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Exogenous expression of marine lectins DlFBL and SpRBL induces cancer cell apoptosis possibly through PRMT5-E2F-1 pathway

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Lectins are widely existed in marine bioresources, and some purified marine lectins were found toxic to cancer cells. In this report, genes encoding *Dicentrarchus labrax* fucose-binding lectin (DlFBL) and *Strongylocentrotus purpuratus* rhamnose-binding lectin (SpRBL) were inserted into an adenovirus vector to form Ad.FLAG-DlFBL and Ad.FLAG-SpRBL, which elicited significant in vitro suppressive effect on a variety of cancer cells. Anti-apoptosis factors Bcl-2 and XIAP were determined to be downregulated by Ad.FLAG-DlFBL and Ad.FLAG-SpRBL. Subcellular localization studies showed that DlFBL but not SpRBL widely distributed in membrane systems. Both DlFBL and SpRBL were shown associated with protein arginine methyltransferase 5 (PRMT5), and PRMT5-E2F-1 pathway was suggested to be responsible for the DlFBL and SpRBL induced apoptosis. Further investigations revealed that PRMT5 acted as a common binding target for various exogenous lectin and non-lectin proteins, suggesting a role of PRMT5 as a barrier for foreign gene invasion. The cellular response to exogenous lectins may provide insights into a novel way for cancer gene therapy.

Lectins are carbohydrate-binding proteins that bind reversibly with mono- or oligosaccharide with high specificity and affinity, thus providing biological recognition molecules for a variety of cell types, including hematopoietic cell subpopulations[1], pluripotent stem cells[2], and various cancer cells[3–6]. In marine bioresources, lectins are widely existed in bacteria, algae, invertebrate animals, and fishes, which could be mainly classified into C-type lectins, galectins, F-type lectins, and rhamnose-binding lectins[7].

F-type lectins, or fucolectins, recognize fucose and have diversified biological functions in marine animals[8,9]. Previously, an F-type lectin was purified from the serum of sea bass *Dicentrarchus labrax*[10], which was further cloned, sequenced, and named as *Dicentrarchus labrax* fucose-binding lectin (DlFBL)[11]. The molecular property of DlFBL showed two tandem carbohydrate-recognition domains displaying F-type sequence motif. In situ hybridization showed that DlFBL distributed in hepatocytes and intestinal cells, as well as in embryonic yolk residues during fish ontogeny[12]. The function of DlFBL in *Dicentrarchus labrax* has been linked to enhancing the phagocytosis of microorganisms by macrophages[11].

Rhamnose-binding lectins (RBLs) are a family of animal lectins showing L-rhamnose binding activity and mainly locate in fish reproductive tissues such as eggs and ovaries, as well as immune systems including mucus and blood cells[7,13–16]. As unique structural motifs, the carbohydrate recognition domains of RBLs (RBL-CRDs) are responsible for sugar binding, and have been found widely distributed in proteins in almost all animals[7]. Diversified functions of RBLs have been found. RBLs in *Botryllus schlosseri* showed immune roles through stimulating phagocytic cells to produce more reactive oxygen species and phagocytose foreign particles, as well as inducing cytotoxic morula cells to secret more cytokines[17]. In addition to rhamnose binding, some RBLs such as sweet fish lectin (SFL), *Silurus asotus* lectin (SAL), and chum salmon lectins (CSLs) showed specific binding affinity with globotriaosylceramide (Gb3), which is also known as the receptor for various toxins such as Shiga toxin[13,18,19]. The binding of RBLs to cell surface Gb3 has been shown to induce a variety of biological functions, which include inducing apoptosis to cancer cells[20], causing disappearance of heat shock protein 70 from cell membrane[21], as well as stimulating cytokine production from macrophages and enhancing phagocytosis[22].

We previously delivered a plant gene encoding mannose binding lectin *Pinellia pedatisecta* agglutinin (PPA) into a variety of cancer cells through an adenovirus vector and achieved significant anti-proliferative effect. The

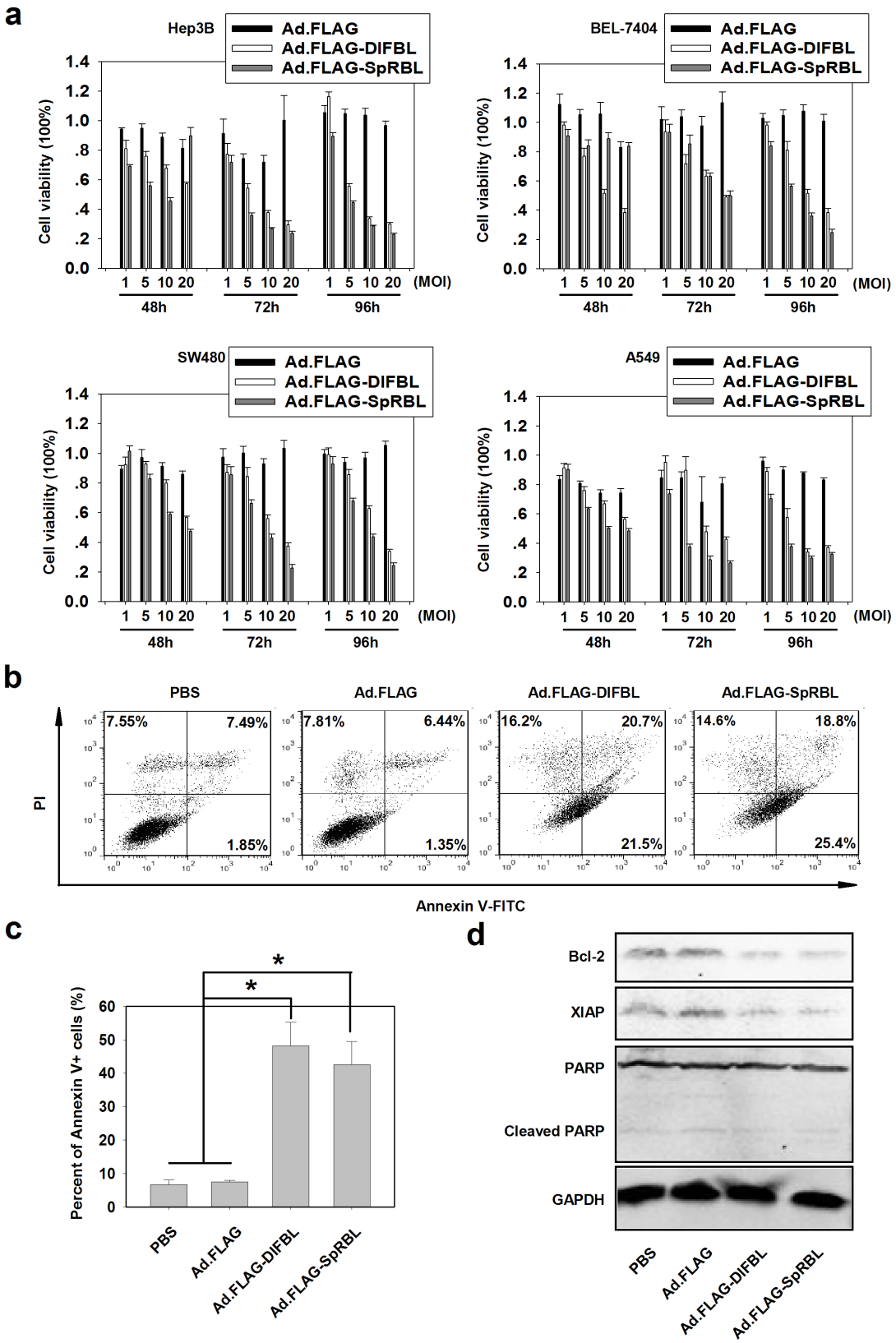

Figure 1 | Ad.FLAG-DlFBL and Ad.FLAG-SpRBL induced apoptosis and suppressed cancer cell proliferation. (a) Hepatocellular carcinoma cell lines Hep3B and BEL-7404, colorectal cancer cell line SW480, and lung cancer cell line A549 were treated with Ad.FLAG, Ad.FLAG-DlFBL, or Ad.FLAG-SpRBL at 1, 5, 10, or 20 MOIs for the time periods indicated. Cell viability was analyzed through MTT assay. Values from at least 6 repeats were calculated as percent of PBS control and presented as mean ± SEM. (b) Hep3B cells treated with Ad.FLAG, Ad.FLAG-DlFBL, or Ad.FLAG-SpRBL at 20 MOI as well as PBS control for 48 h. Cells were then stained with Annexin V-FITC and PI and analyzed under a flow cytometer. (c) The percent of Annexin V-positive cells from 3 repeats were shown as mean ± SEM (*: $p < 0.05$). (d) Cell lysates were analyzed by Western blot for levels of Bcl-2, XIAP, and PARP. GAPDH served as the loading control. Full length blots were shown in Fig. S1.

exogenously expressed PPA was shown to induce cancer cell death through interacting with the methylosome that contains protein arginine methyltransferase 5 (PRMT5) and methylosome protein 50 (MEP50)[23]. Based on these findings, we proposed that lectins widely existed in the natural world may harbor a reservoir of cytotoxic genes against various cancer cells. In this study, genes encoding marine lectins DlFBL and sea urchin *Strongylocentrotus purpuratus* rhamnose-binding lectin (SpRBL) were inserted into a replication-deficient adenovirus vector to generate recombinant adenoviruses Ad.FLAG-DlFBL and Ad.FLAG-SpRBL. The Ad.FLAG-DlFBL and Ad.FLAG-SpRBL induced cytotoxicity and the underlying mechanism were analyzed.

Results

A replication-deficient adenovirus vector was engineered to carry genes encoding FLAG-tagged DlFBL and SpRBL, forming adenoviruses Ad.FLAG-DlFBL and Ad.FLAG-SpRBL. To evaluate the anti-proliferative effect of exogenous DlFBL and SpRBL, various cancer cells including hepatocellular carcinoma cell lines Hep3B and BEL-7404, lung cancer cell lines A549, and colorectal carcinoma cell line SW480 were treated with Ad.FLAG-DlFBL and Ad.FLAG-SpRBL, as well as the control adenovirus Ad.FLAG. As compared to Ad.FLAG, Ad.FLAG-DlFBL and Ad.FLAG-SpRBL significantly suppressed the in vitro proliferation of these cancer cells, as determined by MTT assay (Fig. 1a). The suppressive effect of Ad.FLAG-DlFBL and Ad.FLAG-SpRBL on cancer cells showed both time and dose dependant manner. Furthermore, Ad.FLAG-SpRBL elicited a better anti-proliferative effect than Ad.FLAG-DlFBL after 72 h of infection.

To determine the underlying mechanism of the Ad.FLAG-DlFBL and Ad.FLAG-SpRBL induced anti-proliferative effect, Hep3B cells treated with Ad.FLAG-DlFBL, Ad.FLAG-SpRBL, Ad.FLAG or PBS were stained with Annexin V-FITC and propidium iodide (PI), a common method for apoptotic cell staining, and analyzed under a flow cytometer. Fig. 1b showed that Ad.FLAG-DlFBL and Ad.FLAG-SpRBL induced much higher percent of Annexin V+/PI- and Annexin V+/PI+ cells, compared to PBS and Ad.FLAG controls. Significant differences were achieved as shown in Fig. 1c. Results indicate that exogenous expression of DlFBL and SpRBL suppressed cell proliferation through inducing apoptosis.

To further analyze the underlying mechanism of apoptosis induced by Ad.FLAG-DlFBL and Ad.FLAG-SpRBL, Hep3B cells treated with Ad.FLAG-DlFBL, Ad.FLAG-SpRBL, Ad.FLAG, or PBS were lysed, and apoptotic signaling elements were investigated by Western blot. As shown in Fig. 1d, compared to PBS and Ad.FLAG controls, Ad.FLAG-DlFBL and Ad.FLAG-SpRBL did not significantly induced the cleavage of poly (ADP-ribose) polymerase (PARP), a substrate for various caspases, suggesting that Ad.FLAG-DlFBL and Ad.FLAG-SpRBL did not enhanced the activation of caspases. However, Ad.FLAG-DlFBL and Ad.FLAG-SpRBL significantly suppressed levels of anti-apoptosis element including Bcl-2

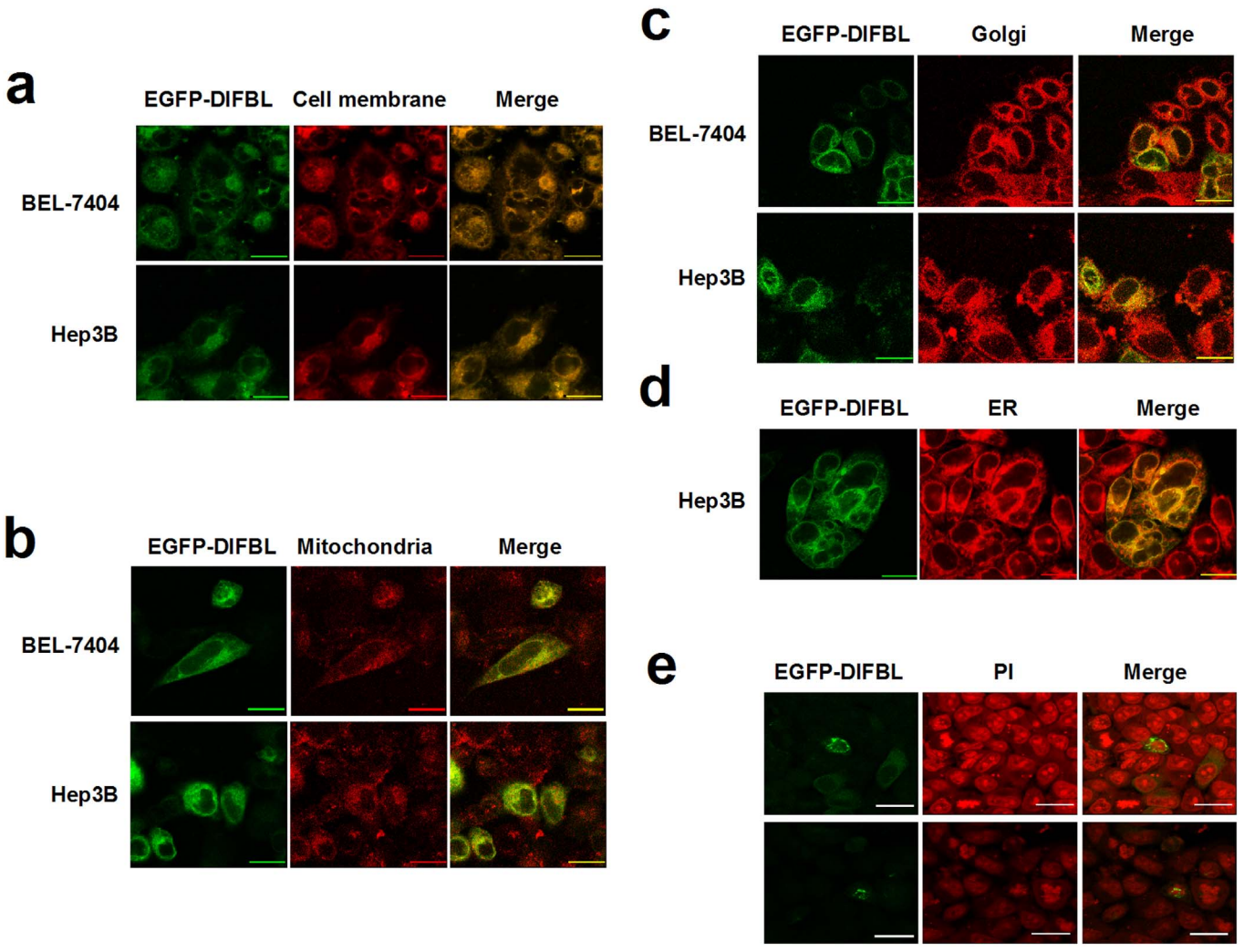

Figure 2 | Subcellular distribution of DlFBL. Hep3B or BEL-7404 cells were transfected with pEGFP-DlFBL-C1. After 24 h, cells were then stained with DiI (a), Mito Tracker Red Mitochondrion-Selective Probe (b), Golgi-Tracker Red (c), ER-Tracker Red (d), and PI (e), and followed by analysis under a confocal laser scanning microscope. Bars show 20 μm.

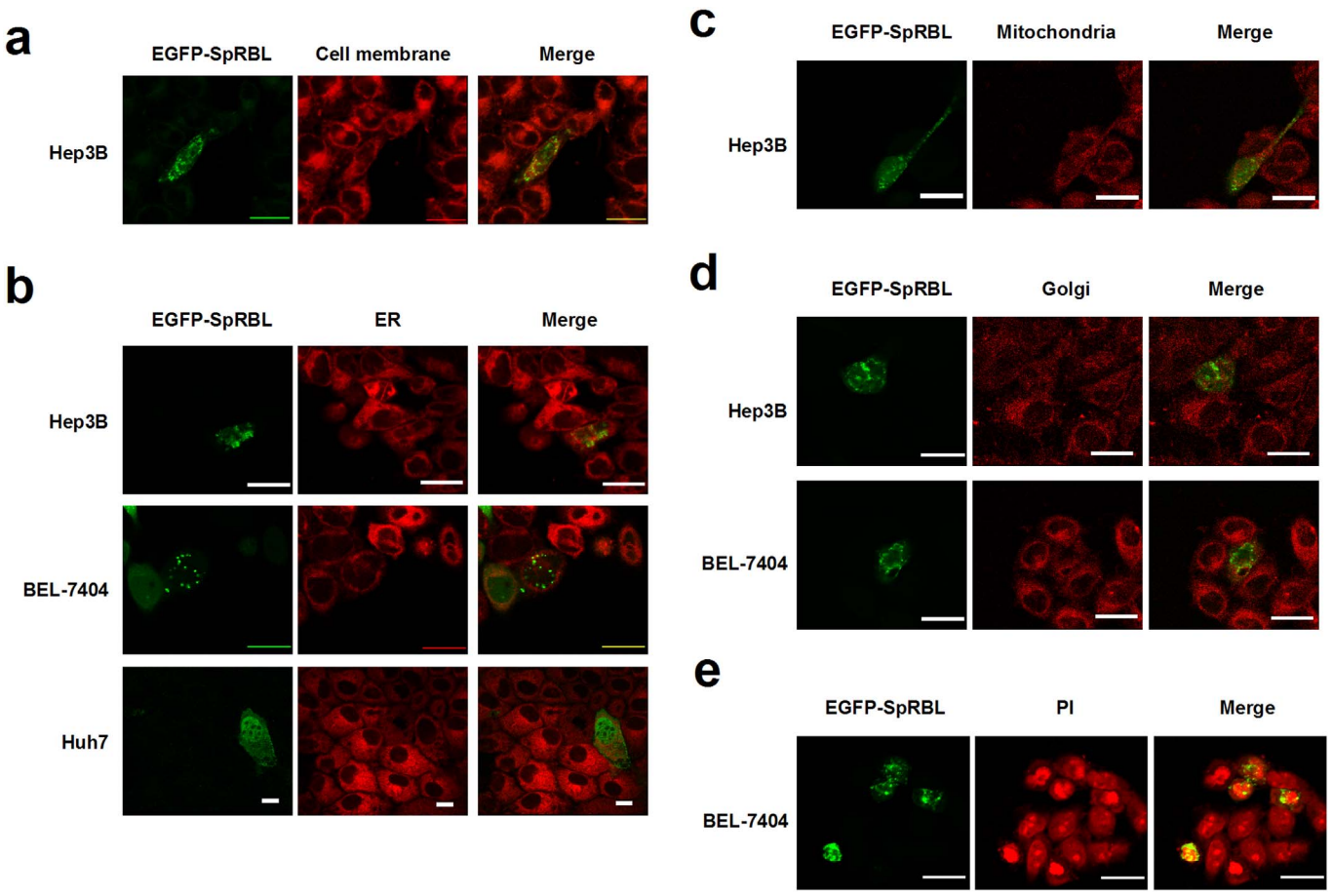

Figure 3 | Subcellular distribution of SpRBL. Hep3B, BEL-7404, or Huh7 cells were transfected with pEGFP-SpRBL-C1. After 24 h, cells were then stained with DiI (a), ER-Tracker Red (b), Mito Tracker Red Mitochondrion-Selective Probe (c), Golgi-Tracker Red (d), and PI (e), followed by analysis under a confocal laser scanning microscope. Bars show 20 μm for Hep3B and BEL-7404 cells, 50 μm for Huh7 cells.

and X-linked inhibitor of apoptosis protein (XIAP). Data suggested that exogenous expression of DlFBL and SpRBL induced apoptosis in Hep3B cells through downregulating anti-apoptosis factors including Bcl-2 and XIAP.

Intracellular distribution of DlFBL and SpRBL was investigated to further study the underlying mechanism of the apoptosis induced by DlFBL and SpRBL exogenous expression. Cancer cells Hep3B, BEL-7404, or Huh7 were transfected with plasmids pEGFP-DlFBL-C1 or pEGFP-SpRBL-C1, followed by subcellular staining and subsequent observation under a confocal laser scanning microscope. As shown in Fig. 2a–d, DlFBL was shown distributed in various membrane systems, including cell membrane, mitichondria, endoplasmic reticulum (ER), and Golgi apparatus. However, as shown in Fig. 3a–d, the majority of SpRBL did not show co-localization with membrane systems including cell membrane, mitichondria, ER, and Golgi apparatus, which was dramatically different from DlFBL. Interestingly, co-localization of DlFBL and SpRBL with PI high-staining areas in BEL-7404 cells was observed (Fig. 2e and Fig. 3e), suggesting the entrance of DlFBL and SpRBL into the nucleus.

Because nuclear translocation also happened to PPA, which utilized the PRMT5 as a target[23], we then analyzed the possible interaction of DlFBL and SpRBL with PRMT5. Plasmids pEGFP-DlFBL-C1 or pEGFP-SpRBL-C1 was co-transfected into Hep3B cells with pdsRed-PRMT5, and the distribution of DlFBL, SpRBL, and PRMT5 was analyzed under a confocal laser scanning microscope. As shown in Fig. 4a and b, obvious co-localization of DlFBL and SpRBL with PRMT5 was determined. Recently, transcription factor E2F-1 was determined to be directly methylated by PRMT5 and acting as a downstream element for PRMT5. We then examined the effect of Ad.FLAG-DlFBL and Ad.FLAG-SpRBL infection on E2F-1 levels. As shown in Fig. 4c, either Ad.FLAG-DlFBL or Ad.FLAG-SpRBL treatment significantly downregulated E2F-1 levels, as compared to PBS and Ad.FLAG controls. Data suggested that the PRMT5-E2F-1 pathway was involved in the DlFBL and SpRBL induced apoptosis.

We then asked whether PRMT5 acts as a common binding target for various exogenous lectins. Genes encoding *Haliotis discus discus* sialic acid binding lectin (HddSBL), *N*-acetyl-*D*-glucosamine binding *Ulva pertusa* lectin 1 (UPL1), mannose binding *Pinellia pedatisecta* agglutinin (PPA), as well as a non-lectin protein Equinatoxin II S105-P142 fragment EqtII (S105-P142), were inserted into pEGFP-C1 plasmid, to form pEGFP-HddSBL-C1, pEGFP-UPL1-C1, pEGFP-PPA-C1, and pEGFP-EqtII (S105-P142)-C1, followed by co-transfection into BEL-7404 and Hep3B cells with pdsRed-PRMT5. A confocal laser scanning microscopy showed that not only lectins including HddSBL, UPL1, and PPA (Fig. 4d), but also non-lectin EqtII (S105-P142) fragment (Fig. 4e), were found co-localized with PRMT5. Data suggested that PRMT5 may form a common binding target for a variety of foreign lectin and non-lectin proteins.

Discussion

We reported here that DlFBL and SpRBL distributed differently in cell membrane and cytoplasm. Since lectins are carbohydrate-specific proteins, the differed distribution of these two lectins may suggest differed glycosylation pattern of various membrane systems. Glycans are linked and modified to proteins and lipids by glycosyltransferases and glycosidases in secretory pathways, which play important roles in regulating cell adhesion, molecular trafficking, and signal transduction[24]. L-fucose is a common component of N-

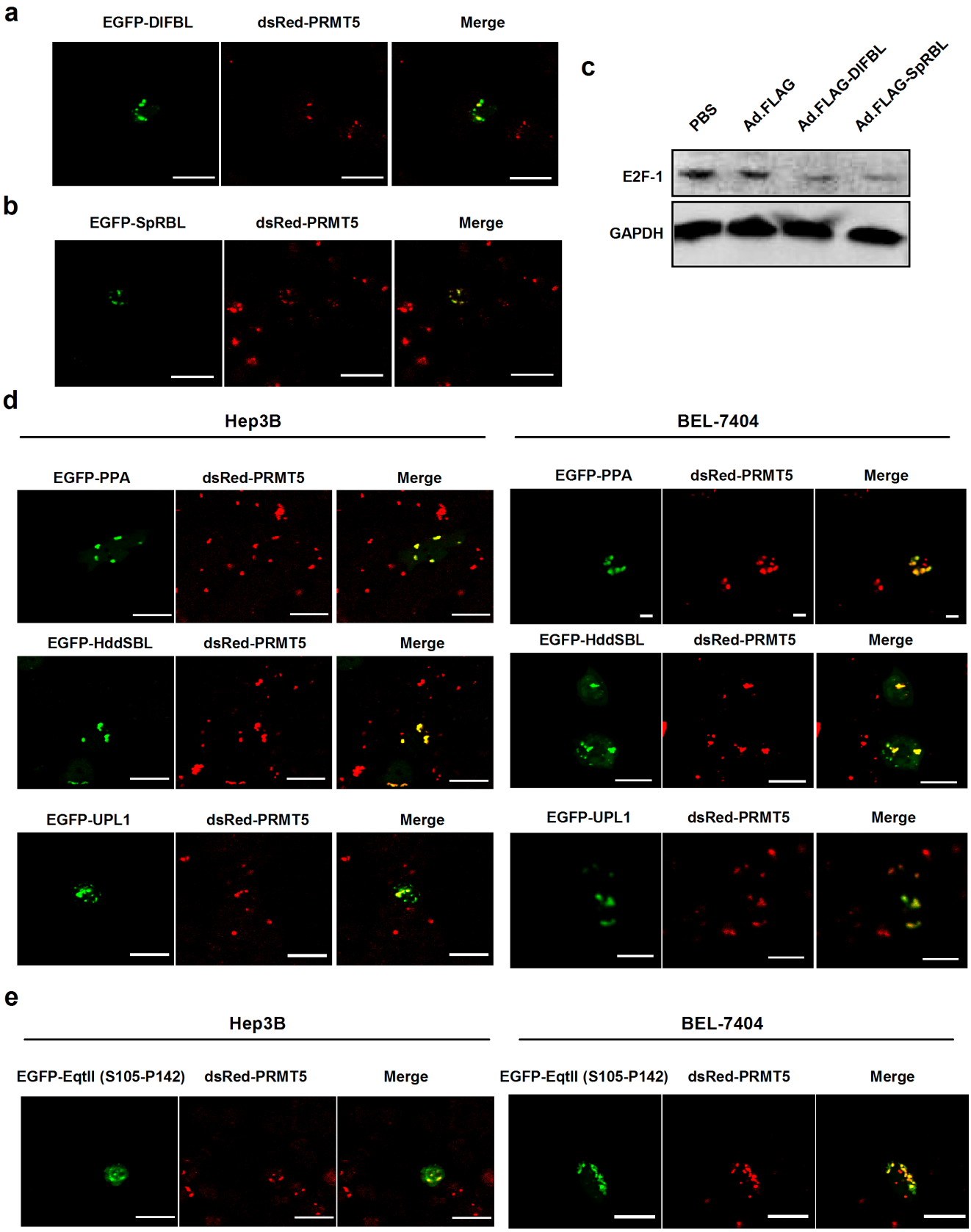

Figure 4 | PRMT5 acts as a common target for various exogenous proteins. (a) Hep3B cells were cotransfected with pEGFP-DlFBL-C1 and pdsRed-PRMT5. (b) Hep3B cells were cotransfected with pEGFP-SpRBL-C1 and pdsRed-PRMT5. (c) Hep3B cells treated with Ad.FLAG, Ad.FLAG-DlFBL, or Ad.FLAG-SpRBL at 20 MOI as well as PBS control for 48 h. Cell lysates were analyzed by Western blot for the levels of E2F-1. GAPDH served as the loading control. Full length blots were shown in Fig. S1. Plasmid pdsRed-PRMT5 was cotransfected into Hep3B cells or BEL-7404 cells with (d) pEGFP-PPA-C1, pEGFP-HddSBL-C1, pEGFP-UPL1-C1, and (e) pEGFP-EqtII (S105-P142)-C1. After 48 h, the co-localization of PPA, HddSBL, UPL1, and EqtII (S105-P142) fragment with PRMT5 was observed under a confocal laser scanning microscope. Bars show 20 μm.

and O-linked glycans and glycolipids in mammalian cells, and frequently exists as terminal residues of glycan structures[25]. Therefore, membrane exposed fucose may provide recognition site for DlFBL, thus resulting in a wide distribution of DlFBL as shown in our data. On the contrary, poor binding of SpRBL with membrane systems suggest that rhamnose is rarely existed as terminal residues of glycan chains during protein and lipid glycosylation.

Transcription factor E2F-1 has been identified as an activator of cell cycle progression, as well as an apoptosis inducer[26]. Overexpression of E2F-1 was shown to promote leukemia cell proliferation in a cytokine –independent manner, and a variety of cell cycle dependant cyclins were maintained by E2F-1 without cytokine stimulation[27]. As a growth-promoting factor, E2F-1 was shown to be associated with adverse prognosis in non-small cell lung cancer[28]. In contrast, E2F-1 induced cell apoptosis through cooperation with either p53[29] or p73[30]. In response to DNA damage, E2F-1 was activated by Chk2 and led to apoptosis[31]. Recently, E2F-1 was determined to be a direct substrate for PRMT1 and PRMT5[32,33], and E2F-1 methylated by PRMT1 augmented cell apoptosis, whereas E2F-1 methylated by PRMT5 favored cell proliferation[33], suggesting a key role of differed arginine methylation in determining E2F-1 biological activities. In our data, both DlFBL and SpRBL were found associated with PRMT5, and the expression levels of E2F-1 were downregulated by DlFBL and SpRBL. Furthermore, the expression of DlFBL and SpRBL also suppressed the levels of anti-apoptotic factors Bcl-2 and XIAP. As E2F-1 has been shown to bind to the promoter of Bcl-2 and positively regulate Bcl-2 expression[34], our results suggest that the association of exogenous DlFBL and SpRBL with PRMT5 may alter the function of PRMT5 and then the downstream events, the methylation pattern of E2F-1 and decreased levels of E2F-1. The suppressed E2F-1 may subsequently result in a downregulation of apoptosis inhibitors such as Bcl-2 and XIAP, which finally led to cell apoptosis. Therefore, the PRMT5-E2F-1-Bcl-2 pathway was suggested to be the target for the exogenous DlFBL and SpRBL induced apoptosis.

PRMT5, a type II protein arginine methyltransferase, has multiple roles involved in cell differentiation, proliferation, survival, as well as tumorigenesis, through acting on a variety of substrates. PRMT5 methylates histone H4R3, which recruits DNA methyltransferase 3A, resulting in gene silencing[35]. Arginine methylation by PRMT5 enhances NF-κB p65 subunit activation and drives gene expression including many cytokines and chemokines[36]. In addition to E2F-1 stated above, HOXA9 is another transcription factor methylated by PRMT5, which has been shown involved in E-selectin induction and endothelial cell inflammatory response[37]. The substrate specificity of PRMT5 was suggested to be dependant on interactors that PRMT5 associated with[38–41]. We previously demonstrated that PRMT5 was co-precipitated with exogenous mannose-binding lectin PPA[23]. Here, we further identified that PRMT5 acted as a common binding target for various exogenous lectins, including DlFBL, SpRBL, PPA, HddSBL, and UPL1, suggesting a complicated glycosylation pattern of PRMT5 complex. The sugar decoration may be responsible for the wide interaction of PRMT5 complex with various endogenous and exogenous proteins. Second, in addition to lectins, non-lectin protein EqtII S105-P142 fragment was also found associated with PRMT5, suggesting that PRMT5 may play an important role as a mediator to interact with exogenous proteins. Taking into consideration the fact that Hep3B cells infected with Ad.FLAG-DlFBL or Ad.FLAG-SpRBL finally underwent apoptosis, we propose here that PRMT5 may play a role as a sensor for the expression of invaded foreign genes, and subsequently affect E2F-1-Bcl-2 pathway to initiate cell apoptosis, thus forming a barrier for foreign gene sustaining.

We show here that the exogenous expression of DlFBL and SpRBL induced apoptosis and suppressed cancer cell proliferation in vitro. PRMT5 acted as a common binding target for various exogenous lectin or non-lectin proteins. Data suggest that the delivery of genes encoding DlFBL and SpRBL lectins may be a novel strategy to induce cancer cell apoptosis. Further cancer-specific controlling of DlFBL and SpRBL expression may provide insights into a novel way for cancer gene therapy.

Methods

Cell culture and transfection. Heptocellulart carcinoma cell lines Hep3B, Huh7, and BEL-7404, lung cancer cell lines A549, and colorectal carcinoma cell line SW480 were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin solution, and 1% L-Glutamine. Appropriate amounts of plasmids were transfected into cells by Thermo Scientific TurboFect Transfection Reagent (Thermo Fisher Scientific Inc, Canada) following the manufacturer's instruction.

Plasmid construction. The plasmid pGH-genes carrying DNA sequences encoding *Dicentrarchus labrax* fucose-binding lectin (DIFBL, GenBank accession number: EU877448), *Strongylocentrotus purpuratus* rhamnose-binding lectin (SpRBL, GenBank accession number: LOC100892700), *Haliotis discus discus* sialic acid binding lectin (HddSBL, GenBank accession no. EF103404), *Ulva pertusa* lectin 1 (UPL1, GenBank accession no. AY433960) were purchased from Shanghai Generay Biotech Co., Ltd, China. The sequences were cut with *Xho*I, and then inserted into the corresponding site of pEGFP-C1 to form plasmids pEGFP-gene-C1.

Adenoviral construction. The FLAG tagged sequences of DlFBL and SpRBL were amplified from pGH-DIFBL and pGH-SpRBL through polymerase chain reaction. The products were inserted with pCA13 to form pCA13-FLAG-DlFBL and pCA13-FLAG-SpRBL. The expression cassettes of FLAG-DlFBL and FLAG-SpRBL were digested from plasmids pCA13-FLAG-DlFBL and pCA13-FLAG-SpRBL with *Bgl*II and inserted into the corresponding site of plasmid pShuttle, forming plasmids pShuttle-FLAG-DlFBL and pShuttle-FLAG-SpRBL, which were subsequently transformed into strain BJ5183. Viral genomes of Ad.FLAG-DlFBL and Ad.FLAG-SpRBL were generated through homologous recombination of pShuttle-FLAG-DlFBL and pShuttle-FLAG-SpRBL with viral skeleton plasmid pAdEasy-1, and transfected into 293A cells after linearized by *Pac*I. Adenoviruses Ad.FLAG-DlFBL and Ad.FLAG-SpRBL were then produced and virus titers were determined by Titer-EZ adenoviral titer detection Reagent (Shang Hai SBO Medical Biotechnology CO., Ltd, Shanghai, China) following the manufacturer's instruction.

Subcellular staining and colocalization study. ER-Tracker Red, Golgi-Tracker Red, propidium iodide (PI), and DiI were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Mito Tracker Red Mitochondrion-Selective Probe was purchased from Invitrogen (Grand Island, NY, USA). Cells were plated on cover glass bottom dish (35 mm) at 1×10^5 per dish one day before transfection with plasmids. Cells were then transfected with plasmids as previously stated for 24 h or 48 h, followed by staining with ER-Tracker Red, Golgi-Tracker Red, Mito Tracker Mitochondrion Selective Probes, PI, or DiI followed by observation under a confocal laser scanning microscope (Nikon, Inc., Japan).

Cytotoxicity detection and Flow cytometry assay. Cells were plated on 96-well plates at 5×10^3 per well one day before infected with adenoviruses. Then cells were infected with adenoviruses at corresponding multiplicity of infections (MOI) for 48 h, 72 h, or 96 h. The cytotoxicity detection assay was carried out as the procedure of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Meanwhile, cells were plated on 6-well plates at 3×10^5 per well one day before infected with adenoviruses. Then cells were collected to detect apoptosis by Annexin V-FITC Apoptosis Detection Kit (KeyGEN Biotech Co., Ltd, Nanjing, China) following the manufacturer's instruction, followed by analyzing under a BD FACSAria flow cytometry (BD Biosciences, San Jose, CA, USA).

Western blotting analysis. The cell extracts were subjected to SDS-PAGE and electroblotted onto nitrocellulose membranes. The membranes were then blocked with Tris-buffered saline and Tween 20 contaning 5% of bovine serum albumin at room temperature for 2 h and incubated with corresponding antibodies overnight at 4°C. The membranes were washed and incubated with appropriate dilution of IRDye 800 donkey anti-mouse IgG or IRDye 700 donkey anti-rabbit IgG (LI-COR, Inc., Lincoln, NA, USA) for 1 h at room temperature. After washing with Tris-buffered saline, the membranes were then analyzed by an Odyssey Infrared Imaging System (LI-COR, Inc.).

The rabbit anti-PARP (H250) antibody was purchased from Santa Cruz biotechnology Inc. (Santa Cruz, CA, USA). Rabbit anti-XIAP antibody was purchased from Epitomics (Burlingame, CA, USA). Rabbit anti-GAPDH antibody and rabbit anti-Bcl-2 antibody were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA).

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Author contributions

G.L. designed experiments. L.W., X.Y., X.D., L.C. performed experiments. G.L. conceived data and wrote the manuscript.

Additional information

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