Identification of differentially expressed transcripts targeted by the knockdown of endogenous IFITM3

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Abstract. Interferon inducible transmembrane protein 3 (IFITM3) is a double transmembrane protein. As a member of the IFITM family, IFITM3 can be upregulated by interferon (IFN) to be involved in various biological processes. In order to determine whether gene expression profiles can be altered by a lack of IFITM3, the present study used shRNAs lentivirus for knocking down the endogenous expression of IFITM3 in human HeLa cells and human whole genome microarrays to obtain gene expression profiles. A total of 1,011 downregulated transcripts and 615 upregulated transcripts were identified using the Agilent expression platform. The identified transcripts were involved in multiple pathways, including the complement pathways, and the antigen processing and presentation pathway. The present study identified the transcripts, which were affected by the downregulation of endogenous IFITM3 and the pathways they were involved in. These findings may lead to an improved understanding of the biological functions of IFITM3.

Introduction

Interferon-induced transmembrane protein 3 (IFITM3) is a member of the gene family encoding IFITM, which can be upregulated by type I IFN (IFN-α and IFN-β) and type II IFN (IFN-γ). In humans, the IFITM gene family comprises at least four members, termed IFITM1, IFITM2, IFITM3 and IFITM5, respectively (1). The IFITM genes are clustered on chromosome 11, encoding for proteins upregulated by IFN. With the exception of IFITM5, the genomic and protein sequence identities of the IFITM genes are high. Their protein structures are similar, each containing two transmembrane domains, one conserved intracellular loop and two extra-cellular terminals. As one of the IFITM family members, IFITM3 has been demonstrated to be pivotal in IFN signaling, as downregulating the expression of IFITM3 using small interfering RNA was observed to reduce the antiviral activities performed by IFN-γ by 40-70% (2).

Endogenous IFITM3 may have important regulatory effects intracellularly. IFITM3 is involved in cellular development and differentiation (3). Previous studies have identified genetic variations or altered expression of IFITM3 that may be associated with immune diseases, such as viral infections (2) schizophrenia (4), autism (5,6), inflammatory bowel disease (7) and cancer (8-10). The human HeLa cell line is one of the most widely used cell line in molecular biology for investigating gene functions (11,12), and the endogenous expression of IFITM3 has been detected in HeLa cells previously (2). To investigate the global transcriptional profile when IFITM3 is downregulated, the present study used a knockdown (KD) approach and identified a series of altered transcripts when IFITM3 was downregulated in HeLa cells. Investigating alterations in the global transcriptional profile may improve current understanding of the molecular mechanism of the antimicrobial function of IFITM3.

Materials and methods

KD of endogenous IFITM3. HeLa cells (America Type Culture Collection, Manassas, VA, USA; cat. no. CRL-2266) at a density of 3-5x10^6 per T75 flask (Greiner Bio-One, Frickenhausen, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM)/15% fetal bovine serum (FBS)/10 mM HEPES (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) 1 day prior to lentiviral infection. Following culture, the IFITM3 short hairpin (sh)RNA lentivirus (Santa Cruz Biotechnology, Inc.; cat. no. sc-97053-V)

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or control shRNA lentivirus (Santa CruzBiotechnology, Inc.; cat. no. sc-108080), each with a multiplicity of infection of 50%, was added to polybrene with a 8 μg/ml final concentration (Sigma-Aldrich; Thermo Fisher Scientific, Inc.; cat. no. S2667) to the cultures and incubated overnight at 37˚C. On the third day, selection medium (DMEM/15%FBS/10 mM HEPES containing 50 μg/ml puromycin) was added for sorting of the stably infected cells. After 1 week, the adherent cells were digested with 0.25% trypsin and 0.02% EDTA, and resuspended for single cell culture at a density of $1.0x10^3$ cells/well in 96-well plates, with the same selection medium as that described above, for monoclonal colony amplification.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Cell selection was performed by adding puromycin to the cell culture medium, following which the resistant monoclonal colonies were identified. The efficiency of endogenous IFITM3 KD in the HeLa cells were confirmed by RT-PCR analysis. The re-cultured HeLa cells were harvested and RNA was extracted using a standard TRIzol procedure (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. 15596-018). The RNA was subjected to cDNA synthesis using M-MLV reverse transcriptase (Promega; Madison, WI, USA; cat. no. M170) in a 20 μl liquid phase reaction, from which 1 μl of cDNA was used for subsequent PCR amplification. The primers used as an internal control for RT-PCR to amplify human 18S were as follows: Forward 5'-GGAAGGGCACCACCAGGAGT and reverse 5'-TGC AGCCCCGGACATCTAAG. The primers used for amplifying the IFITM3 and IFITM3-targeted RNAs via PCR analysis (Opticon® DNA engine; MJ Research, Waltham, MA, USA, cat. no. CFD3200) are listed in Table I. SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd. Dalian, China) were used for quantitative (q) PCR in a 25 μl reaction volume with 95˚C for 30 sec followed by 40 cycles with each cycle consisting of 95˚C for 5 sec and 60˚C 30 sec. Relative expression ratio of targeted genes were analyzed using the $2^{-\Delta\Delta Cq}$ method (13) with 18S as a reference gene.

Analyses of mRNA expression using oligonucleotide arrays. The RNA KD and control (CT) HeLa cells were cultured for mRNA extraction three times independently. The three RNA samples from the KD cells were mixed with equal quality, as were those from the CT cells. Microarray analysis was performed using a Human Whole Genome Oligo Microarray (Agilent Technologies, Inc., Santa Clara, CA, USA; cat. no. G4112A). According to the protocols of the Low RNA Input Linear Amplification and Labeling Kit Plus (Agilent Technologies; cat. no. 5184-3523), double-stranded cDNA was synthesized and applied as a template to label cRNA. Cy3 (scanned as red) was used to label the cRNA from the KD group from that of CT group. The ratio of fluorescence intensity for each probe (KD/CT) was determined to indicate the difference between the RNA-KD and CT cells. Certain transcripts identified as IFITM3-targeted were subjected to quantitative (q)PCR analysis to enable relative quantification. Gene Ontology (GO) enrichment analysis was performed according to the GO database (www.geneontology.org) to identify significant functional categories among differentially expressed genes. Furthermore, pathway analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg) and BioCarta (www.biocarta.com) pathway analysis programs. Each P-value was calculated with Fisher's exact test using the R-package, whereas each q-value was calculated with John Storey's method using the R-package (14).

Results

IFITM3-targeted transcripts. The transcriptional expression profiles of the endogenous IFITM3-KD and CT HeLa cells were investigated using a human Agilent GeneChip microarray platform. Using the 2-fold change as the cut-off, the Agilent microchip revealed 1,011 downregulated and 615 upregulated transcripts. It was noted that certain alterations gathered in gene families, including the histone (HIST), caveolae (CAV), pregnancy-specific β-1-glycoprotein (PSG), calmodulin (CALM), E twenty-six (ETS) and golgin (GOLG) families (Fig. 1).

Validation of IFITM3-targeted transcripts using qPCR analysis. Several IFITM3-targeted transcripts of the HIST family, including HIST cluster 1 (HIST1) H1a, HIST1 H1b, HIST1 H2aa, HIST1 H4j and HIST1 h4k, were further assessed using RT-qPCR analysis. In addition, certain additional transcripts, including mitogen-activated protein kinase 13, myeloid differentiation primary response 88, prostaglandin-endoperoxide synthase 2, transmembrane emp24 protein transport domain containing 7, ubiquitin-conjugating enzyme E2 N, vitamin D receptor and thioredoxin interacting protein, were randomly selected for validation. The gene expression levels determined using RT-qPCR analysis appeared coincident with those of the microarray (Fig. 2).

Analysis of altered pathways. In the present study, when endogenous expression of IFITM3 in the HeLa cells was reduced, several genes in the antigen processing and presentation pathway appeared to be either upregulated or downregulated.

The IFITM3-downregulated targets were subjected to clustering pathway analysis, and the associated gene pathways (21 upregulated and 21 downregulated) are listed in Table II. Alterations of gene expression involving the complement cascades, antigen processing and presentation pathways are detailed independently in Fig. 3. It was found that the majority of the transcripts detected in the complement pathway exhibited a downregulatory trend, particularly transcripts of complement component 3 (C3), complement component 4b (C4B) and complement component 5 (C5). In terms of the antigen processing and presentation pathway, transcripts of heat shock protein 90 kDa α class A member 1, heat shock 70 kDa protein 1A, heat shock 70 kDa protein 1-like, heat shock 70 kDa protein 2, heat shock 70 kDa protein 8, nuclear transcription factor Y (NFY) α and NFY β were upregulated, whereas the transcripts of heat shock 70 kDa protein 5, heat shock 70 kDa protein 6, regulatory factor X-associated protein, killer cell immunoglobulin-like receptor (KIR) three domains and long cytoplasmic tail 3, KIR two domains and short cytoplasmic tail 4, calreticulin

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction analysis.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
IFITM3	ATGAATCACACTGTCCAAACCTTCT	CTATCCATAGGCCTGGAAGATCAG
VDUP1	CGATAGTTTCGGGTCAGG	GATACATAAGTTCAAGGTCCAA
VDR	CAGGGTGGGATGGAGGAGAAG	TGGGTGGTGGAGTGAGAATAAGAA
UBE2N	AACTTTATTTAGACGCTGTAGATGG	AATGTTATTAGTGAGGGCTGTGAT
TMED7	ATTGGATAGCCATCCTAGTCACT	GCTGGTCTTCAAACACCGTAA
PTGS2	TGTCCCTTTACTTCATTCAGTGTTC	ATGACTCCTTTCTCCGCAACA
PLCB4	AAGCCTGCTGTAGTTGAGTTGC	CTTGACGAGTGTTATGCGTGTTT
MYD88	GAATCCCTGTAGGAAATGGTGAAGC	AGGAAGTGGAATGGGCGGTGT
MAPK13	GCCAAATCCTACATCCAGTCCCT	TCCAGCATCTTCTCCAGCAGGT
HIST1H1A1	TCCGTGTCAGAGCTGATCGTG	GCGGCTGTTGTTCTTCTCCAC
HIST1H4J	GATCCGGGACGCCGTGACCTAT	GGGACGCTCAACCACCGAAACC
HIST1H4K	TCCGGGACGCCGTGACCTATA	GGACGCTCAACCACCGAAACC
HIST1H1B1	CTTGCCACCATGTCGGAAACC	CCAGCTTAATGCGGCTGTTATTCTT
HIST1H2AA	GTGTATTTGGCGGCAGTGTTAGA	TGCTTTGGGCTTTATGGTGGT

IFITM3, interferon inducible transmembrane protein 3; VDUP1; vitamin D3 upregulated protein 1; VDR, vitamin D receptor; UBE2N, ubiquitin-conjugating enzyme E2 N; TMED7, transmembrane emp24 protein transport domain containing 7; PTGS2, prostaglandin-endoperoxide synthase 2; PLCB4, phospholipase C β4; MYD88, myeloid differentiation primary response 88; MAPK13, mitogen-activated protein kinase 13; HIST, histone.

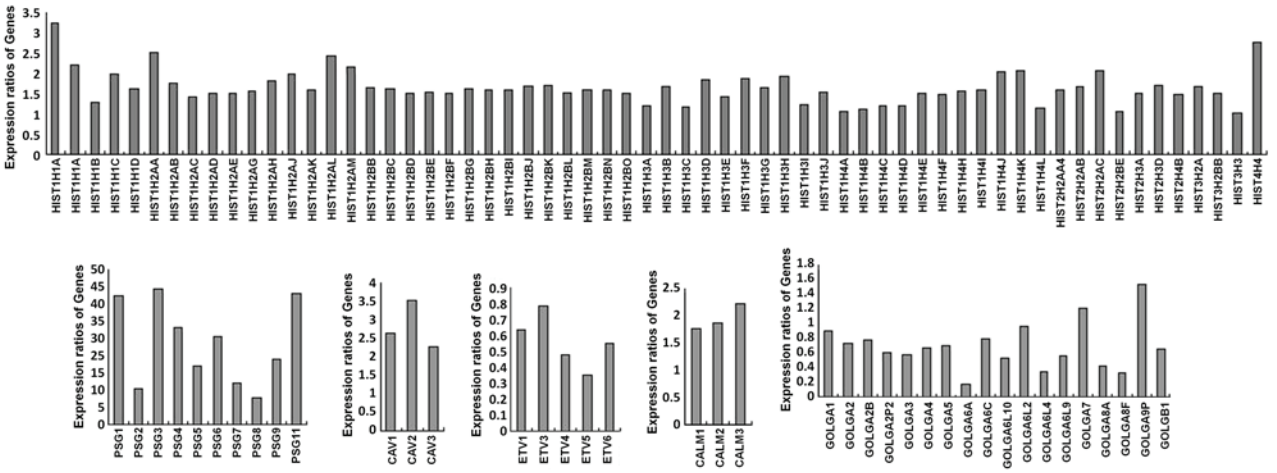

Figure 1. Gene families altered by interferon inducible transmembrane protein 3 knockdown. Expression ratios of altered levels of genes in HIST, CAV, PSG, CALM, ETV and GOLG families between IFITM3 knocked down cells and control cells were showed as columns. HIST, histone, CAV, caveolae; PSG, pregnancy-specific β-1-glycoprotein; CALM, calmodulin; ETV, ets variant; GOLG, golgin.

and major histocompatibility complex class II, DQ α2, were downregulated.

Discussion

Alterations in expression gathered in gene families. When IFITM3 was downregulated by shRNA, the HeLa cells showed an altered gene expression profile. The expression levels of hundreds of gene transcripts were altered and associated with multiple gene pathways. The differentially expressed profile obtained in the present study provided evidence that IFITM3 is involved in regulating a broad range of transcripts, and this is likely to result in different consequences in terms of the biological functions of cells. Of note, similar changes were found to be gathered in families.

It was found that the majority of gene transcripts in the HIST, CAV, PSG and CALM families, were upregulated. Histones are basic nuclear proteins, which are responsible for the nucleosome structure within the chromosomal fiber in eukaryotes. The expression of HIST genes is coupled temporally and functionally with DNA replication, and is controlled at the transcriptional and post-transcriptional levels (15). The PSG family belongs to a member of the immunoglobulin superfamily (16). Evidence shows that it is involved in modulation of the innate immune system (17). All the CALM family gene members (CALM1, CALM2 and

Table II. Associated pathways with altered regulation.

Pathway	Count	P-value	q-value
Upregulated			
Focal adhesion	20	1.2e-15	2.0e-13
Calcium signaling pathway	14	6.5e-10	1.1e-8
Regulation of actin cytoskeleton	13	4.9e-8	5.1e-7
Gap junction	9	9.6e-8	8.9e-7
MAPK signaling pathway	13	7.1e-7	0.0000048
Wnt signaling pathway	10	7.1e-7	0.0000048
Glioma	7	0.0000013	0.0000078
Nitrogen metabolism	5	0.0000015	0.0000090
Cytokine-cytokine receptor interaction	12	0.0000026	0.000013
Melanogenesis	8	0.0000029	0.000014
Pathogenic *Escherichia coli* infection	6	0.0000068	0.000026
ECM-receptor interaction	7	0.0000072	0.000026
Antigen processing and presentation	7	0.000011	0.000034
Axon guidance	8	0.000015	0.000047
Complement and coagulation cascades	6	0.000026	0.000074
Systemic lupus erythematosus	8	0.000032	0.000088
Long-term potentiation	6	0.000041	0.00010
B cell receptor signaling pathway	6	0.000041	0.00010
Phosphatidylinositol signaling system	6	0.000045	0.00011
Prostate cancer	6	0.00010	0.00023
Downregulated			
MAPK signaling pathway	19	4.4e-10	9.1e-9
Arachidonic acid metabolism	9	1.5e-8	1.5e-7
Glycine, serine and threonine metabolism	8	2.4e-8	2.1e-7
Long-term depression	9	1.1e-7	6.8e-7
Calcium signaling pathway	12	0.0000013	0.0000066
Cytokine-cytokine receptor interaction	14	0.0000019	0.0000091
VEGF signaling pathway	8	0.0000023	0.000010
Cell adhesion molecules	10	0.0000031	0.000012
GnRH signaling pathway	9	0.0000036	0.000014
Insulin signaling pathway	10	0.0000040	0.000015
Nitrogen metabolism	5	0.0000062	0.000022
Aminoacyl-tRNA biosynthesis	6	0.0000062	0.000022
Linoleic acid metabolism	5	0.000020	0.000057
Histidine metabolism	5	0.000023	0.000065
Neuroactive ligand-receptor interaction	12	0.000038	0.00010
Antigen processing and presentation	7	0.000067	0.00016
Tryptophan metabolism	5	0.00012	0.00026
Complement and coagulation cascades	6	0.00013	0.00028
Type II diabetes mellitus	5	0.00015	0.00031
Regulation of actin cytoskeleton	10	0.00020	0.00040

MAPK, mitogen-activated protein kinase; ECM, extracellular matrix; VEGF, vascular endothelial growth factor; GnRh, gonadotropin-releasing hormone.

CALM3) encode an identical protein, calmodulin, which modulates a calcium-activated cadherin function. CALM members are considered to be involved in the intracellular invasion and colonization of human intestinal epithelial cells by *Campylobacter jejuni in vitro*. CALM1 was found to exist close to a risk locus of *C. jejuni* colonization in the avian intestine in a population-based genome-wide association study (18).

By contrast, the majority of the gene transcripts in the ETS family and GOLG family were downregulated. The ETS proteins are transcription factors; they regulate several target genes, which modulate cellular functions, including growth, apoptosis, development, differentiation and oncogenic transformation (19). The GOLG family of proteins localize to the Golgi, and appear to be involved in membrane traffic and Golgi structure; individual golgins are found in different locations in the Golgi stack, and are typically anchored to the membrane at their carboxyl termini by a transmembrane domain or by binding a small GTPase (20). Although these altered gene families require further validation to confirm their association with IFITM3, the results of the present study provide insight for an improved understanding of the molecular functions of IFITM3.

Potential role of IFITM against microorganisms. Previously, IFITM3 has been found to inhibit the replication of human immunodeficiency virus-1 (21). In addition, IFITMs, particularly IFITM3, have been confirmed to inhibit viral infections, by influenza A virus, flaviviruses (dengue virus, West Nile virus and hepatitis C) and filoviruses (Ebola virus and Marburg virus) (22-24). In our previous study, a functional polymorphism of IFITM3 was found to contribute to tuberculosis susceptibility (25). Thus, IFITM3 may limit microorganism infection by adjusting the host immune

Table III. Genes detected in the complement pathway.

Gene ID	Symbol	Full name	Probe ID	Systematic name	Fold change
715	C1R	Complement component 1, r subcomponent	A_23_P125423	NM_001733	1.32
716	C1S	Complement component 1, s subcomponent	A_23_P2492	NM_001734	2.1
718	C3	Complement component 3	A_23_P101407	NM_000064	0.42
721	C4B	Complement component 4B	A_23_P42282	NM_001002029	0.41
727	C5	Complement component 5	A_23_P71855	NM_001735	0.3
3627	C7	Complement component 7	A_23_P213857	NM_000587	0.83
733	C8G	Complement component 8G	A_23_P20713	NM_000606	0.77
629	CFB	Complement factor B	A_23_P156687	NM_001710	0.54
8518	CFD	Complement factor D	A_23_P119562	NM_001928	0.72
10747	MASP2	Mannan-binding lectin serine peptidase 2	A_23_P301971	NM_139208	1.27
719	C3AR1	Complement component 3a receptor 1	A_23_P2431	NM_004054	0.64
728	C5AR1	Complement component 5a receptor 1	A_23_P153562	NM_001736	0.69
3689	ITGB2	Complement component 3 receptor 3 and 4 subunit	A_23_P329573	NM_000211	0.64

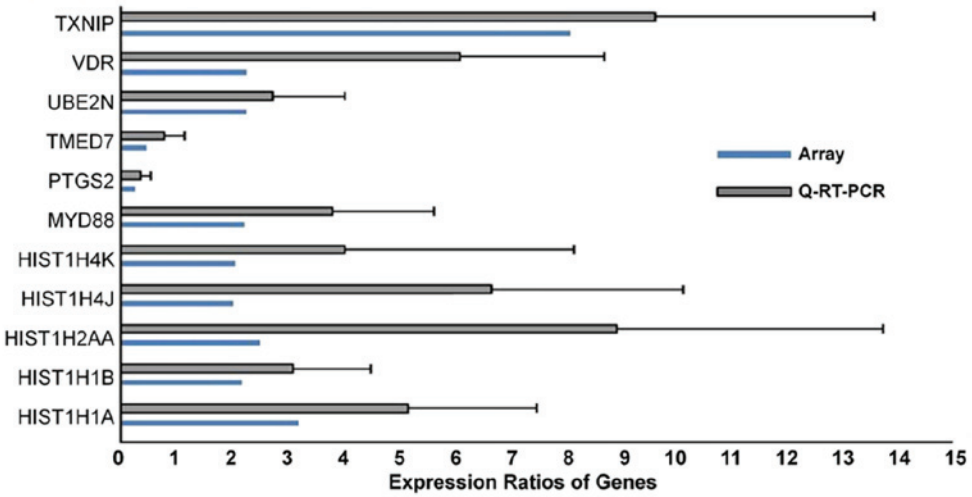

Figure 2. Reverse transcription-quantitative polymerase chain reaction analysis validation. TXNIP, thioredoxin-interacting protein; VDR, vitamin D receptor; UBE2N, ubiquitin-conjugating enzyme E2 N, TMED7, transmembrane emp24 protein transport domain containing 7; PTGS2, prostaglandin-endoperoxide synthase 2; MYD88, myeloid differentiation primary response 88; HIST, histone.

ability. However, the precise mechanisms remain to be fully elucidated.

As is known, the control of pathogen infections is critically dependent on the recognition and elimination of infected cells. As a biological process of the innate immune system, antigen processing prepares antigens for presentation to specific cells of the immune system.

According to the clustering pathway analysis performed in the present study, when the endogenous expression of IFITM3 in HeLa cells was reduced, 21 upregulated and 21 downregulated pathways were identified. Several genes in the antigen processing and presentation pathway appeared to be either upregulated or downregulated. Of note, the majority of the components in complement signaling were downregulated, particularly C3, C4b, and C5 (Table III). Among these altered complement molecules, C5 showed the most marked reduction. As a feature of the innate immune system, the complement system assists in clearing pathogens from an organism. The complement system can be activated by the classical complement pathway, the alternative complement pathway or the mannose-binding lectin pathway (26,27). In all three pathways, a C3-convertase cleaves and activates C3, creating C3a and C3b, and causing a

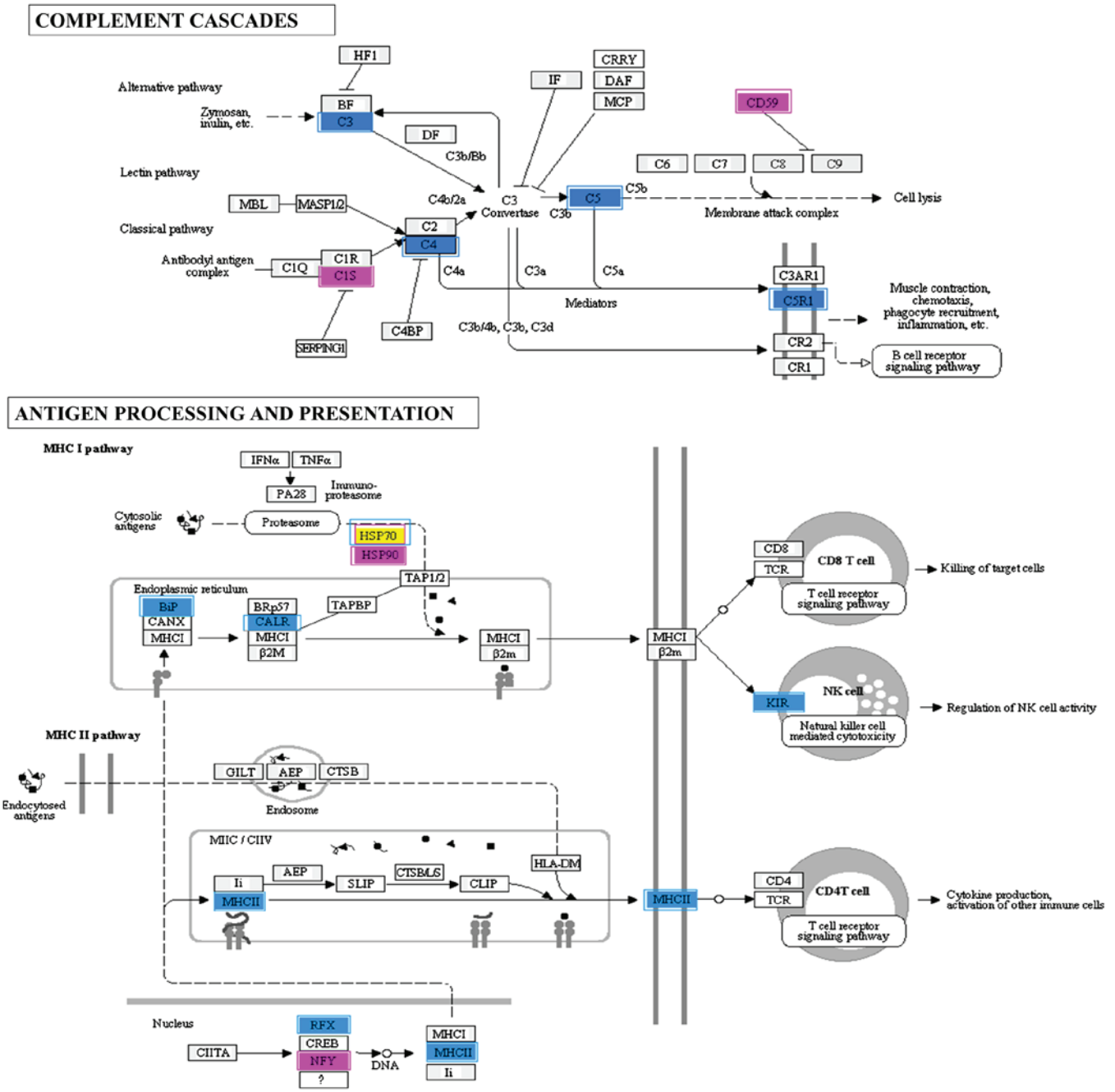

Figure 3. Affected gene pathways associated with interferon inducible transmembrane protein 3 knockdown. Purple, upregulated; yellow, upregulated and downregulated; blue, downregulated.

cascade of further cleavage and activation events. C3b binds to the surface of pathogens, leading to increased internalization by phagocytic cells through opsonization. Individuals with C3 deficiency are susceptible to bacterial infection (28). Increased levels of C3a have been found in the bronchoalveolar lavage fluid (BALF) of mice infected with the pathogenic avian influenza, H5N1 (29). C4b is the basic form of complement factor 4, which is a member of the classical activation pathway. Extensive deposition of its fragment has been found in the lungs in cases of influenza-associated mortality (30). C5 is comprised of α and β polypeptide chains (C5a and C5b), which are linked by a disulfide bridge. C5a is an important chemotactic cleavage product, assisting in the recruitment of inflammatory cells. C5b initiates the membrane attack pathway, by forming in the membrane attack complex, which is the cytolytic end product of the complement cascade and forms a transmembrane channel to cause osmotic lysis of the target cell. Kupffer cells and other macrophage cell types assist in clearing complement-coated pathogens. Individuals with C5 mutation show a propensity for severe recurrent infections. Increased levels of C5a have been found in the BALF of mice infected with H5N1 and influenza virus A (29,31).

The results of the present study suggested that sufficient expression of IFITM3 appeared to be pivotal for the recognition and elimination of infected cells by altering the transcription of genes involving the complement cascade.

As a member of the IFITM protein family, IFITM3 is involved in various biological processes. In the present study, the use of endogenous IFITM3-KD provided information regarding global gene expression alterations, and assisted in identifying the IFITM3-targeted transcripts and pathways. The results of the present study may enable more detailed investigation of the biological functions of IFITM3 in the future.

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