A NOVEL ANTIFIBROTIC MECHANISM OF NINTEDANIB AND PIRFENIDONE: INHIBITION OF COLLAGEN FIBRIL ASSEMBLY

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ONLINE DATA SUPPLEMENT

Material and Methods

Material:

Primers were purchased by MWG Eurofins (Ebersberg, Germany) and are listed in Supplementary Table E1. Used primary antibodies are given in Supplementary Table E2. Secondary HRP-linked antibodies were purchased from GE Healthcare Life Sciences (Freiburg, Germany).

Statistical analysis

Statistical analysis was performed in GraphPad Prism 7.02 (GraphPad Software, San Diego, CA). For quantification of transcript and protein, results are given as mean ± SEM of independent experiments with fibroblasts derived from at least four different IPF patients. Paired t-test was used for statistical analysis of fiber thickness and one way ANOVA (post test: Bonferroni`s multiple comparison test: comparison against control) was used for statistical analysis for all other experiments. Significance is indicated as follows: *$p<0.1$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

MTT Cytotoxicity Assay

To assess cytotoxicity of nintedanib and pirfenidone, 20.000 cells/cm^2 IPF fibroblasts were seeded in 24-well plates in absence and presence of TGF-β1 (R&D Systems, Minneapolis, MN) (2ng/mL) in combination with nintedanib (0.01 µM, 0.1 µM, 1.0 µM) or pirfenidone (100 µM, 500 µM, 1000 µM) (both Selleck, Houston, TX) for 48h in starvation medium. Nintedanib and pirfenidone were dissolved in DMSO. The final DMSO concentration in the medium was always 1%.

After 48h thiazolyl-blue-tetrazolium-bromide (Sigma-Aldrich) in PBS was added to each well (final: 0.5mg/mL) and incubated at 37°C, 5% CO_2 for 30 min. The supernatant was aspirated and crystals were dissolved in 0.5 mL isopropanol/0.1% Triton X-100 for 30 min at room temperature on a shaker. Absorbance (570 nm) was measured using the Sunrise multiplate reader (Tecan; Männedorf, Switzerland).

Isolation and culture of primary human lung IPF and donor fibroblasts (phLF)

Lung specimens from IPF patients and healthy donors were dissected into pieces of 1-2 cm^2 followed by collagenase I (1 mg/mL) (Biochrom, Berlin, Germany) digestion at 37°C for 2 hours, filtration through nylon filters with a pore size of 70 µm (BD Falcon,Bedford, USA) and centrifugation at 400 *g* at 4°C for 5 minutes. Cells were resuspended in DMEM/F-12 medium (Life Technologies; Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (Pan Biotech, Aidenbach, Germany) and penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA) and seeded onto 10 cm cell culture dishes. For expansion, phLF were cultured in DMEM/F12 (Life Technologies) supplemented with 20% FBS (Pan Biotech) and penicillin/streptomycin (Life Technologies). Cells were routinely split when reaching a confluency of 80-90%. For this study, phLF from six different IPF patients were used in passages 5-8.

RNA isolation and Real-Time quantitative Reverse-Transcriptase PCR (qRT-PCR) Analysis

Isolation of RNA from cultured cells was performed by using the peqGOLD RNA isolation kit (Macherey-Nagel, Düren, Germany). Subsequently, RNA was reverse-transcribed according to the manufacturer`s protocol (Life Technologies) in a 40 µL reaction using M-MLV reverse transcriptase and random hexamers. For quantitative real-time PCR (qRT-PCR) SYBR Green PCR master mix (Roche Applied Science, Mannheim, Germany) and primer mixtures given in Table 1 were used (95°C for 5 min, followed by 45 cycles of 95°C for 5 sec, 59°C for 5 sec and 72°C for 10 sec). Relative transcript abundance of a gene is expressed as -ΔCp values ($-\Delta Cp = Cp^{reference} - Cp^{target}$) or as Fold Change derived from the relevant ΔΔCp values, using $2^{-(\Delta\Delta Cp)}$. As endogenous control, DHX8 was used for standardization of relative mRNA expression.

Protein Isolation and Western Blot Analysis

To extract proteins from cultured cells, cells were scraped into Radio-Immunoprecipitation Assay (RIPA) buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1% Triton X100, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS) containing a protease inhibitor and a phosphatase inhibitor cocktail (both Roche), incubated and centrifuged for 15 min at 13.000 rpm at 4°C. The supernatant was used to determine protein concentration via Pierce BCA Protein Assay (Thermo Fisher Scientific, Waltham, USA). After denaturation of the samples with Laemmli buffer (65 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.01% bromophenolblue, 100 mM DTT), proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was blocked for 1 hour at room temperature with 5% milk in TBS-T (0.1% Tween 20, TBS) to prevent nonspecific binding. Then, the membrane was shortly rinsed and washed (three times for 5 min) in TBS-T followed by incubation with

primary antibody (Table 2) overnight at 4°C. After washing (three times for 5 min) and incubation with the secondary antibody for 1 hour at room temperature, the proteins were visualized with either SuperSignal™ West Dura Extended Duration Substrate or SuperSignal™ West Femto Maximum Sensitivity Substrate (both Thermo Fisher Scientific) and analyzed by the ChemiDocXRS+ imaging system (Bio-Rad, Munich, Germany). Band quantification was performed in Image Lab (version 3.0, Bio-Rad, Hercules, CA).

Quantification of secreted collagen

Precipitation of collagen I and III was carried out as follows: 0.2 g/mL solid $(NH_4)_2SO_4$ was added to the supernatant and the solution was incubated on ice for 30 min. After centrifugation at 20.000 *g* for 30 min at 4°C, the pellet was dissolved in 1/10 of the original volume of 0.1 M acetic acid containing 0.1 mg/mL pepsin (Thermo Fisher Scientific) and incubated on ice overnight at 4°C. Subsequently, 5 M NaCl was added to a final concentration of 0.7 M, incubated on ice for 30 min followed by centrifugation at 20.000 g for 30 min at 4°C. The pellet containing collagen type I and III was resuspended in 0.1 M acetic acid and analyzed by Western blot.

Collagen precipitation for post-translational modification analysis

IPF fibroblasts were seeded at a density of 20.000 – 25.000 cells/cm^2 and cultured in DMEM/F12 (20% FBS, 0.1 mM 2-phospho-L-ascorbic acid, penicillin/streptomycin). When cells had reached a confluency of 80% serum-free DMEM/F12 medium (0.1 mM 2-phospho-L-ascorbic acid) was added containing TGF-β1 (2 ng/mL) and nintedanib (1 µM) or pirfenidone (1000 µM) or DMSO for control and incubated for 24h followed by collection of the medium. DMEM/F12 (20% FBS, 0.1 mM 2-phospho-L-ascorbic acid, penicillin/streptomycin) containing TGF-β1 (2 ng/mL) was added for 24h, followed by alternating cycles of serum-free media supplemented with TGF-β1 and nintedanib or

pirfenidone or DMSO and media containing 20% FBS and TGF-β1. Serum-free cell culture supernatants were collected (20mL in each collection cycle) until 100 mL were obtained.

Precipitation of collagens from cell culture supernatant was in principle carried out in a scale-up version as described previously (1). 0.2 g/mL solid $(NH_4)_2SO_4$ was added to the supernatant and the solution was incubated on ice for 30 min. After centrifugation at 20.000 *g* for 30 min at 4°C, the pellet was dissolved in 1/6 of the original volume of 0.1 M acetic acid containing 0.1 mg/mL pepsin (Thermo Fisher Scientific) and incubated on ice overnight at 4°C (Input). Subsequently, 5 M NaCl was added to a final concentration of 0.7 M, incubated on ice for 30 min followed by centrifugation at 20.000 g for 30 min at 4°C. After collection of the supernatant, the pellet was resuspended in 0.1 M acetic acid (0.7 M fraction). 5 M NaCl was added to the supernatant to a final concentration of 1.2 M and incubated on ice for 30 min, followed by centrifugation at 20.000 g for 30 min at 4°C. The supernatant was collected and the pellet was resuspended in 0.1 M acetic acid (1.2 M fraction). Again, 5 M NaCl was added to a final concentration of 2.5 M, incubated on ice for 30 min followed by centrifugation at 20.000 g for 30 min at 4°C. The pellet was resuspended in 0.1 M acetic acid (2.5 M fraction). The different fractions were resolved by SDS-PAGE (Input: 60 µL, 0.7 M fraction 20 µL, 1.2 M - 2.5 M fractions 80 µL) and visualized by Coomassie staining, followed by band excision, collagen digestion, mass spectrometry (MS) and amino acid analysis

Collagen Digestion and MS Analysis

SDS-PAGE bands were subjected to in-gel digestion with trypsin. Digest conditions were 13 ng/µl Promega trypsin in 100 mM ammonium bicarbonate at 37°C for 18h. Identification of tryptic peptides was performed on a Q-TOF Micro mass spectrometer (Waters, Billerica, MA) equipped with an electrospray ionization source. Data were collected with the MassLynx

(version 4.1) data acquisition software (Waters) and processed using Mascot Distiller (Matrix Software, London, UK). High performance liquid chromatography was performed with a nanoACQUITY (Waters) system using a 75 µm x 100-mm 3-µm Atlantis dC18 column as the analytical column and a 180 µm x 20-mm 5-µm Symmetry C18 column as the trapping column. Chromatography mobile phases consisted of solvents A (0.1% formic acid and 99.9% water (v/v)) and B (0.1% formic acid and 99.9% acetonitrile (v/v)). Peptide samples were loaded onto the trapping column and equilibrated for 2 min in 99% solvent A followed by a 120-min gradient to 60% solvent A at a constant flow rate of 1 µl/min. Analysis was performed in survey scan mode. Tryptic peptides were identified from MS/MS spectra by a Mascot search against the National Center for Biotechnology Information (NCBI) nr database (peptide tolerance 1.0 Da, MS/MS tolerance 1.0 Da)

Amino Acid Analysis

Acid Hydrolysis was performed in 6 x 50-mm Pyrex culture tubes placed in Pico Tag reaction vessels fitted with a sealable cap (Eldex Laboratories, Inc., Napa, CA). Samples were placed in culture tubes, dried in a SpeedVac (GMI, Inc. Albertsville, MN). Acid hydrolysis was performed in a reaction vessel that contained 500 µL of 6 M HCl (Pierce). The vessel was then purged with argon and the samples were hydrolyzed under vacuum at 110 °C for 24 h. The acid hydrolyzed samples were then dried under vacuum and reconstituted in 100 mL of 0.02 M HCl containing an internal standard (100 µM norvaline; Sigma). Analysis was performed by ion exchange chromatography with postcolumn ninhydrin derivatization and visible detection (440 nm/570 nm) with a Hitachi L-8800A amino acid analyzer (Hitachi High Technologies America, Inc., San Jose, CA) running the EZChrom Elite software (Scientific Software, Inc., Pleasanton, CA).

Base hydrolysis was performed in a reaction vessel that contained 100 µL of 4M NaOH per sample. The vessel was then purged with argon and the samples were allowed to hydrolyze under vacuum at 110 °C for 24 h. The base hydrolyzed samples were then dried under vacuum and reconstituted in 100 µL of 0.1M pH 9.5 borate buffer. 50 µL of 10 mM 4-fluoro-7-nitrobenzofurazan (NBD-F) in acetonitrile was added and the solution was incubated at room temperature for 6 hours. 100 µL of 0.2 M pH 2.0 tartarate buffer was added to the solution to quench the reaction. Analysis was performed by liquid chromatography on a 2695 HPLC (Waters, Inc. Milford, MA) with a Model 121 fluorometer detector (Ex. 460 Em. 530 Gilson Middleton, WI) running the MassLynx software

Collagen I fibril formation assay

A stock solution of collagen type I in 50 mM acetic acid was diluted to a final concentration of 0.1 µM into 0.1 M sodium bicarbonate buffer (pH 7.8) containing 0.15 M sodium chloride and 1 mM calcium chloride. Nintedanib (0.5 µM, 1 µM), pirfenidone (1.25 µM, 2.5 µM, 10 µM) or the same volume of DMSO (control) was added to the solution to obtain 0.5% final DMSO concentration. The solution was heated up to 34°C and the absorbance (light scattering) was recorded at 313 nm as a function of time.

Table E1. Primer table for qRT-PCR. Primers were synthesized by MWG Eurofins (Ebersberg, Germany).

Target	**Species**	**Forward primer** (5′-3′)	**Reverse primer** (5′-3′)
COL1A1	human	TACAGAACGGCCTCAGGTACCA	ACAGATCACGTGATCGCACAAC
COL3A1	human	ATCAACACCGATGAGATTAT	AGTATTCTCCACTCTTGAGTTC
COL5A1	human	CTTCAAGGTTTACTGCAAC	CCCTTCGGACTTCTTG
FN1	human	CCGACCAGAAGTTTGGGTTCT	CAATGCGGTACATGACCCCT

FKBP10	human	CGACACCAGCTACAGTAAG	TAATCTTCCTTCTCTCTCCA
SERPINH1	human	ATGTTCTTCAAGCCACAC	TCGTCGTCGTAGTAGTTGTA
PAI-1	human	GACATCCTGGAACTGCCCTA	GGTCATGTTGCCTTTCCAGT
DHX8	human	TGACCCAGAGAAGTGGGAGA	ATCTCAAGGTCCTCATCTTCTTCA

Table E2. Primary antibodies. Primary antibodies which were used for Western Blot analysis. Secondary HRP-linked antibodies were purchased from GE Healthcare Life Sciences (Freiburg, Germany).

Target	**Antibody**	**Provider**
ACTB	HRP-conjugated anti-ACTB antibody	Sigma Aldrich, Louis, MO, USA
AKT	rabbit polyclonal anti AKT antibody	Cell Signaling, Boston, USA
Collagen type I	rabbit polyclonal anti-Collagen I antibody	Rockland, Gilbertsville, PA, USA
Collagen type III	rabbit polyclonal anti-Collagen III antibody	Rockland, Gilbertsville, PA, USA
Collagen type V	rabbit polyclonal anti-Collagen V antibody	Santa Cruz, Dallas, TX, USA
ERK1	mouse monoclonal anti-ERK1 antibody	BD Biosciences, New Jersey, USA
Fibronectin	rabbit polyclonal anti-Fibronectin	Santa Cruz, Dallas, TX, USA

	antibody	
FKBP10	rabbit polyclonal anti-FKBP10 antibody	ATLAS, Stockholm, Sweden
HSP47	mouse monoclonal anti-HSP47 antibody	Enzo Life Sciences, Inc., USA
p-AKT	rabbit monoclonal anti-pAKT (Ser473) antibody	Cell Signaling, Boston, USA
p-ERK	rabbit monoclonal anti-pERK1/2 (Thr202/Thr204)	Cell Signaling, Boston, USA

Figure E1: Effect of nintedanib and pirfenidone on the viability of phLF from IPF patients

Effect of increasing concentrations of nintedanib (0.01 µM, 0.1 µM, 1µM) and pirfenidone (100 µM, 500 µM, 1000 µM) on the viability of phLF of IPF patients after 48h treatment in combination with and without TGF-β1 (2 ng/mL), as measured by MTT assay. n=2.

Figure E2: Nintedanib reduces FN1 expression similarly in IPF and donor fibroblasts

(**A-D**) Quantitative reverse transcriptase-polymerase chain reaction analysis of phLF isolated from (**A, C)** IPF patients or (**B, D**) healthy donors treated for 48h with increasing concentrations of nintedanib (0.01, 0.1, 1 µM) or pirfenidone (100, 500, 1000 µM) in absence or presence of TGF-β1 (2 ng/mL). Transcript levels of FN1 are shown as -ΔCt values (**A, B**) as well as as transcript fold changes (**C, D**) to show the effect normalized to control. DEAH (Asp-Glu-Ala-His) Box Polypeptide 8 (DHX8) was used as endogenous control. Data are based on 7 (IPF) or 3 (donor) completely independent experiments and are given as mean ± SEM. Statistical significance between control and different concentrations of nintedanib or

pirfenidone is indicated by horizontal brackets and asterisks for -ΔCt values and asterisks only for fold changes relative to 1.

(**E, F**) Western Blot analysis of phLF isolated from (**E**) IPF patients or (**F**) healthy donors treated for 48h with increasing concentrations of nintedanib (0.01, 0.1, 1 µM) or pirfenidone (100, 500, 1000 µM) in absence or presence of TGF-β1 (2 ng/mL). Densitometric analysis and representative blots show the effect of nintedanib and pirfenidone on FN1 protein expression relative to β-actin (ACTB). Data are based on 8 (IPF) or 3 (donor) completely independent experiments and are given as mean ± SEM. Statistical significance between control and different concentrations of nintedanib or pirfenidone is indicated by horizontal brackets and asterisks.

Statistical analysis was performed by One-Way ANOVA (post test: Bonferroni`s multiple comparison test: comparison against control). (*p<0.1, **p<0.01, ***p<0.001, ****p<0.0001). The well-known effect of TGF-β1 on these transcripts and proteins was significant, but is not specified in the interest of clarity. ctrl = control; TGF-β1 = transforming growth factor β1.

Figure E3: *PAI-1* transcripts are downregulated by nintedanib in IPF and donor fibroblasts

(**A-D**) Quantitative reverse transcriptase-polymerase chain reaction analysis of phLF isolated from (**A, C)** IPF patients or (**B, D**) healthy donors treated for 48h with increasing concentrations of nintedanib (0.01, 0.1, 1 µM) or pirfenidone (100, 500, 1000 µM) in absence or presence of TGF-β1 (2 ng/mL). Transcript levels of PAI1 are shown as -ΔCt values (**A, B**) as well as as transcript fold changes (**C, D**) to show the effect normalized to control. DEAH (Asp-Glu-Ala-His) Box Polypeptide 8 (DHX8) was used as endogenous control. Data are based on 7 (IPF) or 3 (donor) completely independent experiments and are given as mean ±

SEM. Statistical significance between control and different concentrations of nintedanib or pirfenidone is indicated by horizontal brackets and asterisks for -ΔCt values and asterisks only for fold changes relative to 1. The well-known effect of TGF-β1 on these transcripts was significant, but is not specified in the interest of clarity. ctrl = control; TGF-β1 = transforming growth factor β1. Statistical analysis was performed by One-Way ANOVA (post test: Bonferroni`s multiple comparison test: comparison against control). (*p<0.1, **p<0.01, ***p<0.001, ****p<0.0001). The well-known effect of TGF-β1 on these transcripts and proteins was significant, but is not specified in the interest of clarity. ctrl = control; TGF-β1 = transforming growth factor β1.

Figure E4: Selected PTMs are neither affected by nintedanib nor by pirfenidone

SDS gel electrophoresis of different fractions of collagens precipitated out of 100 mL cell culture media of primary human IPF fibroblasts treated with nintedanib (1 µM) or pirfenidone (1000 µM) in combination with TGF-β1 (2 ng/mL) (A). SDS gel bands (indicated by red boxes) corresponding to the α1-chain of type I collagen (upper red boxes) and the α2-chain of type I collagen (lower red boxes) of the 0.7 M and the 1.2 M fraction were extracted and trypsin digested (A). MS/MS data of the A1 site (Pro-986) of α1-chain of type I (B), A3 site (Pro-707) of α1-chain of type I collagen (C), A3 site (Pro-707) of α2-chain of type I collagen (D) and glycosylation site of hydroxylysine (Lys-174) (E) of control, nintedanib- and pirfenidone-treated samples show no qualitative difference in intensity of prolyl-3-hydroxylation or glycosylation at these sites.

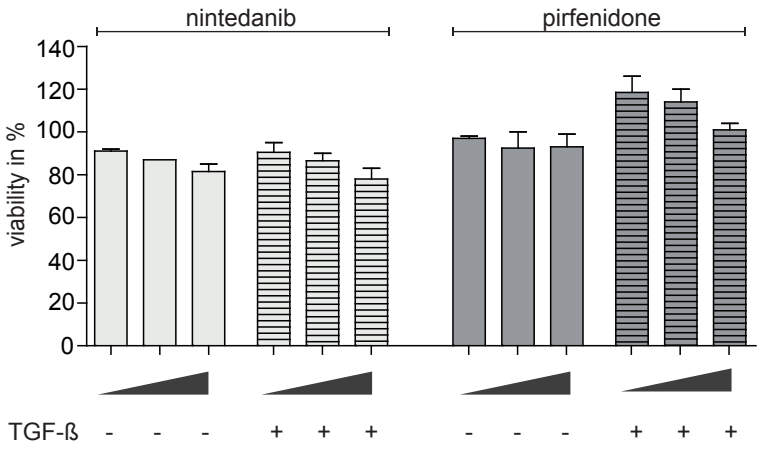
Figure E5: After 48 h, the RTK inhibitor nintedanib consistently inhibits PDGFR/AKT signaling in IPF and donor fibroblasts but not FGFR/ERK signaling

(**A-D**) Western Blot analysis of phLF isolated from (**A, C**) IPF patients or (**B, D**) healthy donors treated for 48h with increasing concentrations of nintedanib (0.01, 0.1, 1 µM) in absence or presence of TGF-β1 (2 ng/mL). (**A, B**) Densitometric analysis and representative blots show the effect of nintedanib and pirfenidone on AKT phosphorylation relative to total AKT levels. (**C, D**) Densitometric analysis and representative blots show the effect of nintedanib and pirfenidone on ERK phosphorylation relative to total ERK levels. Data are based on 8 (IPF) or 3 (donor) completely independent experiments and are given as mean ± SEM. Statistical significance between control and different concentrations of nintedanib or pirfenidone is indicated by horizontal brackets and asterisks. Statistical analysis was performed by One-Way ANOVA (post test: Bonferroni`s multiple comparison test: comparison against control). (*p<0.1, **p<0.01, ***p<0.001, ****p<0.0001).

REFERENCES

1. Staab-Weijnitz CA, Fernandez IE, Knüppel L, Maul J, Heinzelmann K, Juan-Guardela BM, Hennen E, Preissler G, Winter H, Neurohr C, Hatz R, Lindner M, Behr J, Kaminski N, Eickelberg O. FK506-Binding Protein 10, a Potential Novel Drug Target for Idiopathic Pulmonary Fibrosis. *Am J Respir Crit Care Med* 2015; 192: 455-467.

Figure S1


nintedanib
pirfenidone
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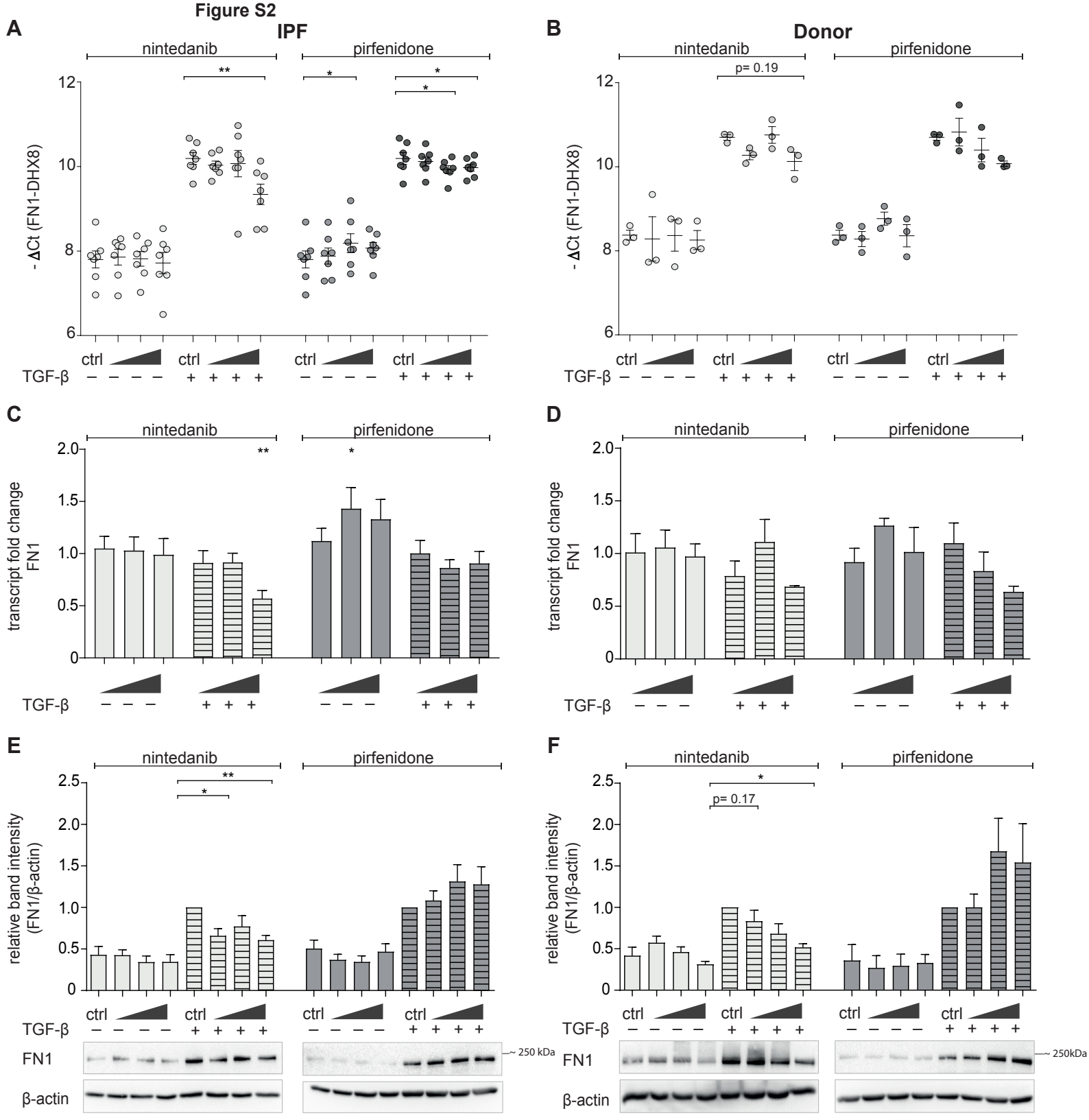
100
80
60
40
20
0
TGF-ß - - - + + + - - - + + +

Figure S2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IPF
nintedanib
pirfenidone
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ctrl
TGF-β
B
Donor
p= 0.19
C
transcript fold change FN1
D
E
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β-actin
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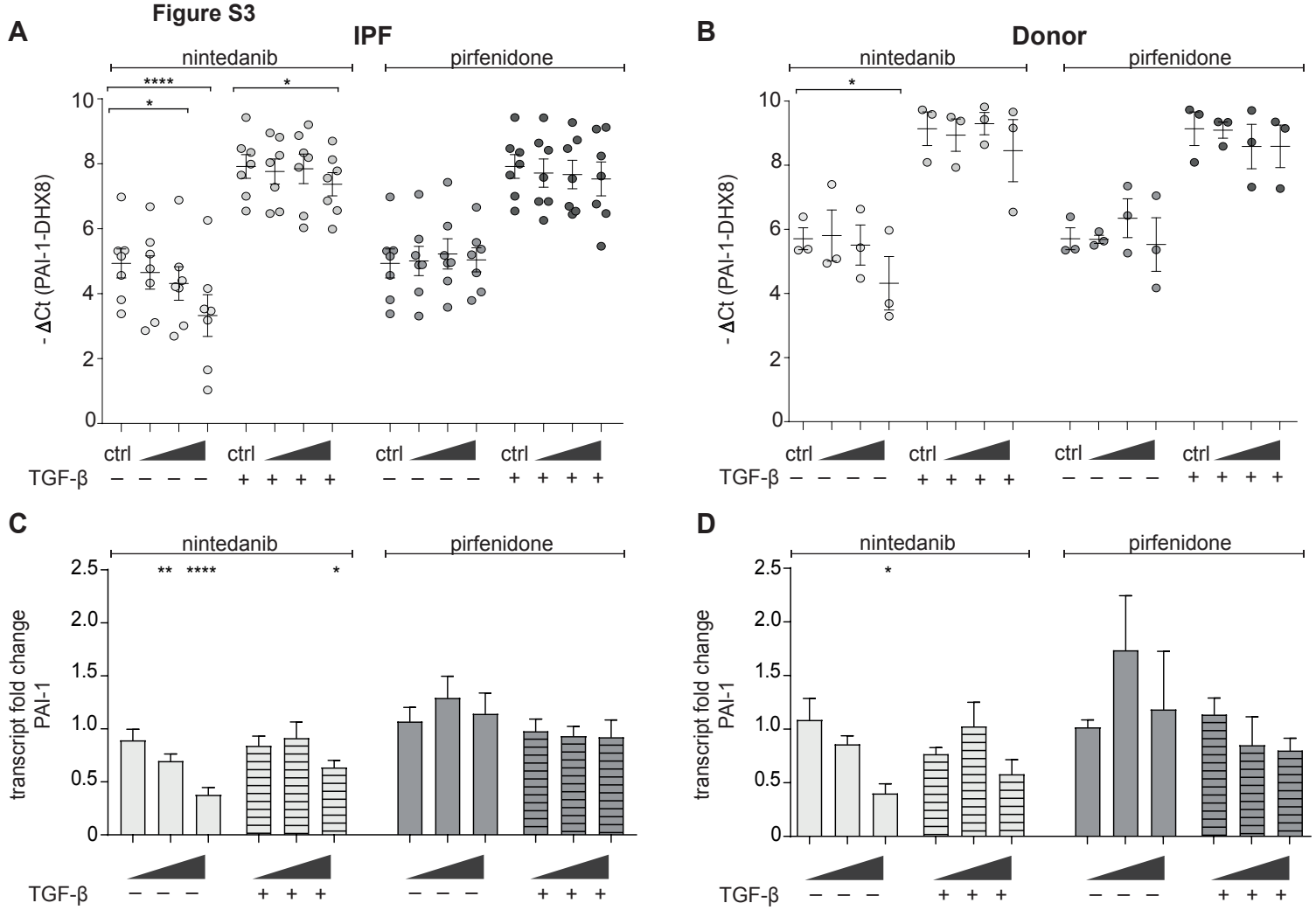
F
p= 0.17
~ 250kDa

Figure S3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IPF
nintedanib
pirfenidone
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2
4
6
8
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ctrl
ctrl
ctrl
ctrl
TGF-β
– – – – + + + + – – – – + + + +
B
Donor
nintedanib
pirfenidone
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- ΔCt (PAI-1-DHX8)
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transcript fold change PAI-1
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D
nintedanib
pirfenidone
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transcript fold change PAI-1
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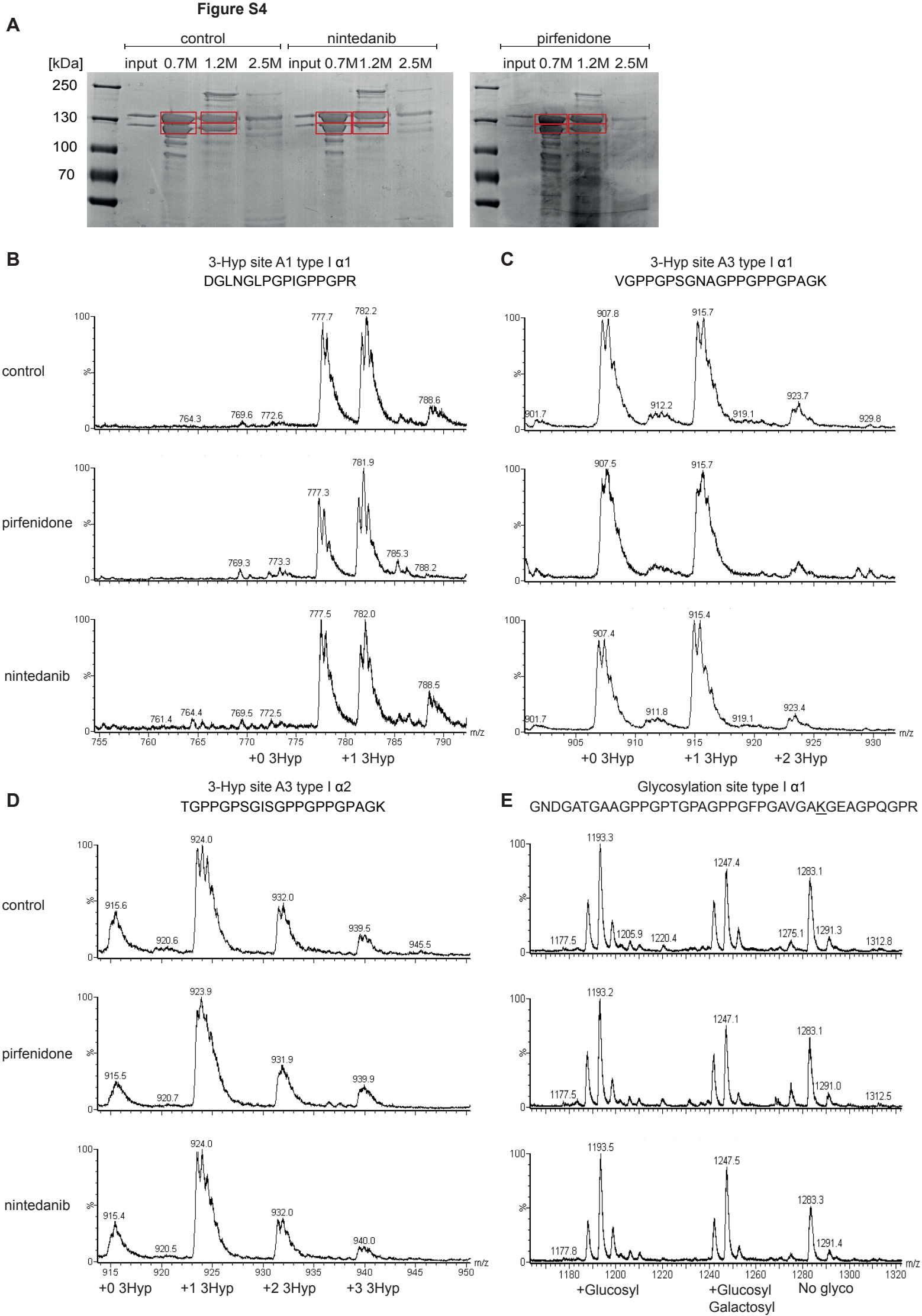
1.5
2.0
2.5
TGF-β
– – – + + + – – – + + +

Figure S4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nintedanib
pirfenidone
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130
100
70
B
3-Hyp site A1 type I α1
DGLNGLPGPIGPPGPR
control
pirfenidone
nintedanib
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+1 3Hyp
C
3-Hyp site A3 type I α1
VGPPGPSGNAGPPGPPGPAGK
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+2 3Hyp
D
3-Hyp site A3 type I α2
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E
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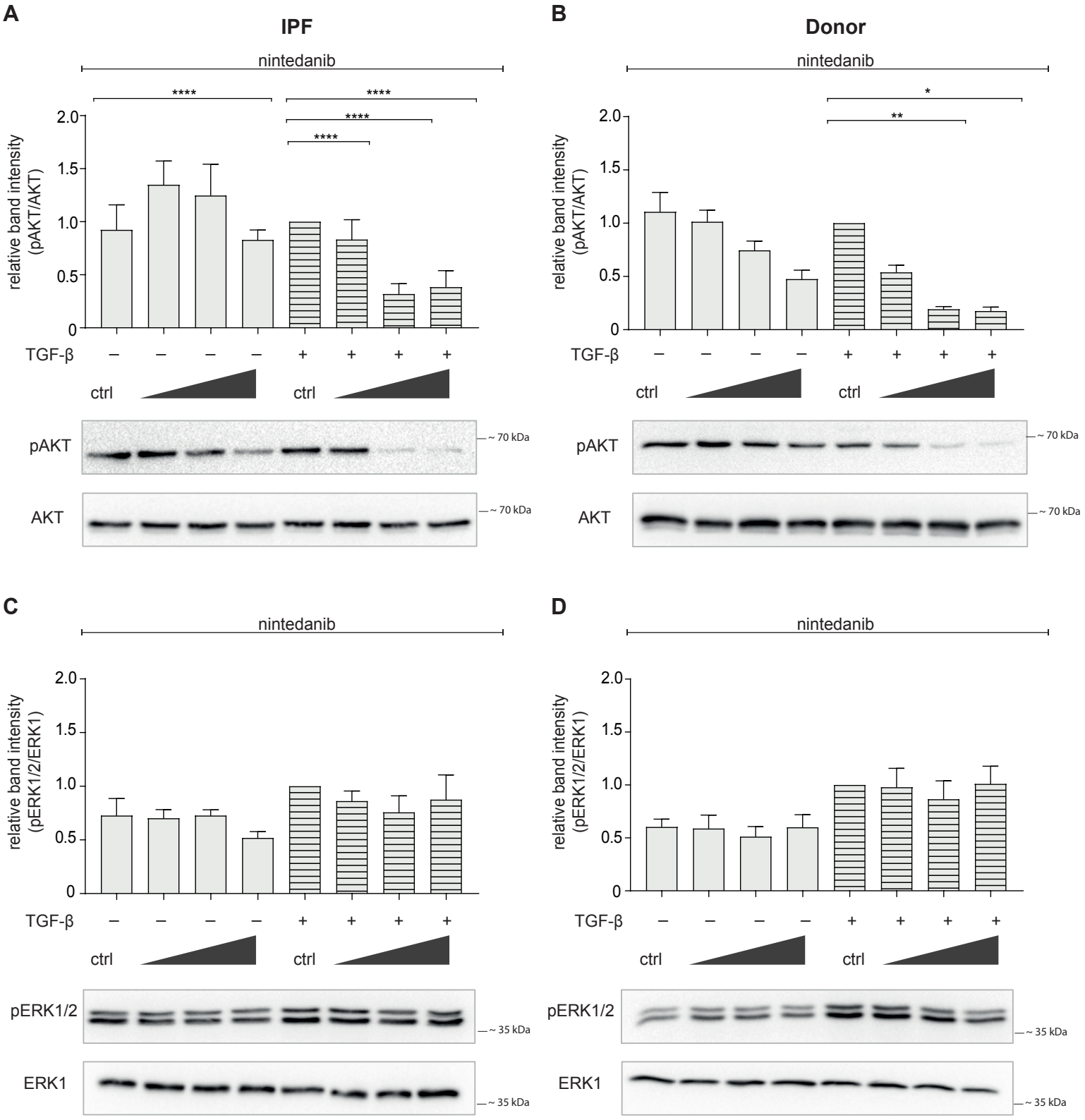
Figure S5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IPF
nintedanib
****
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relative band intensity (pAKT/AKT)
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1.5
1.0
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0
TGF-β – – – – + + + +
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ctrl
pAKT
~ 70 kDa
AKT
~ 70 kDa
B
Donor
nintedanib
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relative band intensity (pAKT/AKT)
TGF-β – – – – + + + +
ctrl
ctrl
pAKT
~ 70 kDa
AKT
~ 70 kDa
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nintedanib
relative band intensity (pERK1/2/ERK1)
TGF-β – – – – + + + +
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