition, nuclear galectin-3 expression appears to be an independent predictive factor of recurrence for both adenocarcinomas and squamous cell carcinomas of the lung (Mathieu et al. 2005). Recently, Yoshimura et al. (2003) also described differential expression between small-cell lung carcinoma (SCLC) and non-SCLC (NSCLC) when measured by real-time quantitative RT-PCR.

Lung cancer worldwide constitutes 16% of all malignant tumors and accounts for 28% of cancer deaths (Greeblee et al. 2001). The secular trend is that women are more susceptible than men to tobacco-induced carcinogenesis and may show higher risk than men for lung cancer development from smoking because estrogens may be involved in lung carcinogenesis directly by stimulating the transcription of estrogen-responsive genes in the nucleus of lung cells and transactivating growth factor signaling pathways, in particular, the epidermal growth factor pathway (Alberg et al. 2005). The third trend in lung cancer histology indicates that the proportion or incidence of lung adenocarcinoma (AD) has been increasing markedly over the past two decades, surpassing the squamous cell carcinoma (SQ) as the most common histological subtype of lung cancer in many countries (Stabile and Siegfried 2004). Gene-environment interaction is postulated to involve in this change, especially increased nitrate levels from filter tobacco smoke and air pollution.

A potent tobacco carcinogen, 4-(methylnitrosamino)-1-(3-pyridyle)-1-butanone (NNK), is most likely to contribute to the induction of human lung cancer in combination with polycyclic aromatic hydrocarbons (PAHs) and strongly related to human pulmonary adenocarcinoma. Animal experiments indicated that NNK induces lung adenocarcinoma in mice, rats, and Syrian golden hamsters, independent of administration route and the lung is an important target organ for NNK-induced carcinogenesis in rodents. NNK is known to be activated well in the lung, with production of methylating and pyridyloxobutylating agents that attack DNA and cause mutations (Nettesheim 1991). With lung carcinogenesis models, NNK may help to gain insights into basic lung tumor biology and etiology, assist in finding markers for early diagnosis, and finally promote anti-lung cancer therapies.

Several reports have stressed the importance of high-density oligonucleotide microarrays for rapid simultaneous analysis of expression levels of large numbers of genes, overcoming the limitations inherent in analysis of single genes (Mcpherson et al. 2001). In addition, studies of expression profiles of adenocarcinomas of the lung using different commercially available chips identified different classes of tumors, albeit with some overlap (Bhattacharjee et al. 2001). Hsu et al (2000) generated galectin-3 knock-out mouse model through the disruption of its exon-5 using CD1 mouse and found that the galectin-3-deficient mice exhibit attenuated peritoneal inflammatory responses and phagocytic clearance of apoptotic thymocytes by peritoneal macrophages, developed accelerated glomerulopathy, non-alcoholic fatty-liver disease in male mice and dramatically lower the basal level of JNK1 protein in bone marrow-derived mast cells (Hsu et al. 2000; Pugliese et al. 2001; Sano et al. 2003; Stitt et al. 2005; Chen et al. 2006; Nomoto et al. 2006; Silva-Monteiro et al. 2007). Here, we established lung tumor model induced by NNK in female galectin-3 knock out (gal3−/−) CD1 and wild-type (gal3+/+) mice, and clarify the molecular mechanisms of galectin-3 and NNK in female lung carcinogenesis with high-density oligonucleotide microarrays and GeneSpring analysis, followed by real-time PCR and immunohistochemistry.

Materials and methods

Animals and treatments

Galectin-3 knock-out (gal3−/−) mice and wild-type littermates (gal3+/+) generated on a CD1 background were generously provided by Dr. Hsu's group (Hsu et al. 2000). Every three mice were housed in a plastic cage with paper chips for bedding. All had access to standard rodent food (CE-2, CLEA Japan, Tokyo, Japan) and water ad libitum, and were housed under pathogen-free conditions in a temperature-controlled animal room with a 12-h light/dark illumination cycle. Animal use procedures were in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Committee on Animal Experimentation of the University of Toyama. NNK (Toronto Research Chemicals Inc., North York, Canada) was dissolved in 0.9% sterile NaCl at a concentration of 2 mg/0.2 ml and administered via intraperitoneal (IP) injection to two groups of 18 gal3+/+ and 28 gal3−/− mice at 2 mg (10 μmol)/mouse/week for 6 consecutive weeks and sacrificed at 28 weeks after the first injection. The 48 gal3+/+ and 49 gal3−/− CD1 female mice were treated as above as the second group, but sacrificed at 32 weeks after the first injection. In each case, control groups of 14 mice were also maintained only with intraperitoneal injection of physiological saline, respectively.

Histological examination

After animals were killed under sodium pentobarbital anesthesia, lungs of all animals were carefully inspected grossly. One portion of normal lung or lesion was immediately frozen in liquid nitrogen and then stored at –80°C until used for RNA extraction. The other portion was fixed with 10% formalin, embedded in paraffin, incised into 4 μm sections, and stained with hematoxylin and eosin for histopathological examination. Lung lesions were independently examined and diagnosed by three pathologists (Takano Y, Takahashi H, and Abdel-Aziz H. O.) according to the International Classification of Rodent Tumors (Dungworth et al. 2001). We diagnosed lung proliferative lesions as hyperplasia and tumors, and did not subclassify the tumors into adenomas and adenocarcinomas because of the difficulty in evaluating malignancy (Rehm et al. 1994).

RNA preparation

Each frozen sample was homogenized with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was extracted according to the recommendation with the treatment of DNase I (RNase-free DNase kit, Qiagen, Valencia, CA, USA) for 15 min at room temperature to remove residual genomic DNA.

Affymetrix GeneChip hybridization

For investigation of gene expression, the Affymetrix® mouse expression 430 A array was applied in the present study. Sample preparation was carried out as described in the Affymetrix GeneChip® Expression Technical Manual. Briefly, 5 μg of total RNA was used to synthesize double-strand cDNA with a GeneChip® Expression 3′-Amplification Reagents One-Cycle Cdna Synthesis Kit (Affymetrix, Santa Clara, CA, USA). Biotin-labeled cRNA was then synthesized using GeneChip® Expression 3′-Amplification Reagents for IVT Labeling (Affymetrix). After fragmentation, the biotinylated cRNA was hybridized to a GeneChip array at 45°C for 16 h. The chip was washed, stained with streptavidin-phycoerythrin, scanned with a GeneChip scanner 3000 (Affymetrix), and the results were analyzed using GeneChip Analysis Suite Software (Affymetrix). Hybridization intensity data were converted into presence/absence calls for each gene and changes in gene expression between experiments were detected by comparison analysis. The data were further analyzed using GeneSpring version 7.3 (Silicon Genetics, Redwood City, CA, USA) to extract significant genes and determine the gene ontology, including biological processes, cellular component, and molecular functions. A difference of twofold or more was applied to select up-regulated and down-regulated genes.

Real-time PCR

Ten genes included in the highly significant canonical pathways (Wnt/β-catenin, EGF, PDGF, β-cell receptor signaling, and PPAR) were chosen for further validation by real-time PCR. Total lung complementary DNA (cDNA) was synthesized from 400 ng aliquots using TaqMan Reverse Transcription Reagents (Applied Biosystems, Tokyo, Japan). PCR and analyses were carried out using an Mx3000P QPCR System (Stratagene, La Jolla, CA, USA) with denaturation for 10 min at 95°C, followed by 50 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 30 s. The amount of mRNA was calculated using GAPDH as an endogenous control. All primers and probes used for analysis were designed at Nippon EGT (Toyama, Japan) as given in Table 1.

Network, gene ontology, and canonical pathway analysis

Genes identified by GeneSpring were used for network and gene ontology analyses. Gene accession numbers were imported into the Ingenuity Pathway Analysis version 3.1 (IPA) software (Ingenuity Systems, Mountain View, CA, USA) and the gene products were categorized based on location, cellular components, and reported or suggested biochemical, biologic, and molecular functions using the software. Mapping to genetic networks available in the Ingenuity database was also performed with ranking by score, the probability that a collection of genes equal to or greater than the number in a network could be achieved by chance alone. A score of three indicates that there is a 1/1,000 chance that the focus genes are in a network due to random chance. Therefore, scores of three or higher have a 99.9% confidence level of not being generated by random chance alone. This score was used as the cut off for identifying gene networks.

Statistical analysis

Statistical evaluation was performed using Spearman test to analyze the rank data, Fisher test to compare the positive rate and Mann–Whitney test to differentiate non-parametric means of different groups. $P < 0.05$ was considered as statistically significant. SPSS 10.0 software was employed to analyze all data.

Results

Histopathology

Regardless of gal3−/− and gal3+/+ mice, NNK administration caused alveolar cell hyperplasia, alveolar adenoma or

Table 1 Nucleotide sequences of TaqMan primers and probes for target genes used in quantitative real-time PCR

Genes	Orientation	Nucleotide sequence (position)	Tm	Length
BCL-6	1343 Forward	5′TATGGAGCCCGCGAACCT3′	47.5	18
	1405 Backward	5′GAGTCCTCCCCACTGGCA3′	49.7	18
	1364 Probe	5′TCTCCAGTCCCCGACCAAGCTCA3′	55.5	23
CDH2	2031 Forward	5′GAACAGGGTGGACGTCATTGT3′	58	21
	2108 Backward	5′GCCGCATTCCAGGCC3′	58	15
	2058 Probe	5′CCTAACTGTCACGGACAAAGATCAGCCC3′	68	28
CNSK2A2	1226 Forward	5′AGCCCGGAGGCCCTAGA 3′	59	17
	1294 Backward	5′TTTGGCGGTCAATCTCTGTTG 3′	60	21
	3403 Probe	5′TCTTGACAAGCTCCTGCGGTACGACC3′	69	26
PDGFα	564 Forward	5′CGCTGCACTGGCTGTTGT3′	47.5	18
	631 Backward	5′ACACTGCGGTGGTGGACC3′	49.7	18
	587 Probe	5′CAGCAGCGTCAAGTGCCAGCCTT3′	55.5	23
JUN	920 Forward	ACTGCAAGATGGAAACGACCT	47.9	22
	998 Backward	AGCCGTAGGCACCGCTCT	49.7	18
	949 Probe	CGATGCCCTCAACGCCTCGTTC	55.3	22
Mouse MAPK1	3373 Forward	5′CCATGCCTTCAAGTGACACG 3′	59	20
	3451 Backward	5′TCGTCATTTTCGGCCTTTTC3′	59	20
	3403 Probe	5′TCAAGTCCGAAGTCGCCGTCCTCTC3′	69	25
Mouse GAPDH	565 Forward	5′AGGGATGATGTTCTGGGCAG3′	59	20
	632 Backward	5′AGACTGTGGATGGCCCCTC3′	58	19
	589 Probe	5′ACGGCCATCACGCCACAGCTTT3′	69	22
SFRP2	1051 Forward	5′CGTGAAACGGTGGCAGAAG3′	59	19
	1117 Backward	5′TTGCAGCTTGCGGATGC3′	59	17
	1074 Probe	5′AGAGAGAGTTCAAGCGCATCTCCCGC3′	69	26
Mouse STAT1	1228 Forward	5′AGGTGTTGTCAGATCGAACCTTC3′	58	23
	1307 Backward	5′CATGCACGGCTGTCGTTCTA3′	59	20
	1254 Probe	5′CTCTTCCAGCAGCTCATTCGGAGCTC3′	68	26
TGFB3	936 Forward	5′TCCAGGGACTGGCGGAG3′	59	17
	1008 Backward	5′TTGAAACGAAAAACCTTAGAGGTAATTC3′	59	28
	957 Probe	5′ATGAACTGGCCGTCTGCCCCAAA3′	69	23
TGFB type 2 receptor	618 Forward	5′CCGGAAGCTGATGGATTTCA3′	60	20
	683 Backward	5′CTGATGTCGGAGCGGTCG3′	60	18
	641 Probe	5′ACAACTGCGCCATCATCCTGGAGG3′	69	24

well-differentiated adenocarcinoma after 28 weeks, although the lesion was too small (Fig. 1). However, incidence of lung tumors (adenomas or adenocarcinoma) was comparatively high in gal3+/+ than gal3−/− mice at 28 weeks (33.3 vs 21.4%), whereas significantly at 32 weeks (52.1 vs 28.6%, $P < 0.05$) (Table 2). As summarized in Table 2, the tumor number and incidence were high in gal3+/+ mice, compared with gal3−/− mice only at 32 weeks ($P < 0.05$).

Affymetrix GeneChip analysis

The expression of 22,690 mouse transcripts was assessed using the Affymetrix® mouse expression 430 A array. We found that the expression of 935 genes was up-regulated and the expression of 1,148 genes was down-regulated only with the treatment of PBS in gal3−/− mice, compared with gal3+/+ ones. Up-regulated expression of 166 genes and down-regulated expression of 181 genes were found in gal3−/− mice after 32 weeks of NNK administration in comparison of PBS treatment. NNK-induced up-regulated expression of 532 genes and down-regulated expression of 824 genes in lung tumors of gal 3+/+ versus gal3−/− mice after 32 weeks of treatment.

Functional network and gene ontology analysis

To refine the significance of the altered genes, we next investigated the biological interactions using the IPA tool

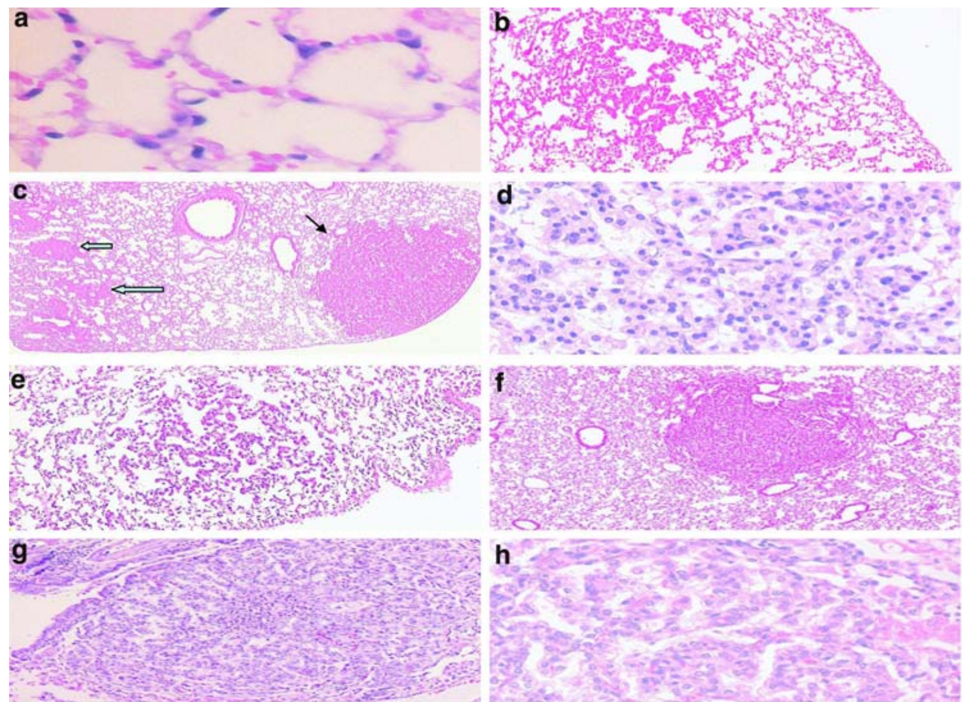

Fig. 1 Histopathology of lung lesions induced by NNK. Gal3+/+ mice: **a** normal lung tissue, **b** alveolar cell hyperplasia, **c** *thin arrow* papillary lung tumor and *thick arrows* solid lung tumor, and **d** higher magnification of the papillary tumor. Hematoxylin and eosin staining, original magnification: **b** and **c** ×100, and **a** and **d** ×400. Gal3−/− mice: **e** alveolar cell hyperplasia, **f** solid lung tumor, **g** papillary lung tumor, and **h** higher magnification of the papillary. Original magnification: **e**–**g** ×100, and **h** ×400

Table 2 Distribution of NNK-induced tumor number and frequency in gal+/+ and gal−/− mice

Group		*n*	TN = 0	TN = 1	TN = 2	TN ≥ 3	PR (%)
28 week	Gal+/+	18	12	3	2	1	33.3
	Gal−/−	28	22	5	1	0	21.4
32 week	gal+/+	48	23	11	6	8	52.1
	gal−/−*	49	35	11	2	1	28.6**

*,** compared with gal+/+ at 32 week, $P < 0.05$ (rs = 0.286, $\chi^2 = 4.640$)

TN tumor number in one mouse' lung, *PR* positive rate

and found the genes to map to genetic networks with functional relationships. Fourteen networks were found in gal3+/+ versus gal3−/− PBS-treated that were highly significant in that they had more of the identified genes present than would be expected by chance. Five networks with high scores (>15) had fifteen and more genes affected, being related to cellular growth and proliferation, cancer and cell movement functions the network of high scoring function is illustrated in Fig. 2a. The shaded genes are those identified by oligonucleotide microarray analysis, green for down-regulated and red for up-regulated. We also performed gene ontology analysis using the IPA tool. Twenty functions were identified as having high scores. The function with the highest *P* values was related to cancer ($P = 6.10e^{-3}$–$4.81e^{-2}$). Gene ontology analysis of the highly significant network revealed top-scoring functions of cellular growth and proliferation (Fig. 2a, $P < 10^{-8}$). After NNK administration of gal3−/− mice, 11 networks were found to be highly significant in that they had more of the identified genes present than would be expected by chance, compared with PBS control. Four networks with high scores (>15) of with 15 and more genes affected, being related to immune response, cellular movement, cellular growth and proliferation, cellular assembly and organization, cellular development and death, and cancer. The network of high-scoring functions for gal3−/− NNK-induced lung tumors is illustrated in Fig. 2b. Gene ontology analysis using the IPA tool was also performed. Nineteen functions were identified as high-level. Out of them, the top was immunological disease related function (Table 3, $P = 7.28e^{-4}$–$3.41e^{-2}$).

Gene ontology analysis of the highly significant network in NNK-induced lung tumors in gal3−/− mice revealed top-scoring function for the immune response (Fig. 2b, $P < 10^{-6}$). Furthermore, 112 networks were found in NNK-induced lung cancers in gal3+/+ versus gal3−/− mice. Fifty-three networks were highly significant in that they had more of the identified genes present than would be expected by chance. Networks with high scores (>18) and more than half of the genes affected, were associated with cell to cell signaling, cellular movement, cancer, gene expression, cell signaling molecular transport, cellular DNA replication recombination and repair, cell cycle, and cell death. Gene ontology analysis was also performed using the IPA tool. Twenty-eight functions were identified as high-level. Out of them, the top was the cancer related function ($P = 3.72e^{-4}$–$4.82e^{-2}$). Gene ontology analysis of the highly significant, network in gal3+/+ mice lung tumors revealed the top-scoring functions for cell to cell signaling

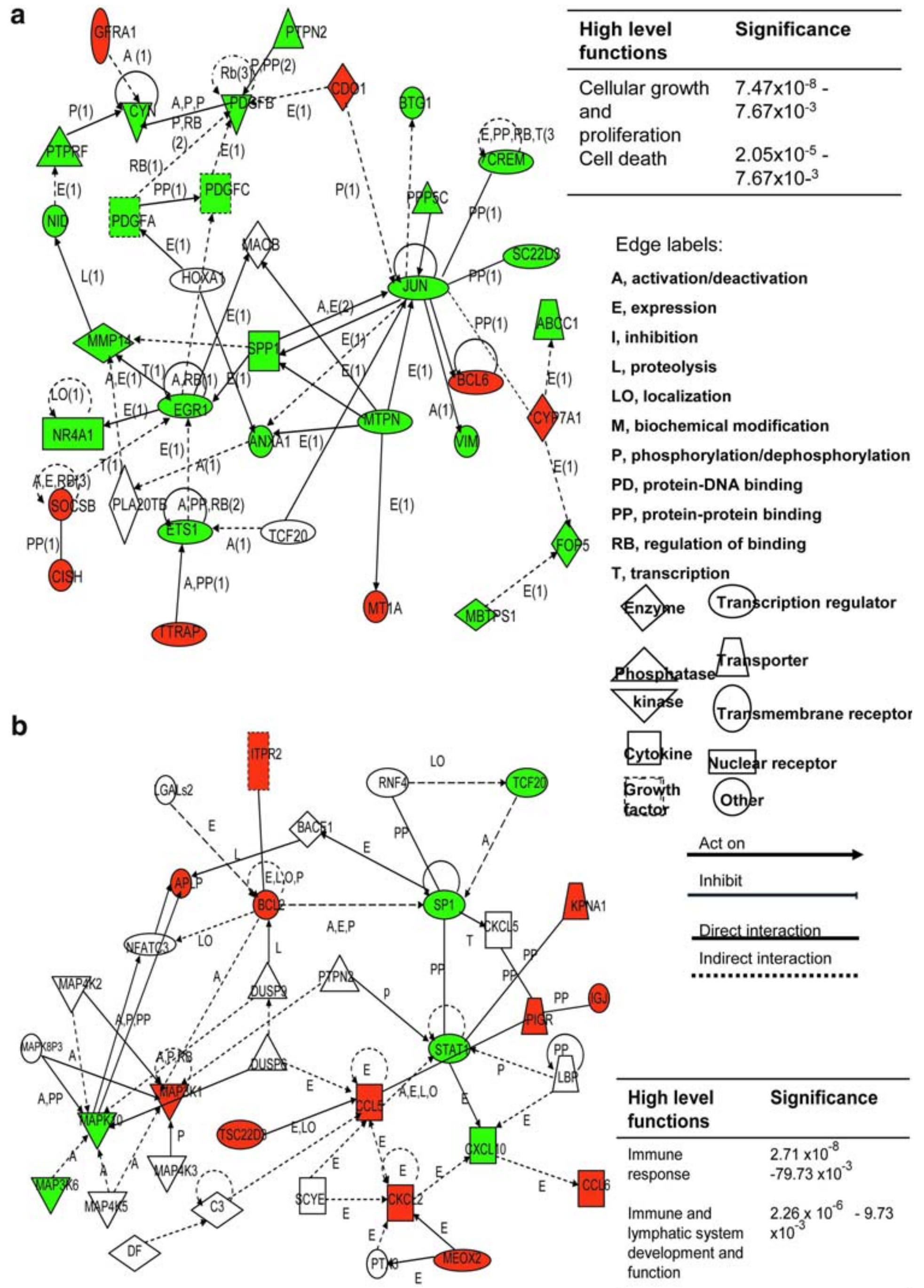

Fig. 2 **a** Top-scoring normal lung tissue of gal3+/+ induced gal3−/− CD-1 lung tumor network composed of multiple interacting genes. **b** Top-scoring NNK-induced gal3−/− CD-1 lung tumor. *Nodes* represent genes, with *their shape* representing the functional class of the gene product, and *edges* indicate the biological relationships between the nodes, color coded according to their *d* score (*red* over expression, *green* under expression)

and interaction, and cell death ($P < 10^{-5}$). A considerable number of genes were revealed to have developmental functions, which partly overlapped with those that were cancer related.

Canonical pathway analysis

We identified three canonical pathways, for B-cell receptor signaling ($P = 0.0121$), ERK/MAPK signaling ($P = 0.017$), and PPAR signaling ($P = 0.0321$) in PBS-treated gal3+/+ versus gal3−/− mice. The Wnt/β-catenin signaling pathway was the highest canonical pathway of significance in gal3+/+ NNK-induced lung tumors versus gal3+/+ PBS-treated tissue ($P = 0.0338$), previously described (Dungworth et al. 2001). Two canonical pathways, EGF signaling ($P = 0.0023$) and PDGF signaling ($P = 0.047$), were found significant in gal3−/− NNK-induced lung tumors versus the control samples (Fig. 3a, b). Tables 4 and 5 summarize the related genes identified as up-regulated or down-regulated with the Affymytrix geneChip. However, we could not find any significant canonical pathway between NNK-induced gal3+/+ and gal3−/− lung tumors.

Table 3 Ontology analysis of the genes affected in gal3$^{-/-}$ NNK-induced mouse lung tumors

Related functions and diseases	Significance	Associated genes
Cancer genes		
Cell to cell signaling and interaction	$3.62e^{-3}$–$3.41e^{-2}$	8
Cancer	$6.90e^{-3}$–$3.41e^{-2}$	9
Cell cycle	$6.90e^{-3}$–$3.41e^{-2}$	3
Cell death	$6.90e^{-3}$–$4.95e^{-2}$	12
Cellular movement	$6.90e^{-3}$–$4.95e^{-2}$	14
Cell signaling	$1.02e^{-2}$–$2.70e^{-2}$	9
Cellular growth and proliferation	$2.06e^{-2}$–$2.70e^{-2}$	8
Developmental genes		
Immunological and lymphatic system development and function	$7.28e^{-4}$–$4.35e^{-2}$	14
Connective tissue development and function	$1.40e^{-2}$–$2.70e^{-2}$	4
Tissue development	$1.83e^{-2}$–$4.02e^{-2}$	10
Cellular development	$2.70e^{-2}$–$4.02e^{-2}$	5
Others		
Immunological disease	$7.28e^{-4}$–$3.41e^{-2}$	5
Molecular transport	$7.28e^{-4}$–$4.55e^{-2}$	18
Immune response	$1.45e^{-3}$–$4.55e^{-2}$	28
Cellular assembly and organization	$4.21e^{-3}$–$3.41e^{-2}$	13
Respiratory disease	$6.90e^{-3}$–$3.41e^{-2}$	2
Gene expression	$2.60e^{-2}$–$2.70e^{2}$	5
Amino acid metabolism	$2.70e^{-2}$–$3.89e^{-2}$	3
Cellular function and maintenance	$2.70e^{-2}$–$2.70e^{-2}$	3

Validation of gene expression with real-time PCR

In order to confirm the results of the ingenuity pathway analysis, we measured the expression of ten genes included in the highly significant canonical pathways. The genes in the Wnt/β-catenin pathway include *CDH2*, *SFRP2*, *TGFβ3*, and *TGFβ2R*, whereas we chose *CSNK2A1*, *MAP3K1*, and *STAT1* for both EGF and PDGF signaling pathways. We have also examined *BCL-6*, *Jun*, and *PDGFα* for B-cell receptor and PPAR signaling pathways. Their differential expression profiles were verified by quantitative real-time PCR using total RNA from the same samples used for microarray analysis. mRNAs for *SFRP2* and *TGFβ3* were significantly increased in gal3+/+ NNK-induced mouse lung cancers, while decrease was noted for *TGFβR2* when compared with gal3+/+ controls. mRNAs for *CSNK2A1* and *MAP3K1* genes were also increased while that for the *STAT1* gene was decreased. mRNA for *BCL-6* was significantly increased in gal3+/+ normal lung tissues compared with the normal lung tissue of gal3−/− and was decreased for both *Jun* and *PDGFα* (Fig. 4). Generally, real-time PCR data correlated positively with the microarray data although mismatches were also found. For example *CDH2* gene was increased in the real-time PCR results although it has been shown as a down-regulated gene in the microarray data and the IPA. This may be at least partly due to differences in methodology (Yuen et al. 2002).

Discussion

Here, we succeeded in establishing the animal model of lung lesions using gal3+/+ and gal3−/− mice induced by NNK, including alveolar hyperplasia, adenoma, and adenocarcinoma. These results suggested NNK from tobacco and air pollution played an important role in the carcinogenogenic pathways from hyperplasia to adenocarcinoma through adenoma (Mori et al. 1996). To focus on the role of galectin-3 in lung oncogenesis, we employed gal3−/− mouse in the present study. Upon treatment with NNK, lung tumors were detected at significantly lower incidence in galectin-3 (gal3−/−) knock-out mice at 32 weeks after the first intraperitoneal injection of NNK than galectin-3 wild-type (gal3+/+) ones. Although the NNK-induced tumors are of no morphological difference in gal3+/+ and gal3−/− mice, the genetic alteration for tumors of both the mice are remarkably distinct because of galectin-3 deficiency in knock-out mice. In agreement with this observation, a large body of evidences indicates that transfection of galectin-3 cDNA promotes cell transformation and cancer

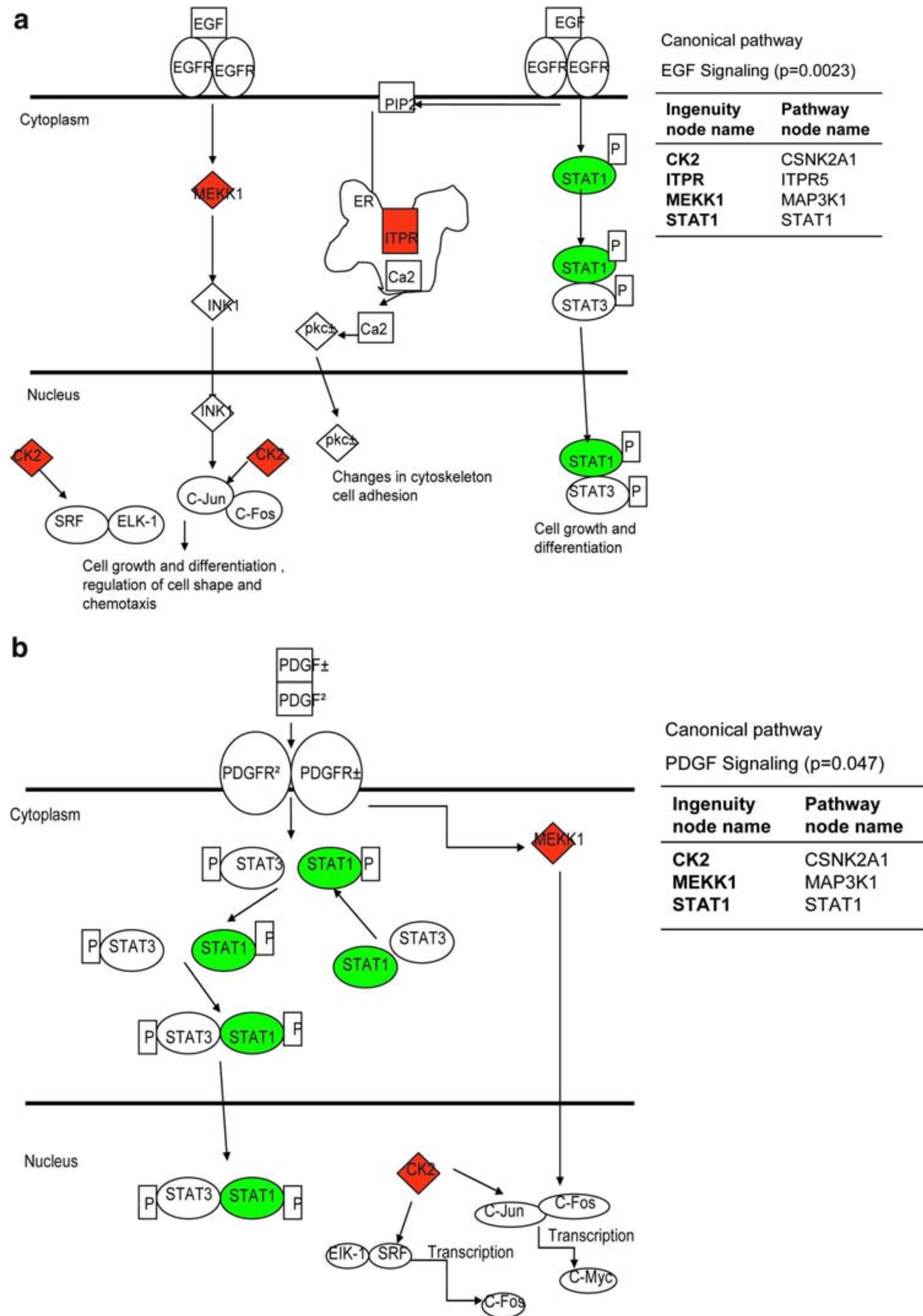

Fig. 3 **a** EGF signaling pathway is the canonical pathway of highest significance in our data set of gal3−/− NNK-induced mouse lung tumors. *Red* indicates up-regulated and *green* indicates down-regulated genes, and *white* indicates that the genes were not user specified, but incorporated into the network through relationships. **b** The PDGF signaling pathway is the second canonical pathway significant in our data

Table 4 Genes in the PDGF and EGF signaling pathways differentially expressed between gal3$^{-/-}$ NNK-induced lung tumors and gal3$^{-/-}$ PBS-treated control

Gene symbol	Gene name	PBS-treated (control)	NNK-induced (lung tumor)	Fold change
CSNK2A1	Casein kinase II, alpha 1 polypeptide	0.479143	1.287242	2.68655
MAP3K1	Mitogen activated protein kinase kinase kinase 1	0.933181	1.993685	2.13644
ITPR5	Inositol 1,4,5-triphosphate receptor 5	0.86665493	1.9747999	2.278646
STAT1	Signal transducer and activator of transcription 1	2.553142	1.0592791	0.414892

Table 5 Genes in the B-cell receptor signaling and PPRA (peroxidase proliferator-activated receptor) signaling pathways differentially expressed between gal3$^{+/+}$ and gal3$^{-/-}$ control lung tissues which have been checked by quantitative real-time PCR

Gene symbol	Gene name	gal3$^{+/+}$ PBS-treated (control)	gal3 $^{-/-}$ PBS-treated (control)	Fold change
BCL-6	B-cell CLL/lymphoma 6 (Zink finger protein 51)	1.45062	0.724847	2.001276
JUN	v-jun sarcoma virus 17 oncogene homolog (avain)	0.408211	2.52032	0.161968
PDGFα	Platelet derived growth factor alpha polypeptide	0.269108	2.007077	0.13408

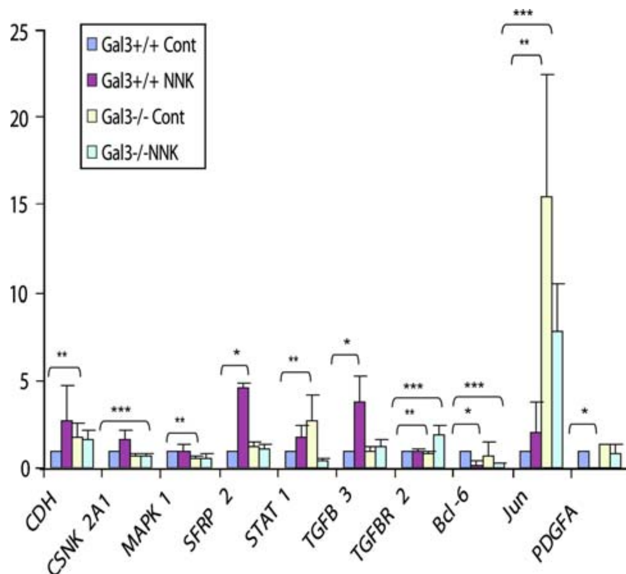

Fig. 4 mRNA levels of the altered genes in the significant canonical pathway (Wnt/β-catenin, EGF, PDGF, B-cell receptor, and PPAR) signaling pathways were determined by real-time polymerase chain reaction, normalized to glyceraldehyde-3-phosphate dehydrogenase, and expressed as fold induction relative to gal3+/+ control and gal3−/− control. The values are expressed as mean ± SEM of four mice in each group. *$P < 0.05$ for gal3+/+ NNK versus gal3+/+ cont, **$P < 0.05$ for gal3−/− cont versus gal3+/+ cont, ***$P < 0.05$ for gal3−/− NNK versus gal3+/+ cont

progression. Indeed, transfection of galectin-3 cDNA in fibroblasts results in acquisition of anchorage-independent growth, while the same transfection in weakly metastatic fibrosarcoma cells caused an increase of experimental lung metastasis in mice (Gururajan et al. 2006). These effects were reversed by transfection of antisense galectin-3 cDNA. Furthermore, oral administration of pH-modified citrus pectin, that blocks galectin-3 binding to its ligands, inhibits tumor growth, angiogenesis, and metastasis in nude mice (Nangia-Makker et al. 2002).

In our recently published paper (Abdel-Aziz et al. 2007), we succeeded in identifying more than 800 genes, which were differentially expressed in NNK-induced gal3+/+ lung tumors. In addition, we clarified the top functions to be related to cellular growth and proliferation. Additionally, the Wnt/β-catenin signaling pathway was described as the canonical pathway of highest significance in NNK-induced gal3+/+ mouse lung tumors. In an extension of this work, we here confirmed our results by quantitative real-time PCR for significantly altered genes in the Wnt/β-catenin signaling pathway. Although the gene spring analysis provided information regarding changes in large categories of genes, we were interested in understanding how individual genes were integrated into specific regulatory networks by the IPA. As a background, we therefore compared gal3+/+ versus gal3−/− untreated lung tissues. Several networks were identified to show differences, some being related to cellular growth and proliferation and cancer. This adds support to roles of galectin-3 in carcinogenesis and might explain the decreased incidence of lung tumors in the gal3−/− group. Moreover the three canonical pathways of significance, for B-cell receptor signaling, Extracellular signal-Related Kinase/Mitogen Activated Protein Kinase (ERK/MAPK) signaling and the Peroxidase Proliferator-Activated Receptor (PPAR) are all cancer related signaling pathways (Gururajan et al. 2006; Miglietta et al. 2006; Fukumoto et al. 2005).

Comparison between gal3−/− NNK-induced lung tumors and gal3−/− PBS-treated lung tissues identified several networks with the difference, some of which were involved in the immune response. Expression levels of 21 genes (60%) in the top function network were altered significantly, although to a lower extent than that in gal3+/+ NNK-induced mouse lung tumors (100%) (Abdel-Aziz et al. 2007). The highest-level function of the top network of the gal3−/− NNK-induced lung tumors, which is to immune response, is in agreement with the known extracellular function of galectin-3, which is an immune-related function (Liu 2005). In addition, recent studies have revealed that galectin-3 is expressed in a variety of cell types in the immune system, participating in the activation and differentiation of immune cells (Chen et al. 2005). This finding raises the possibility that the immune system acts as a two-edged weapon.

In addition, the PDGF and EGF signaling pathways were significantly affected in NNK-induced lung tumors of gal3−/− mice. Aberrant PDGF signaling is a hallmark of a number of solid tumors and studies of breast, lung, and colon cancers have shown a tight relationship between deregulated paracrine PDGF signaling and cancer progression (Kawai et al. 1997). Recently, it was indicated that disrupting PDGFRα-mediated signaling results in significant

inhibition of tumor growth in vivo and inhibition of this signaling may prove to be an effective method for influencing the growth of several human solid tumors by targeting recruitment of tumor stromal fibroblasts (Tejada et al. 2006). The EGF-family of peptide growth factor is well-known to have a central role in the pathogenesis and progression of different carcinoma types (Normanno et al. 2006). It is not known whether galectin-3 is an inhibitor of the PDGF and the EGF signaling pathways. Our novel findings in this respect therefore need further attention using in vivo animal models. Accordingly, other animal models targeting both galectin-3 and PDGF or EGF signaling pathways might be of a great value in decreasing the incidence of lung tumors. Hence may add more knowledge about gene therapy of human lung cancer. A new trend of targeting multiple signal transduction pathways has been mentioned as cancer therapy of lung cancer (Kim et al. 2003b). It was previously reported that blockade of EGFR signaling in tumor cells and tumor-associated endothelial cells could produce significant therapeutic effects against prostate cancer bone metastasis (Kim et al. 2004) and the simultaneous blockade of PDGFR and EGFR signaling, coupled with administration of chemotherapy, significantly suppresses experimental human prostate cancer bone metastasis in nude mice (Bianco et al. 2006). Three genes were shown here to be up-regulated in these pathways. Casein kinase 2, alpha 1 polypeptide (*CSNK2A1*), has been reported to be an independent predictor of outcome in patients with squamous cell carcinoma of the lung (O-charoenrat et al. 2004). Elevated activity of CK2, the protein kinase encoded by *CSNK2A1*, is associated with malignant transformation of several tissue types including lung tissue (Daya-Makin et al. 1994). It was documented that CK2 over expression may contribute to tumorigenesis via regulation of the Wnt/β-catenin pathway with *c-myc* as a downstream target (Landesman-Bollag et al. 2001). The second gene is inositol triphosphate receptor 5 (*ITPR5*). The inositol 1, 4, 5 triphosphate receptor type 2 gene that encodes for a constituent of calcium channels located in the endoplasmic reticulum is thought to be involved in cell transformation and progression (Paterlini-Brechot et al. 2003). Although its role in lung oncogenesis is still obscure, our study suggests that *ITPR5* may be involved in gal3−/− mouse lung progression due to its induction of cell transformation. The third is the mitogen activated protein 3 kinase 1 (*MAP3K1*), which is a tumor promoter target in initiated cells that express oncogenic Ras (Warmka et al. 2004). Although the expression of gal-3 in macrophages was found to be controlled by the Ras/MAP kinase pathway (Kim et al. 2003a), it is not known how disrupted-galectin-3 might lead to up-regulation of *MAP3K1* in lung tumors. Signal transducer and activator of transcription 1 (*STAT1*) was found to be down-regulated in the present study. Dysregulation of *STATs* is associated with many cancers but to our knowledge no role of *STAT1* in human tumor progression has been demonstrated. In a recent study, *STAT1* was documented to function as a tumor suppressor in squamous cell carcinogenesis. Silencing of *STAT1* gene via promoter methylation may contribute to squamous cell carcinogenesis of the head and neck (SCCHN) (Xi et al. 2006).

In summary, our in vivo model of induction of lung tumors in gal3+/+ and gal3−/− mice, and gene expression assessment clarified the role of galectin-3 in lung tumorigenesis using a high-density oligonucleotide microarray and IPA. The differences between the NNK-induced lung tumors in both the wild-type and the knock-out mice versus the corresponding control mechanisms also provided important pointers. Firstly, the number of altered genes is less in lung tumors of gal3−/− than gal3+/+ mice (347 vs 876). Secondly, the top network in the gal3+/+ lung tumors, which was related to cellular growth and proliferation, has 100% affected genes, whereas that of gal3−/− lung tumors was related to the immune response with 60% of affected genes. Thirdly, the canonical pathways are different: being the Wnt/β-catenin signaling pathway with seven genes affected in gal3+/+ lung tumors, but the PDGF and EGF signaling pathways with only four genes affected in case of gal3−/− lung tumors. Consequently, our data provide not only a new molecular basis for understanding the biologic properties of galectin-3 in lung carcinogenesis but also could be useful resources for future development of anti-cancer therapeutic strategies, a way to challenge the terrible curse that is lung cancer.

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