Regulation of TGF-β1 driven lung fibrosis by galectin-3

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ONLINE DATA SUPPLEMENT

Online data supplement

METHODS

Reagents

Tissue culture reagents were purchased from Life Technologies (Paisley, Scotland, UK). Tissue culture plastics were obtained from Costar (Loughborough, Leicestershire, UK) and Falcon (Runcorn, Cheshire, UK). Cytokines and mouse galectin-3 and TGF-β1 ELISA kits were purchased from R&D Systems (Abingdon, Oxon, UK). Bleomycin was from Nippon Kayaku Co. Ltd (Tokyo, Japan). All other reagents were from Sigma-Aldrich Company Ltd (Poole, Dorset, UK) unless otherwise stated. Baculovirus derived recombinant mouse galectin-3 was purified as described previously (1).

TD139

Bis (3-deoxy-3-(3-fluorophenyl-1*H*-1,2,3-triazol-1-yl)-β-D-galactopyranosyl) sulfane TD139 was synthesized by Cu(I)-catalyzed cycloaddition between bis (2,4,6-tri-*O*-acetyl-3-deoxy-3-azido-β-D-galactopyranosyl) sulfane (2) and 3-fluorophenylacetylene, Zemplen de-*O*-acetylation, and purification by preparative hplc as described for analogous compounds (2). ^{1}H-NMR (CD_3OD, 400 MHz) d 8.59 (s, 3H, triazole-H), 7.63 (br d, 1H, 7.6 Hz, Ar-H), 7.57 (br d, 1H, 8.4 Hz, Ar-H), 7.41 (dt, 1H, 6,0 and 8.0 Hz, Ar-H), 7.05 (br dt, 1H, 2.4 and 6.4 Hz, Ar-H), 4.93 (dd, 1H, 2,4 and 10.4 Hz, H3), 4.92 (d, 1H, 10.4 Hz, H1), 4.84 (1H, 10.4 Hz, H2), 4.18 (d, 1H, 2.4 Hz, H4), 3.92 (dd, 1H, 4.2 and 7.6 Hz, H5), 3.84 (dd, 1H, 7.6 and 11.4 Hz, H6), 3.73 (dd, 1H, 4.2 and 11.4 Hz, H6); FAB-HRMS m/z calculatedd for $C_{28}H_{30}F_2N_6NaO_8S$ (M+Na), 671.1712 found, 671.1705. Dissociation constants for TD139 and galectin-1 and 3 were determined with an in detail published competitive fluorescence polarization assay to be 12±3 and 14±3 nM, respectively.(3, 4).

AdTGFβ preparation and model of pulmonary fibrosis

AdTGFβ refers to porcine TGF-β_1 adenovirus (AdTGF$\beta_1^{223/225}$), an adenovirus construct containing a mutation of cysteine to serine at positions 223 and 225, rendering the expressed TGFβ_1 biologically active (5). Control virus (Ad-DL70-3) was constructed as previously described (5).

The TGFβ and control virus were shown to be replication competent adenovirus **(**RCA) free using a modified supernatant rescue assay described by Dion *et al* (6) and modified by Hehir *et al* (7) and Zhu *et al* (8). The first amplification is modified to use A549 cells, which are infected with vector preparations at maximum multiplicity of infection (MOI) of 10 (serial dilutions) and monitored for seven days (1^{st} round – only RCA can amplify under these conditions). On day 7, a crude lysate is prepared from these A549 cells and used to infect fresh monolayers of A549 cells (2^{nd} round). Cells are fed and monitored for cytopathic effect (CPE) over the following 20 days. Any CPE that is seen at this stage has come from at least 1 RCA present in the original materials.

Model of bleomycin-induced lung fibrosis

Female mice 10-14 weeks old were anaesthetized with halothane, and bleomycin or saline was administered intratracheally (33 μg in 50 μl of saline). Mice were culled on days 15, 21 or 32 by terminal anaesthesia, the trachea were cannulated, and the lungs lavaged with 3 x 0.8 ml sterile PBS pH 7.4 containing 0.1 mM EDTA. Lungs were removed and the left lobe and the upper right lobe were tied off and snap frozen in liquid nitrogen for collagen and RNA analysis. The remaining lung was inflated with Methyl Carnoy's solution (60% methanol, 30% chloroform, 10% glacial acetic acid). After 24 h lungs were transferred into 70% ethanol prior to paraffin wax embedding and sectioning for histology and immunohistochemistry.

Determination of lung fibrosis and inflammation

Histological lung inflammation and fibrosis score was carried out in Masson's trichrome stained sections as described (9). Inflammation (peribronchiolar, perivascular, and alveolar wall thickness) was scored in > 5 random fields at magnification X630 using the following system (peribronchiolar and perivascular, 1 = no cells, 2 = <20 cells, 3 = 20 – 100 cells, 4 = > 100 cells; alveolar wall thickness, 1 = 1 cell, 2 = 2 – 3 cells thick, 3 = 4 – 5 cells thick, 4 = > 5 cells thick). The combined inflammatory score was the sum of these scores. Fibrosis score was evaluated as the area of the section positively stained for collagen (1 = none, 2 = <10%, 3 = < 50%, 4

= > 50%). Only fields where the majority of the field is composed of alveoli were scored.

Determination of lung collagen by sircol assay

The left lobe was minced in 5 ml of 3 mg/ml pepsin in 0.5 M acetic acid and incubated with shaking at 4^{o}C for 24 h. Cleared lung extract (0.2 ml) was incubated with 0.8 ml sircol reagent for 1 h at room temperature and precipitated collagen centrifuged at 10,000g for 5 min at 4^{o}C. Pellets were solubilised in 1 ml 1 M NaOH and absorbance measured at 570 nm alongside collagen standards.

Primary Type II murine alveolar epithelial cell isolation

Wild type and galectin-3$^{-/-}$ mouse type II lung alveolar epithelial cells (AECs) were extracted following the method originally described by Corti *et al* (10) which gave rise to AEC yields of >95% purity. Briefly, 1 ml of 50 U/ml dispase (BD Biosciences) was administered intratracheally into perfused lungs followed by instillation of 0.5 ml of 1% low melting point agarose. The agarose within the upper airways was allowed to set on ice for 2 minutes and the lungs were placed in 4 ml 50 U/ml dispase for 45 min at room temperature. The lung lobes minus the upper airways were then dispersed in DMEM containing 50 µg/ml DNAse I (Sigma-Aldrich, UK). The cell suspension was passed through a 100-µm cell strainer and the cells were washed in DMEM followed by resuspension in DMEM containing 10% FCS. The cell suspension was plated onto tissue culture plastic for 1 h to allow any contaminated fibroblasts and macrophages to adhere. Non-adherent epithelial cells were counted and cultured for 2 days on tissue culture plastic or cover-slips pre-coated with 5 µg/ml collagen (AMS Biotechnology) and 10 µg/ml fibronectin (Sigma-Aldrich), Cells were washed three times in PBS before treatment. Epithelial cells were either incubated in DMEM containing 10% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin and 5 µg/ml L-glutamine or transferred to complete mouse media (DMEM/F-12 containing 0.25% BSA, 10 nM hydrocortisone, 5 µg/ml Insulin-Transferrin-Sodium-Selenite (ITS) and supplemented with 0.1 mg/ml sodium succinate, 75 µg/ml succinic acid and 1.8 µg/ml choline bitartrate).

Tcf/Lef reporter assay

The Tcf reporter plasmid kit (Cat No. 17-285) containing the TOPflash and FOPflash reporter plasmids was purchased from Millipore. To examine the effects of galectin-3 on tcf transcriptional activity, WT and galectin-$3^{-/-}$ AECs in six-well plates were transiently transfected with 1 μg of TOPflash and FOPflash plasmids together with 0.2 μg renilla luciferase (pRL-TK Promega) using Polyfect as per manufacturers instructions (Qiagen). After 48 h, cells were harvested for determination of luciferase activity, which was measured using the Promega Dual Luciferase assay system.

Western Blotting

Cells were lysed in 25 mM HEPES pH 7.4, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5% triton X-100, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate and protease inhibitors (Boehringer Mannheim, Sussex, UK; prepared as per manufacturers instructions). Lysates were equilibrated for protein using Pierce BCA protein assay reagent (Pierce) and resolved on 12% SDS-PAGE gels. Western blot analysis was undertaken using the following primary antibodies; mouse monoclonal anti-α-SMA antibody clone 1A4 (Sigma, UK), mouse monoclonal anti-galectin-3 antibody clone A3A12 (Alexis Biochemicals, UK), rabbit polyclonal anti-Smad3 (Cell Signaling Technology) rabbit polyclonal anti-Smad3 phosphospecific (ser 423/425) (Biosource, UK). Rabbit anti phospho(tyr654)-β-catenin, rabbit anti anti-GSK3β, rabbit anti-Smad2 phosphospecific (ser 465) (Abcam), rabbit anti-Smad2/3, rabbit anti β-catenin, mouse anti-E-cadherin antibody clone 36/E (BD Bioscences), rabbit polyclonal anti-β-actin antibody (Sigma, UK), mouse anti-active (ABC) β-catenin, rabbit anti-GSK3β-(ser9) (Millipore), rabbit anti AKT (Santa Cruz Biotechnology), anti-pAKT(S473) (Invitrogen).

Real-time RT-PCR

Total RNA from whole lung and cultured cells was extracted using RNeasy kit (Quiagen) and reverse transcribed into cDNA by using random hexamers (applied Biosystems). A SYBR green-based quantitative fluorescence method (applied Biosystems):

mouse β-actin: forward 5'-AGAGGGAAATCGTGCGTGAC-3',
reverse 5'-CAATAGTGATGACCTGGCCGT-3';
α-SMA: forward 5'-TCAGCGCCTCCAGTTCCT-3',
reverse 5'-AAAAAAAACCACGAGTAACAAATCAA-3'

A549 cell culture

A549 cells (ATCC) were cultured in DMEM supplemented with 10% (v/v) FCS, 50 U/ml penicillin, 50 μg/ml streptomycin and 5 μg/ml L-glutamine in a humidified atmosphere of 5% CO_2:95% air at 37^{o}C. For siRNA treatment, cells in 6 well plates at 70% confluency were washed with 10 mM lactose and transfected with siRNA (final concentration 100nM) against the target sequence GAAGAAAGACAGTCGGTTT (11) using Oligofectamine (Invitrogen, UK). Control duplex was siCONTROL non-targeting siRNA No. 2 (Dharmacon Research, Lafayette, US). Cells were incubated for 48 h in complete media (in the presence of 1 mM lactose for siRNA cells).

Wounding assay

Wounds were induced in confluent cultures of AECs by scoring with a plastic pipette tip. Cells were incubated in serum free DMEM in the presence or absence of TGF-β and migration of cells into the scored region was observed by phase contrast microscopy.

TGF receptor binding

A549 cells (control or transfected with galectin-3 siRNA) in 6 well plates were washed and placed in serum free media overnight. Cells were washed in fresh media and incubated at 37^{0}C for 3 h. Cells were washed to remove any TGF-β1 and placed in binding buffer (DMEM containing 0.1% BSA 10 mM HEPES pH 7.4) with 20 pM [^{125}I]-TGF-β1 (Perkin Elmer 4000 Ci/mmol) and various concentrations of unlabelled

TGF-β1 at 4°C for 3 h. Cells were washed x3 in PBS 0.1% BSA and once in PBS without BSA and bound TGF was extracted in 1% Triton and counted by scintillation spectroscopy. Binding parameters K_d and B_{max} were calculated as described (12).

TGF receptor flow cytometry

A549 cells transfected with siRNA to galectin-3 or control duplex were treated with or without 10 ng/ml TGF-β for 2h. Cells were trypsinised and incubated on ice with goat anti-human TGFR-II (R and D Systems, AF-241-NA) followed by FITC conjugated anti-goat IgG (Dako). Cell surface TGFR-II was determined by FACS analysis on a BD FACSCalibur flow cytometer and analyzed using Flowjo software.

Fibrocyte recruitment to lungs following bleomycin

Mice were given 0.033mg bleomycin intratracheally and lungs harvested at 21 days. Lungs were finely minced and digested with 4 mg/ml collagenase D (Roche Applied Sciences, UK) in PBS containing 0.5% bovine serum albumin (FACS) buffer at 37°C for 1 hour. Cells were washed with FACS buffer and red cells were lysed using standard ACK buffer (0.15 M NH_4CL, 1 mM $KHCO_3$ 0.1 mM EDTA) for 5 minutes on ice then strained using a 40 μm cell strainer. Cell suspensions were washed in FACS buffer containing 0.5% triton X-100 and blocked with Fc block™ (rat anti-mouse CD16/CD32, 553142, BD Europe). Cells were stained with rabbit anti mouse collagen-1 (Millipore 1:100) for 30 min on ice followed by anti-rabbit FITC (Dako 1:200). Cells were then incubated with PE anti-mouse CD34 and PerCP-Cy5.5 anti-mouse CD45.2 (Biolegend 1:100) for 30 min on ice. Washed cells were suspended in 5% neutral buffered formalin prior to analysis using FACSCaliber (Beckton Dickenson). Data was analyzed using FlowJo software.

Supplemental figure legends

Figure E1. Galectin-$3^{-/-}$ mice show similar acute inflammation following $2x10^8$ PFU TGF-β1-adenovirus. A) Levels of active TGF-β1 in the BAL fluid of wild type (WT) and galectin-$3^{-/-}$ mice treated with TGF-β1 adenovirus (closed bars) compared to control adenovirus (open bars). B) Cell counts and C) protein in BAL fluid from TGF-β1 adenovirus treated mice. D) Galectin-3 levels in the BAL fluid was enhanced by TGF-β1-adenovirus in wild type mice. E) Combined inflammatory score obtained from Masson's trichrome stained sections from mouse lung. Results are expressed as mean ± SEM (n = 6).

Figure E2 A) Galectin-3 deletion has no effect on fibroblast proliferation. Fibroblast proliferation was measured by total cell protein. Results represent the mean SEM of 3 experiments. B) AECs were treated with 5 ng/ml TGF-β for 24 or 48h and galectin-3 released into the supernatant was measured by ELISA.

Figure E3 Wild type and galectin-$3^{-/-}$ AECs were cultured for 48hrs with TGF-β1 in the presence or absence of 25 μg/ml recombinant galectin-3. Cells were lysed and western blots probed for active and total β-catenin and β-actin.

Figure E4 No effect of TD139 in non-fibrotic lung. Mice received 0.05 ml saline intratracheally followed by TD139 (10 μg) or saline on days 18, 20, 22 and 24 and lungs were harvested on day 26 as described in materials and methods. Serial sections of mouse lung were stained with Masson's trichrome, galectin-3 and active β-catenin (scale bar = 100 μm) as indicated.

Figure E5 No difference in fibrocyte recruitment in galectin-$3^{-/-}$ and WT mice following bleomycin. Mice were given 0.033mg intratracheal bleomycin and lungs harvested at 21 days. A) Lung digests were stained with anti CD45, anti-CD34 and anti-collagen-1 antibodies and analyzed by flow cytometry. CD45 positive gated cells were examined for CD34 and col-1 staining. B) There was no difference in the percentage of triple positive cells in WT vs galectin-$3^{-/-}$ lungs, n=6 per group. C)

Lung sections were stained for CD34 (red) and Col-1 (green) and examined by fluorescence microscopy. Dual positive orange cells are indicated (arrows). Bar = 50μm.

Figure E6 Schematic showing the regulation of TGF-β induced β-catenin activation and EMT by galectin-3. Galectin-3 secretion is stimulated in epithelial cells by TGFβ. Galectin-3 pentamers bind to poly N-acetyl lactosamine (LNac) residues on TGF receptors causing cell surface retention and promotes signaling to Smads and AKT. Activation of AKT causes phosphorylation and inhibition of GSK3β leading to activation of β-catenin. The concomitant nuclear accumulation of Smads and β-catenin leads to transcription of mesenchymal genes. See text for details.

Online Supplemental References

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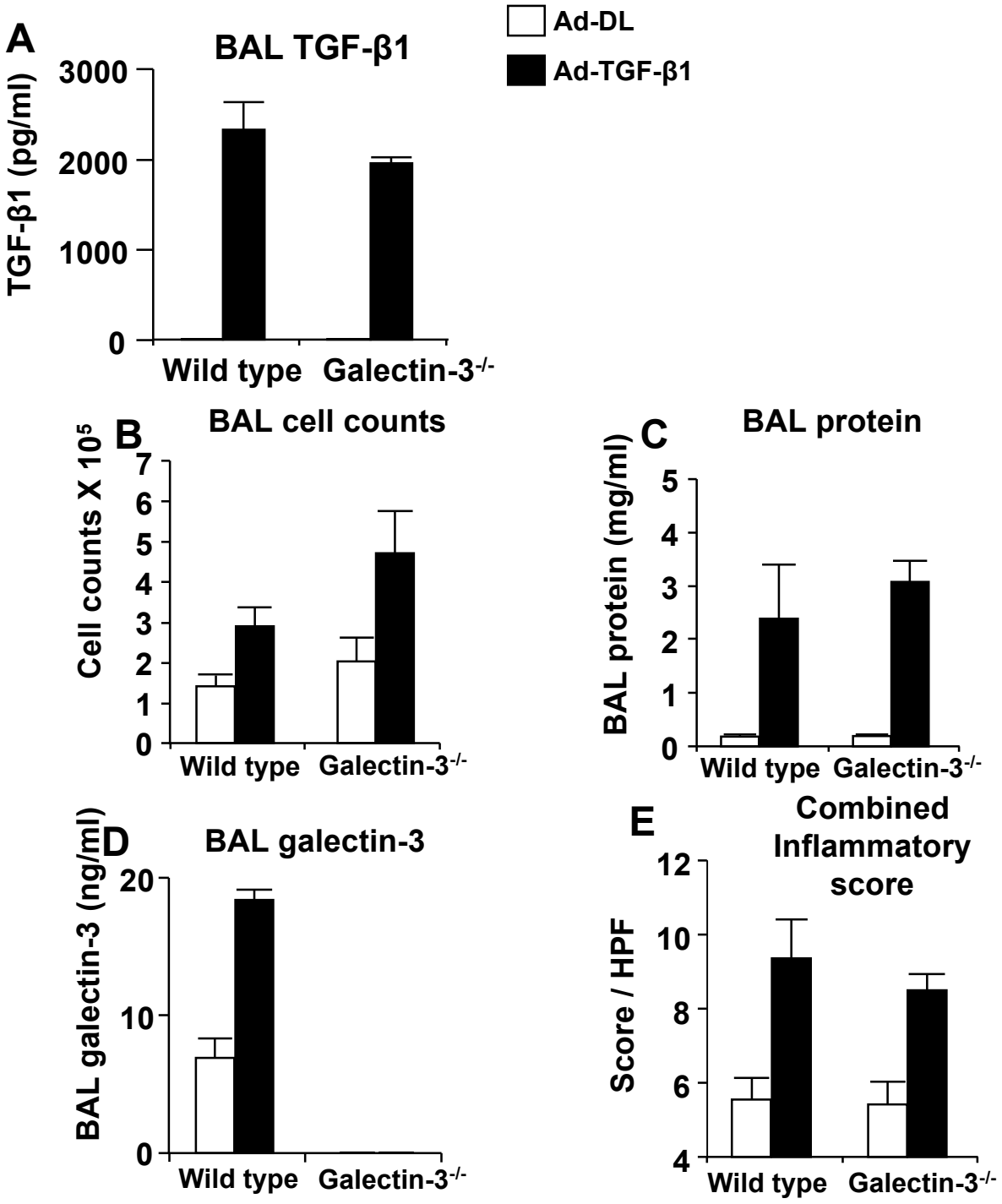
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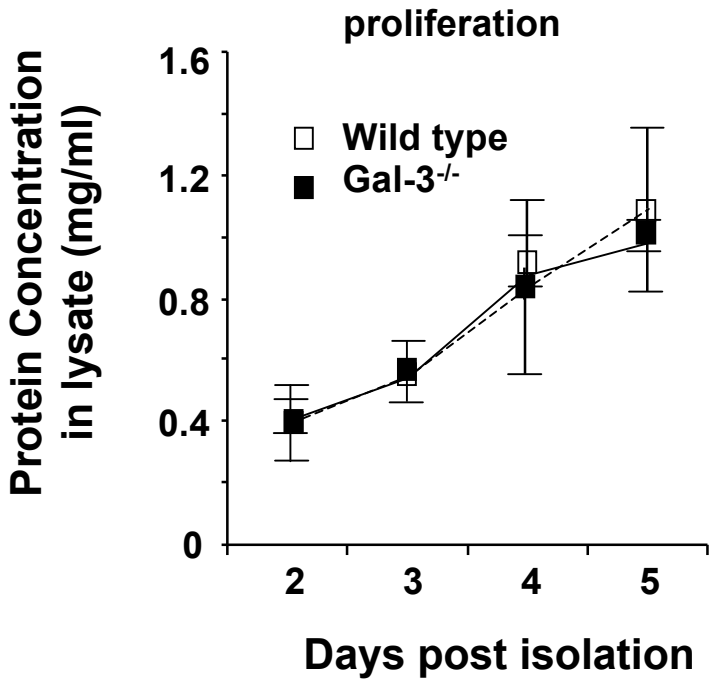
BAL TGF-β1
Ad-DL
Ad-TGF-β1
TGF-β1 (pg/ml)
3000
2000
1000
0
Wild type
Galectin-3-/-
B
BAL cell counts
Cell counts X 10⁵
7
6
5
4
3
2
1
0
Wild type
Galectin-3-/-
C
BAL protein
BAL protein (mg/ml)
5
4
3
2
1
0
Wild type
Galectin-3-/-
D
BAL galectin-3
BAL galectin-3 (ng/ml)
20
10
0
Wild type
Galectin-3-/-
E
Combined Inflammatory score
Score / HPF
12
10
8
6
4
Wild type
Galectin-3-/-

A


proliferation
Protein Concentration in lysate (mg/ml)
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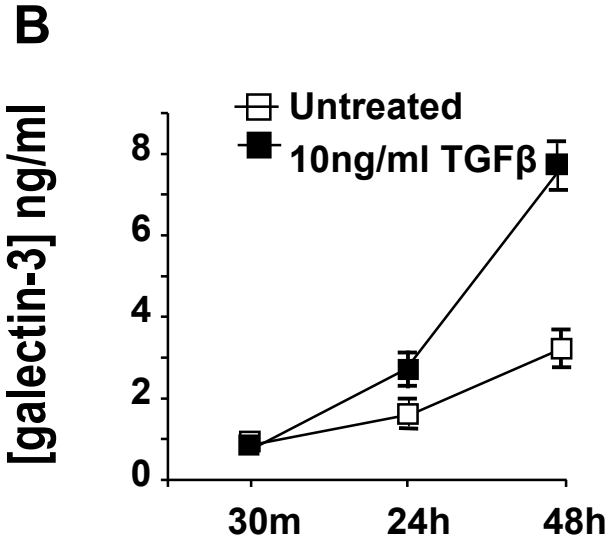
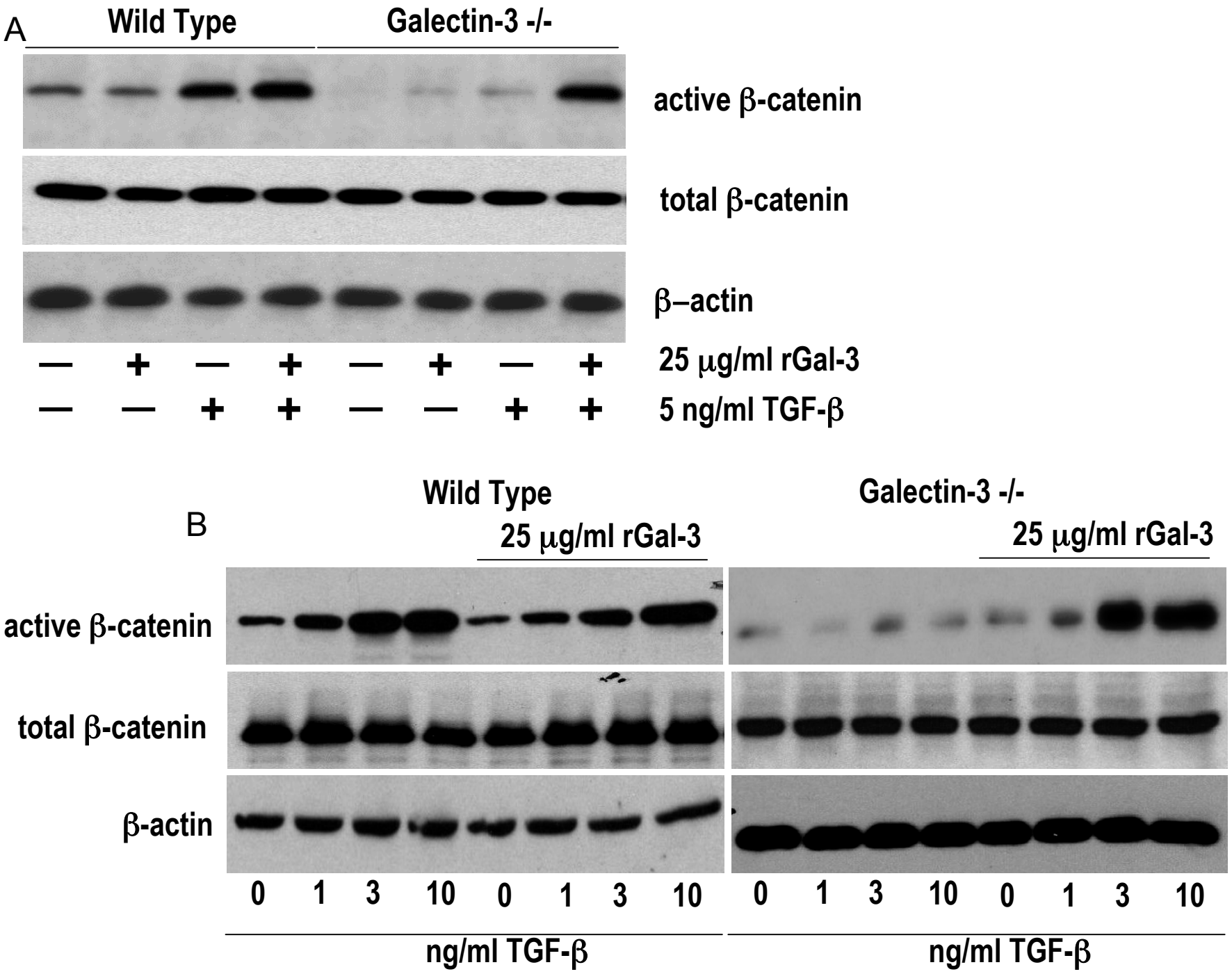
1.2
0.8
0.4
0
Wild type
Gal-3-/-
2
3
4
5
Days post isolation

B


[galectin-3] ng/ml
Untreated
10ng/ml TGFβ
8
6
4
2
0
30m
24h
48h


A
Wild Type
Galectin-3 -/-
active β-catenin
total β-catenin
β–actin
— + — + — + — +
25 μg/ml rGal-3
— — + + — — + +
5 ng/ml TGF-β
B
Wild Type
25 μg/ml rGal-3
Galectin-3 -/-
25 μg/ml rGal-3
active β-catenin
total β-catenin
β-actin
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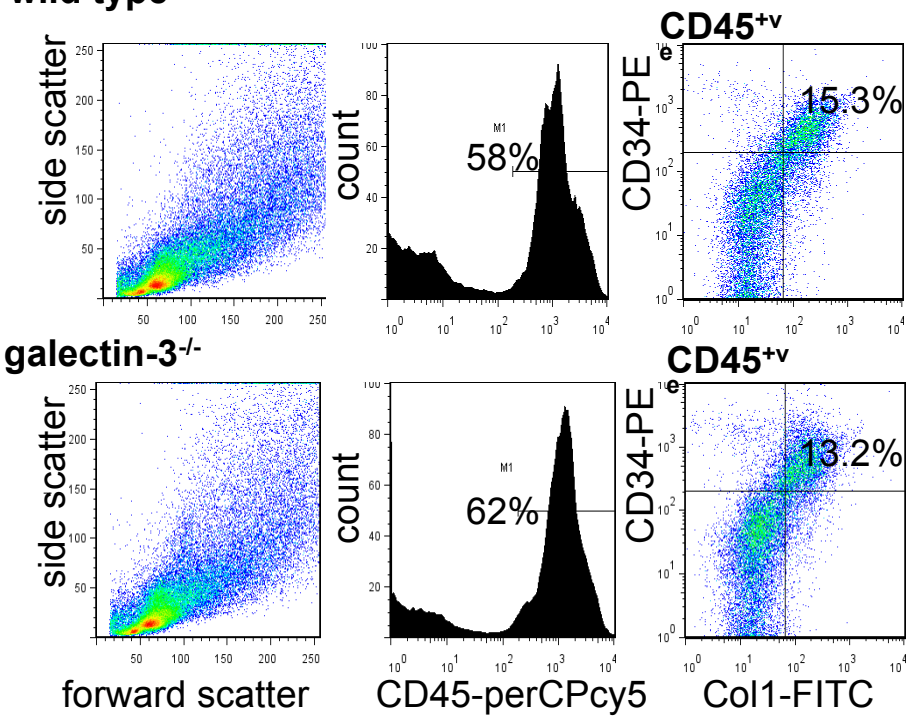
ng/ml TGF-β
0 1 3 10 0 1 3 10
ng/ml TGF-β

A


wild type
side scatter
count
58%
CD34-PE
CD45+ve
15.3%
galectin-3-/-
side scatter
count
62%
CD34-PE
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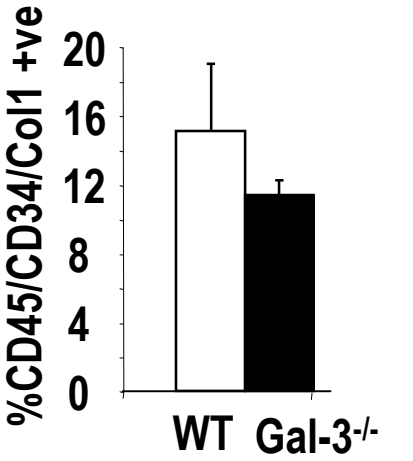
CD45-perCPcy5
Col1-FITC

B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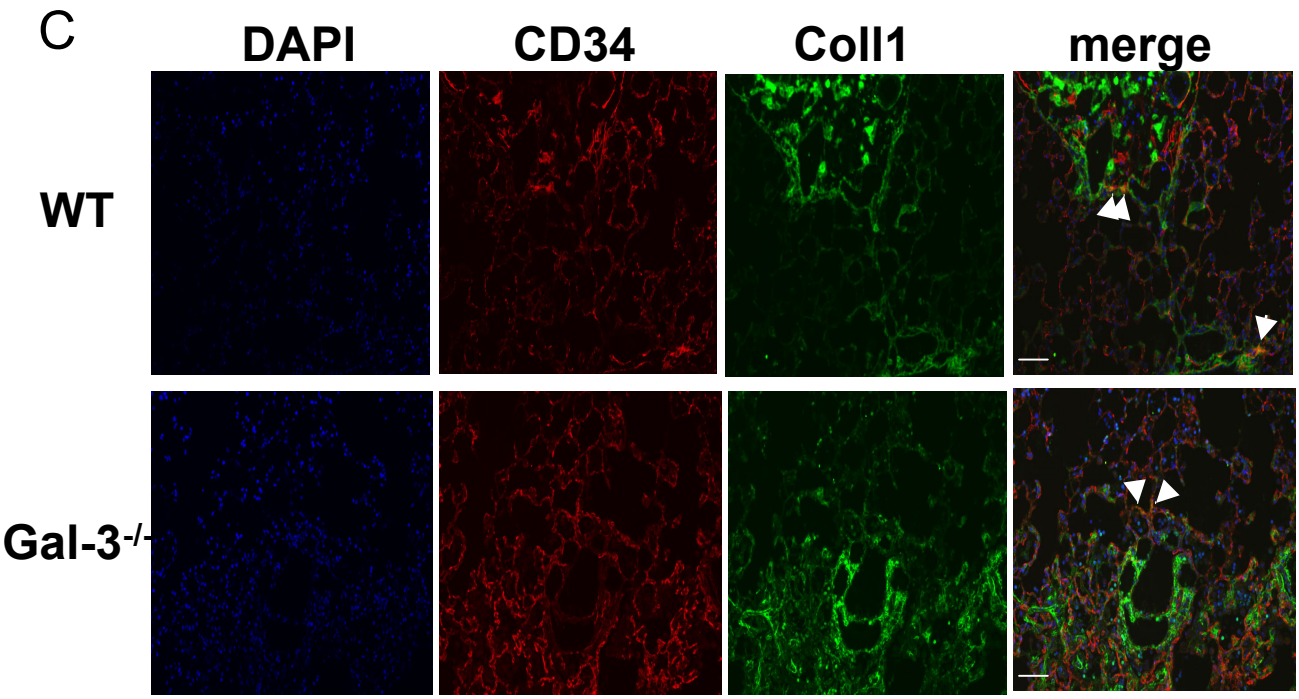
20
16
12
8
4
0
WT
Gal-3-/-

C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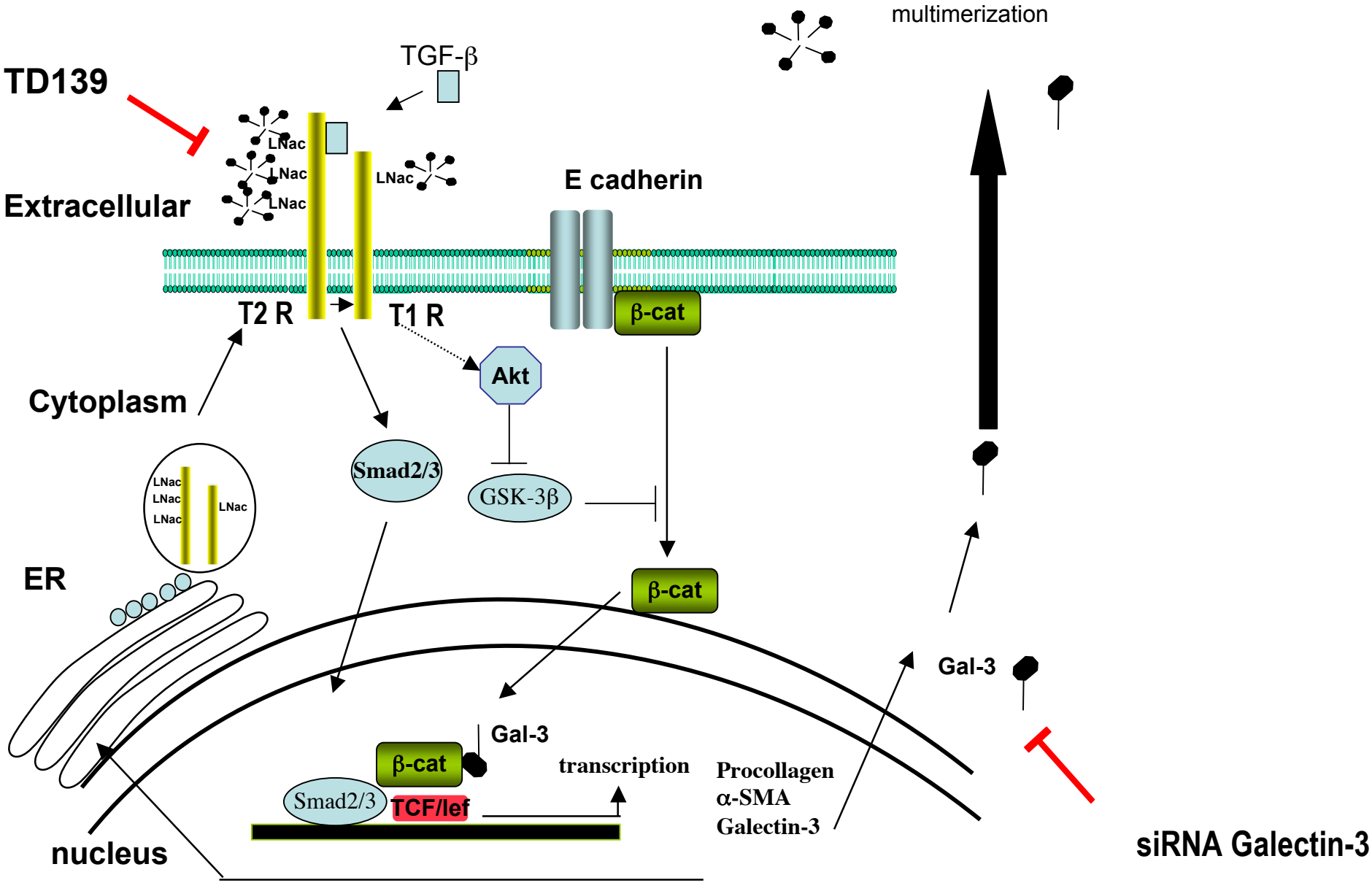
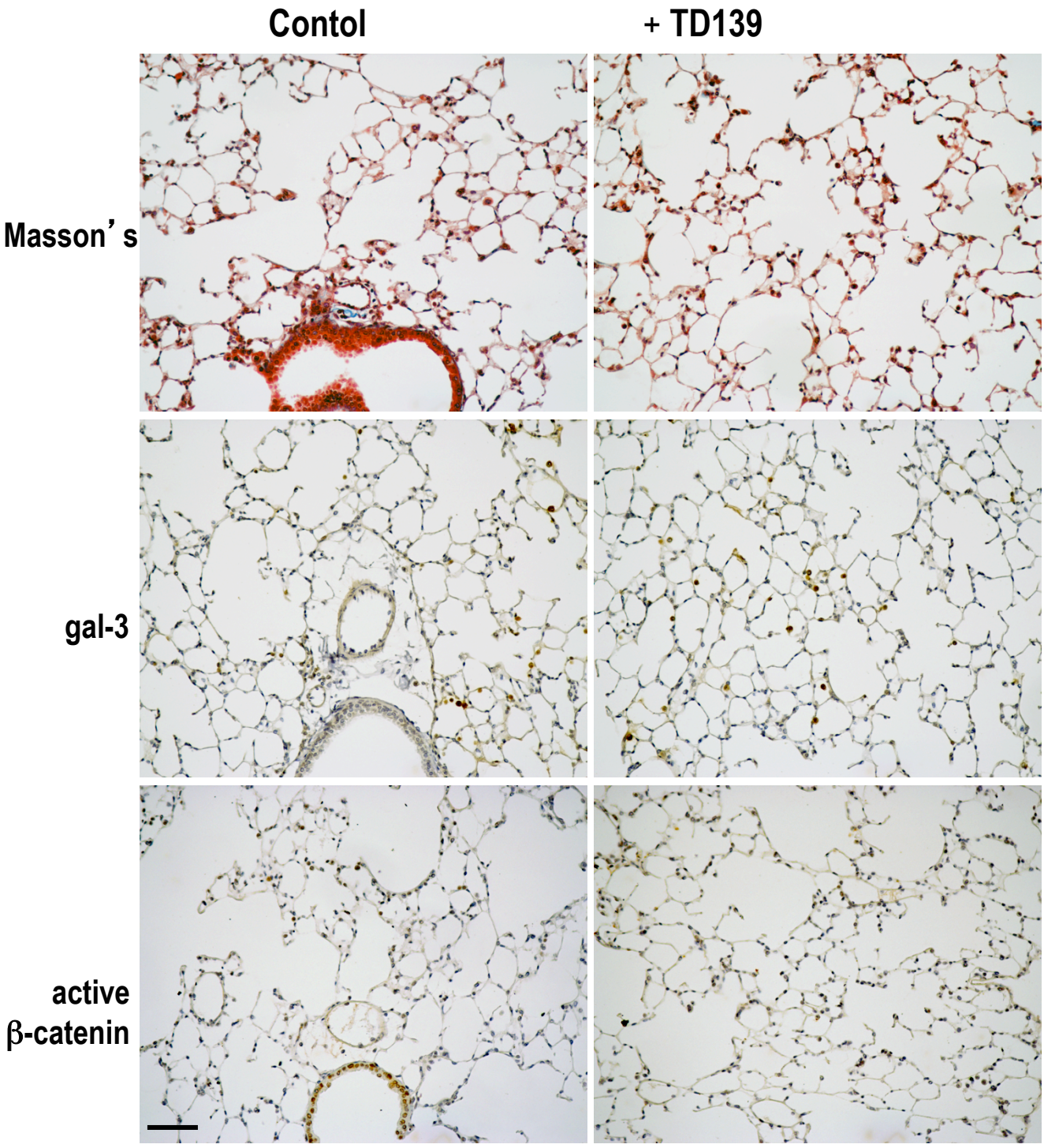
CD34
Coll1
merge
WT
Gal-3-/-

Regulation of EMT by galectin-3




Contol
+ TD139
Masson's
gal-3
active
β-catenin