Identification of senescent-cell surface targetable protein DPP4

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Running title: Targetable senescence cell surface marker DPP4

SUPPLEMENTAL MATERIALS AND METHODS

Cells, cell culture, ionizing radiation (IR), and measurement of SA-β-galactosidase activity

Human diploid fibroblasts (HDFs) WI-38 and IMR-90, from Coriell Cell Repositories, and mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotics, antimycotics, and non-essential amino acids (Gibco and Invitrogen). Human umbilical vein endothelial cells (HUVEC) were cultured in endothelial basal medium (EBM) supplemented with EGM SingleQuots™ Kit with growth factors and supplements (Lonza). Human aortic endothelial cells (HAEC) were cultured in EBM-2 supplemented with EGM-2 SingleQuots™ Kit with growth factors and supplements (Lonza).

Measurement of SA-β-galactosidase activity and transfection of siRNA

Senescence-associated β-galactosidase (SA-β-gal) activity in WI-38 cells was assessed using a kit from Cell Signaling. In silencing experiments, WI-38 cells at PDL39-PDL45 were transfected with 50 nM of *in vitro*-synthesized Ctrl siRNA UUCUCCGAACGUGUCACGU (IDT) or DPP4 siRNA GGUCACCAGUGGGUCAUAA (Ambion) using Lipofectamine 2000 (Invitrogen).

Isolation of cell membrane and cell surface proteins, and mass spectrometry and Western blot analyses

To extract membrane proteins and cell surface proteins, cell lysates were prepared and processed from proliferating (PDL23) and senescent (PDL59) WI-38 fibroblasts using the Mem-PER™ Plus Membrane Protein Extraction Kit (Thermo Scientific) or the Pierce™ Cell Surface Protein Isolation Kit (Thermo Scientific). Mass spectrometry analysis was performed by Poochon Scientific. Briefly, after in-gel trypsin digestion, peptides were analyzed by liquid chromatography-coupled tandem MS (LC-MS/MS) using a Q-Exactive hybrid quadrupole orbitrap mass spectrometer (Thermo Scientific) with a Dionex UltiMate 3000 RSLCnano system. Peptide identification and protein assembly were performed on a Thermo Proteome Discoverer 1.4.1 platform. For each tandem mass spectrometry dataset, search was performed against the corresponding UniProtKB/Swiss-Prot database using the SEQUEST and Percolator algorithms.

Protein lysates were prepared using RIPA buffer [25 mM Tris-Cl (pH 7.5), 1% NP40, 1% sodium deoxycholate, 0.1% SDS, and 150 mM NaCl], supplemented with a phosphatase and protease inhibitor cocktail. After transfer to membranes, primary antibodies were used to detect proteins DPP4 (Abcam), EGFR (Santa Cruz), CAV1 (Caveolin-1; Santa Cruz), HSP90 (Santa Cruz), SIRT1 (Abcam), GAPDH (Santa Cruz), p16 (BD Biosciences), p21 (Millipore), ACTB (Santa Cruz), HRAS (Calbiochem), and p53

(Santa Cruz). Secondary antibodies were from GE Healthcare, and Western blotting signals were detected using enhanced Chemiluminescence (Millipore).

Oligomers for quantitative (q)PCR

Reverse transcription (RT) and quantitative (q)PCR analysis were carried out as described (Noh et al. 2016). Gene-specific primers used (sense and antisense in each case) were as follows: CAAATTGAAGCAGCCAGACA and CACACTTGAACACGCCACTT for *DPP4* mRNA, CAAATTGAAGCAGCCAGACA and CGACTCGCTTACCATATGTC for *DPP4* pre-mRNA, and CATGTACGTTGCTATCCAGGC and CTCCTTAATGTCACGCACGAT for *ACTB* mRNA. Primers to amplify *p16* mRNA, *IL1A* mRNA, *IL1B* mRNA, *IL6* mRNA, *IL8* mRNA, and *CCL13* mRNA, *p21* mRNA, and *18S* rRNA were previously reported (Kim et al. 2012; Herranz et al. 2015; Noh et al. 2015).

Immunofluorescence

Proliferating and senescent WI-38 cells were fixed in 100% methanol (Fisher Scientific) for 10 min at -20°C and then incubated with 10% normal goat serum (Thermo Scientific) for 1 hr at room temperature. DPP4 was detected following incubation with a primary antibody recognizing DPP4 (Cell signaling) at 4°C for 16 hrs, following by incubation (1 hr, 37°C) with an Alexa Fluor 568-conjugated secondary antibody (Thermo Scientific). Nuclei were stained using ProLong® Gold Antifade Mountant with DAPI (Thermo Scientific) and signals were visualized using a confocal microscope (ZEISS 710 LSM or 880 LSM).

Separation of DPP4-positive and -negative PBMCs by magnetic-activated cell sorting (MACS)

For MACS analysis, magnetically labeled cells were loaded on MS column (Miltenyi Biotec Inc.) and DPP4-positive cells (DPP4-PE labeled) were separated from DPP4-negative cells (not labeled). DPP4-negative cells were run through and collected, while DPP4-positive cells remained in the column. Columns were washed three times using MACS buffer (Miltenyi Biotec Inc) and the DPP4-positive cells were eluted using MACS buffer. Total RNA was extracted from both DPP4-positive and DPP4-negative cells.

ROS measurement

The level of intracellular reactive oxygen species (ROS) was measured by using a DCFDA cellular ROS detection assay kit (Abcam) following the manufacturer's instructions. Briefly, 48 h after transfection of DPP4 siRNA or Ctrl siRNA, WI-38 cells were seeded at 2×10^4 cells/well on 96-well plates (black/clear bottom, PerkinElmer) and cultured overnight in complete DMEM without phenol red (Life Technologies) at 37°C with 5% CO_2. When cells reached ~80% confluence, cells were washed and incubated with 20 μM

DCFDA in phenol red-free DMEM at 37°C for 45 min. Fluorescence intensity was read at 485/535 nm (excitation/emission) by using a VICTOR3 microplate reader (PerkinElmer).

Thymidine incorporation assay

WI-38 cells were incubated with [methyl-^{3}H]-thymidine (NET027250UC, Perkin Elmer) for 16 hrs. After washes with 1× PBS and harvest, the incorporated radioactivity was measured using liquid scintillation counting. Radioactivity counts were normalized to total protein amount.

HRAS^{G12V} expression in MEFs

MEFs were transduced using a control lentivirus or a lentivirus expressing HRAS^{G12V}. Transduced cells were selected using hygromycin (100 μg/mL) for 5 days, cultured in fresh DMEM without hygromycin for an additional 5 days, and harvested for analysis.

Assessment of BrdU incorporation in WI-38 fibroblasts by flow cytometry

WI-38 cells were incubated with BrdU for 24 hrs, whereupon they were harvested and incubated with BrdU-FITC antibody for 30 min. FACS analysis was performed using a Canto II flow cytometer (BD Biosciences) and the data were analyzed using the FlowJo software (FlowJo v10.2).

SUPPLEMENTAL REFERENCES

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1. DPP4 is highly expressed in ionizing radiation (IR)-induced senescent cells.
(*A*) Peptide sequences identified by mass spectrometry analysis (*yellow*) aligned with the sequence of the DPP4 protein. (*B*) *Top left*, timeline of the IR treatment to elicit senescence. *Top right*, SA-β-gal staining of WI-38 human diploid fibroblasts that were either left untreated (Control) or subjected to IR. In WI-38 cells that were either left untreated or rendered senescent by IR, the steady-state levels of the mRNAs indicated were quantified by RT-qPCR analysis and normalized to *18S* rRNA levels (*bottom left*), while the levels of DPP4 and loading control ACTB in whole-cell lysates were assessed by Western blot analysis (*bottom right*). (*C*) *Left*, SA-β-gal staining of IMR-90 human diploid fibroblasts that were either left untreated (Control) or subjected to IR. *Center*, in untreated and IR-treated IMR-90 cells, the steady-state levels of the mRNAs indicated were quantified by RT-qPCR analysis and normalized to *18S* rRNA levels. *Right*, in whole-cell lysates prepared from IMR-90 cells that were either left untreated or treated with IR, the levels of DPP4, p21 and loading control ACTB were assessed by Western blot analysis. The graphs (*B*, *C*) represent the means ± S.E.M. from three independent experiments; *, *P*-value<0.05

Figure S2. DPP4 levels rise in other models of senescence.
(*A, B*) HUVEC and HAEC cultures were treated with 4 Gy or left untreated and 14 days later, the levels of *DPP4*, *p21*, and *ACTB* mRNAs were quantified by RT-qPCR analysis and normalized to the levels of *18S* rRNA in HUVEC (*A*) and HAEC (*B right*) cultures. The activity of senescent marker SA-β-gal was assessed in HAEC cultures (*B left*). (*C*) The steady-state levels of *DPP4*, *p21*, and *ACTB* mRNAs in WI-38 cells (PDL25) treated with doxorubicin (2 μg/mL) for 24 hrs and cultured in fresh media for an additional 7 days, were assessed by RT-qPCR analysis and normalized to the levels of *18S* rRNA. (*D*) Western blot analysis of DPP4 levels in a model of oncogene-induced senescence (OIS): mouse embryonic fibroblasts (MEFs) rendered senescent by expression of $HRAS^{G12V}$ via lentiviral transduction for 5 days, and additional culture for 5 days before analysis. SA-β-gal activity was detected in $HRAS^{G12V}$-expressing and control MEFs (*left*) and the levels of mouse (m)DPP4, HRASG12V and loading control mouse (m)ACTB as determined by Western blot analysis (*right*) are indicated (Fig. S2D, *left*). The graphs in (*A*-*C*) represent the means ± S.E.M. from three independent experiments; *, *P*-value <0.05.

Figure S3. Higher abundance of DPP4 in PBMCs from older human donors.
Flow cytometry analysis of the expression of DPP4 in three young PBMC donors (27-36 years of age) and three old PBMC donors (78-88 years of age). Blue numbers, percentage of DPP4-positive cells used to calculate the mean values shown in Fig. 3D.

Figure S4. Control IgG antibody does not affect the viability of proliferating or senescent cells as assessed by ADCC.

Proliferating and senescent WI-38 cells were incubated with rabbit (r)IgG antibody (0, 0.05, 0.5, 5 μg/mL) and NK cells per well for 4 hrs. After removing NK cells, WI-38 cells were incubated for another 18 hrs and cell viability was measured by the MTT assay. Cell viability was compared with that in cultures that received no rIgG antibody (0 μg/mL).

Table S1. Mass spectrometry results of membrane-associated and cell surface proteins in proliferating and senescent cells.

Mass spectrometry analysis from proliferating cells and senescent cells. PSM, peptide spectrum match count. (*A*) Membrane fraction. (*B*) Surface fraction.

Kim302570_FigS1

A

1352311 RecName: Full=Dipeptidyl peptidase 4

MKTPWKVLLG LLGAAALVTI ITVPVVLLNK GTDDATADSR KTYTLTDYLK NTYRLKLYSL RWISDHEYLY KQENNILVFN AEYGNSSVFL ENSTFDEFGH
SINDYSISPD GQFILLEYNY VKQWRHSYTA SYDIYDLNKR QLITEERIPN NTQWVTWSPV GHKLAYVWNN DIYVKIEPNL PSYRITWTGK EDIIYNGITD
WVYEEEVFSA YSALWWSPNG TFLAYAQFND TEVPLIEYSF YSDESLQYPK TVRVPYPKAG AVNPTVKFFV VNTDSLSSVT NATSIQITAP ASMLIGDHYL
CDVTWATQER ISLQWLRRIQ NYSVMDICDY DESSGRWNCL VARQHIEMST TGWVGRFRPS EPHFTLDGNS FYKIISNEEG YRHICYFQID KKDCTFITKG
TWEVIGIEAL TSDYLYYISN EYKGMPGGRN LYKIQLSDYT KVTCLSCELN PERCQYYSVS FSKEAKYYQL RCSGPGLPLY TLHSSVNDKG LRVLEDNSAL
DKMLQNVQMP SKKLDFIILN ETKFWYQMIL PPHFDKSKKY PLLLDVYAGP CSQKADTVFR LNWATYLAST ENIIVASFDG RGSGYQGDKI MHAINRRLGT
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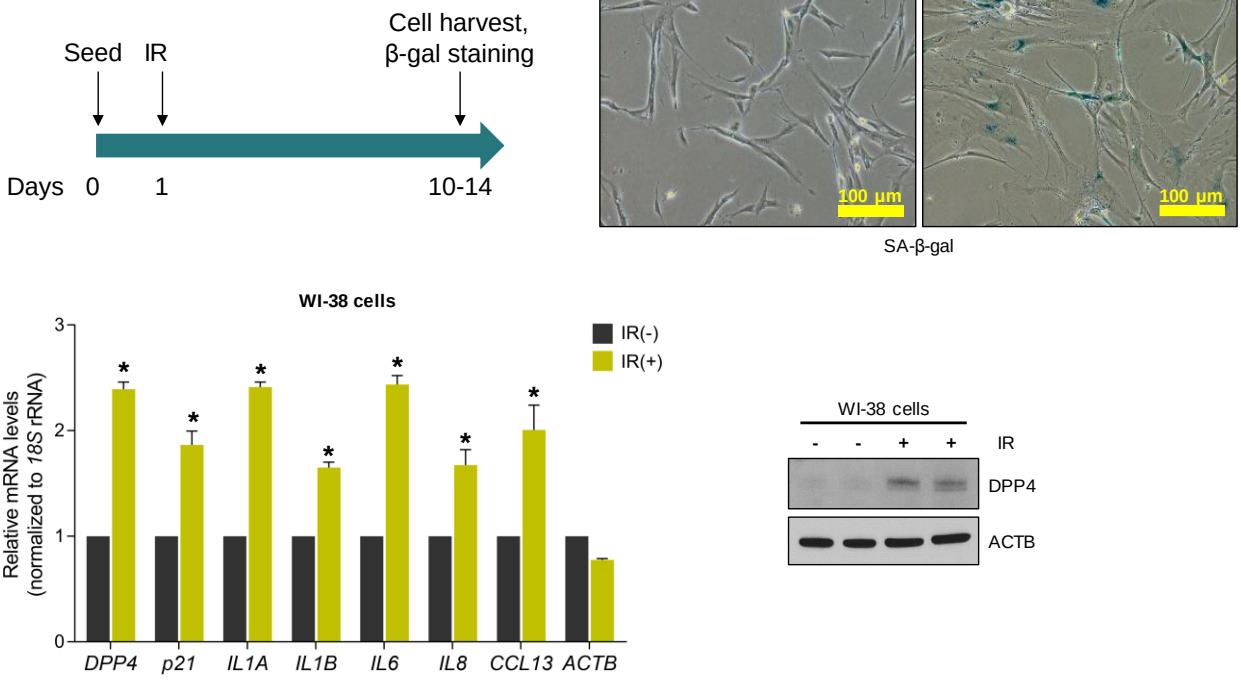
B



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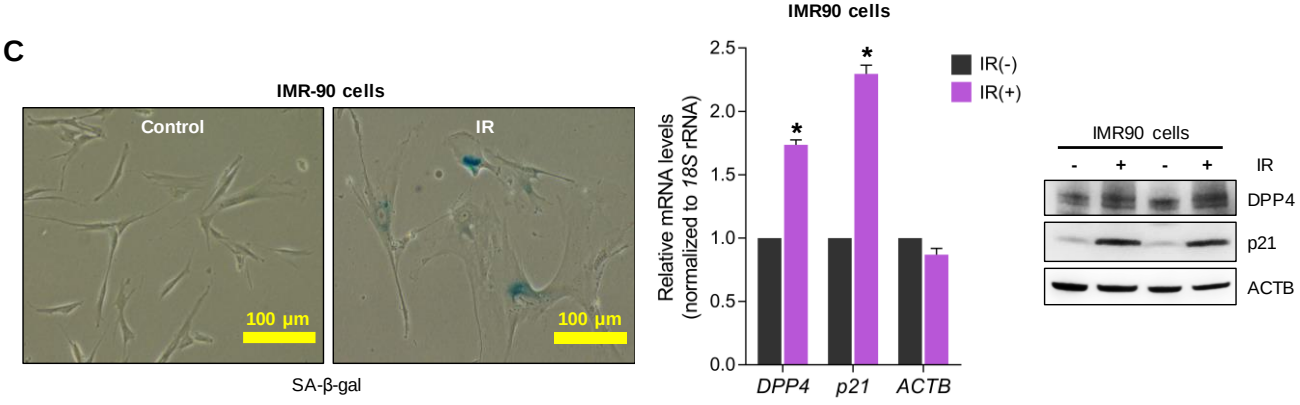

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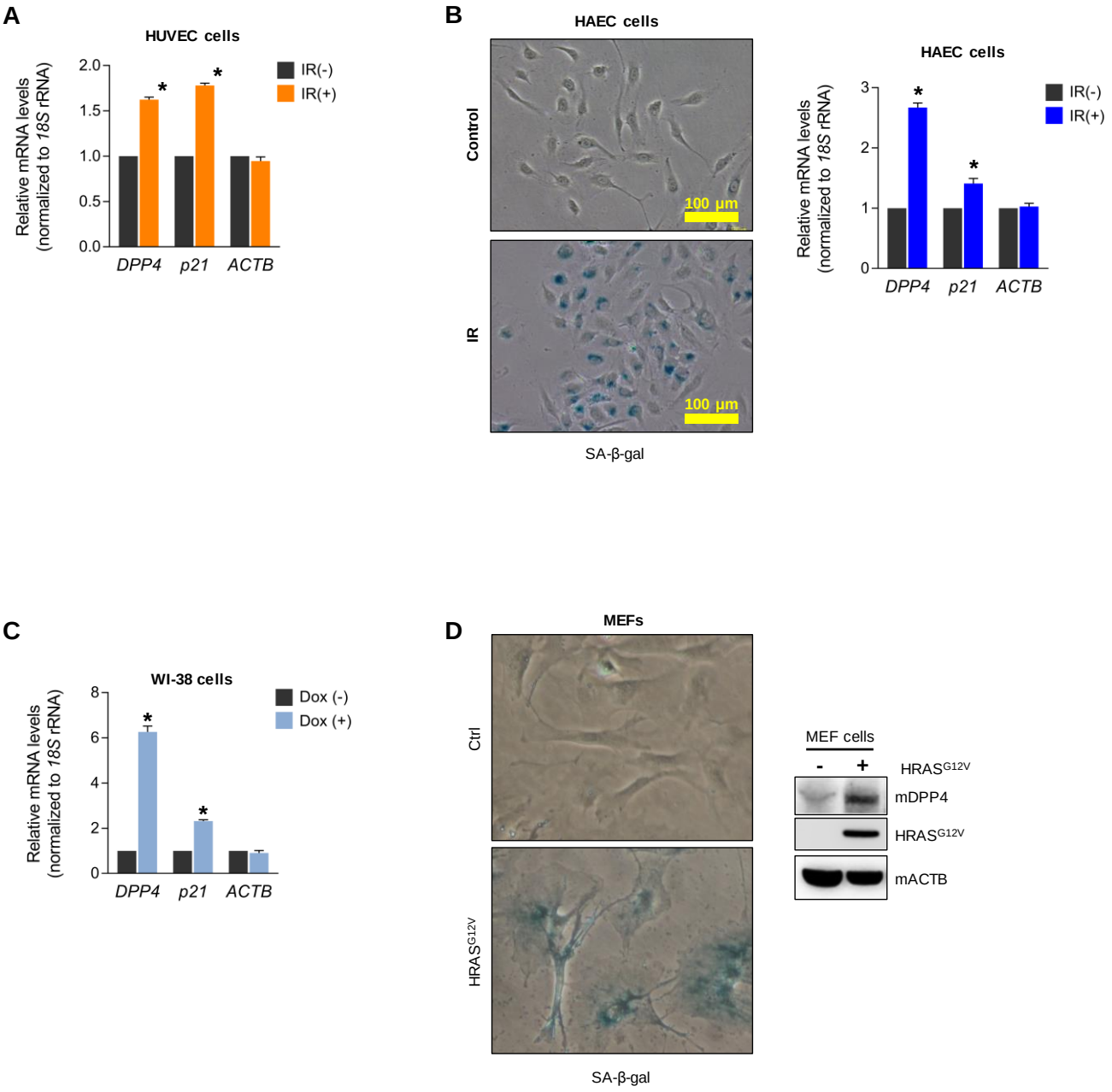

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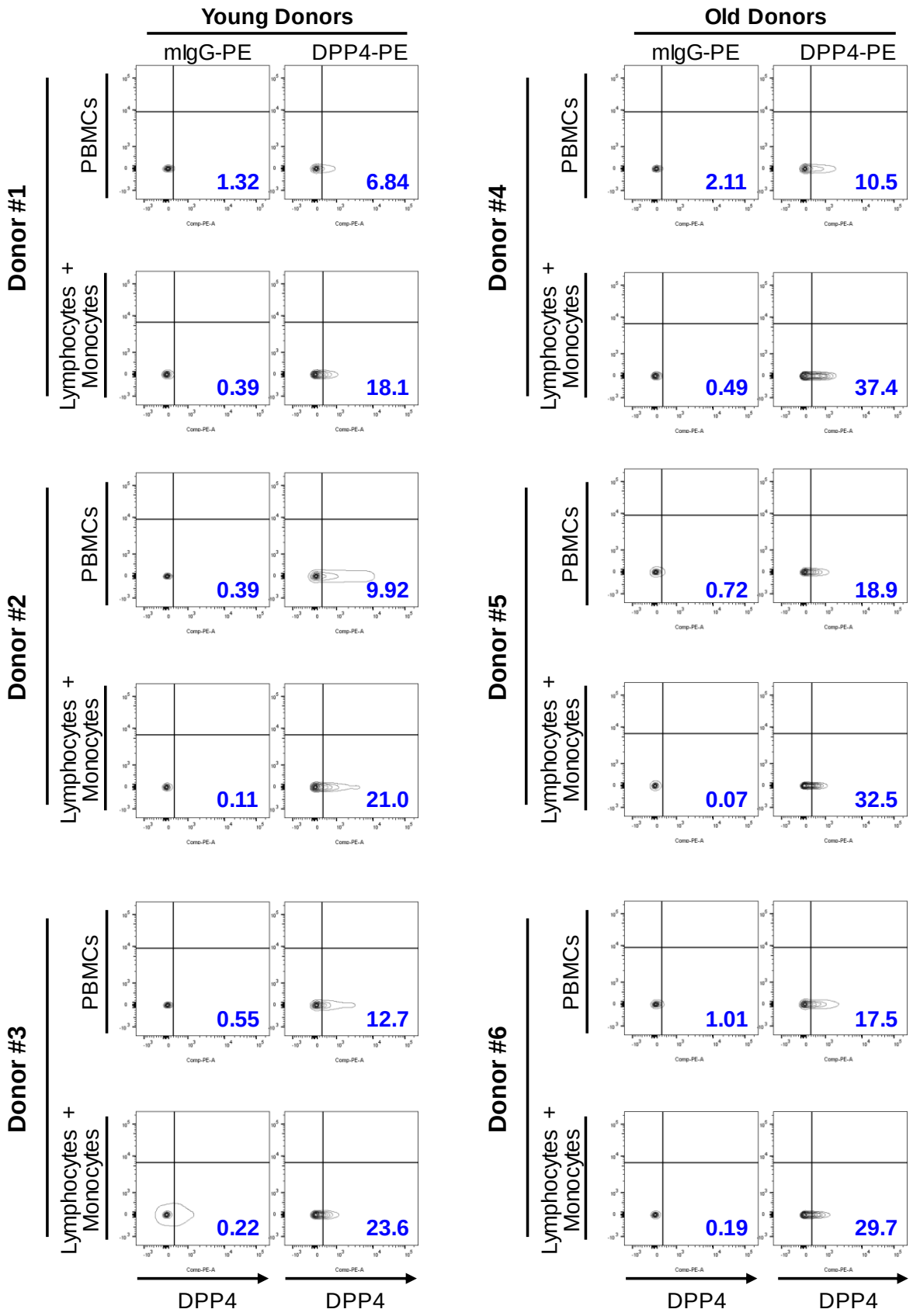

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