THE PIM1 KINASE PROMOTES PROSTATE CANCER CELL MIGRATION AND ADHESION VIA MULTIPLE SIGNALLING PATHWAYS

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ABSTRACT

The ability of cells to migrate and form metastases is one of the fatal hallmarks of cancer that can be conquered only with better understanding of the molecules and regulatory mechanisms involved. The oncogenic PIM kinases have been shown to support cancer cell survival and motility, but the PIM-regulated pathways stimulating cell migration and invasion are less well characterized than those affecting cell survival. Here we have identified the glycogen synthase kinase 3β (GSK3B) and the forkhead box P3 (FOXP3) transcription factor as direct PIM targets, whose tumour-suppressive effects in prostate cancer cells are inhibited by PIM-induced phosphorylation, resulting in increased cell migration. Targeting GSK3B is also essential for the observed PIM-enhanced expression of the prostaglandin-endoperoxide synthase 2 (PTGS2), which is an important regulator of both cell migration and adhesion. Accordingly, selective inhibition of PIM activity not only reduces cell migration, but also affects integrin-mediated cell adhesion. Taken together, these data provide novel mechanistic insights on how and why patients with metastatic prostate cancer may benefit from therapies targeting PIM kinases, and how such approaches may also be applicable to inflammatory conditions.

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Keywords: PIM1; GSK3B; FoxP3; PTGS2; integrins; prostate cancer

INTRODUCTION

The ability of cancer cells to leave the primary tumour sites to migrate and invade to other tissues is one of the major challenges for cancer therapy, since approximately 90% of human cancer deaths are due to metastases (1). Therefore, it is important to investigate the signalling pathways regulating cancer cell motility to reveal the most critical molecules and mechanisms with therapeutic implications.

PIM kinases are constitutively active serine/threonine kinases that promote cell survival, proliferation and migration in different types of malignancies (2-9). Their roles in cell survival and proliferation have been extensively studied (2-4), but the mechanisms through which they regulate cell motility are less well known. We have recently shown that PIM-selective kinase inhibitors reduce prostate cancer cell migration as well as invasion both in cell culture and under *in vivo* conditions, and that overexpression of PIM family members stimulates the formation of lung metastases from orthotopically induced prostate tumours in mice (5-6). So far we have connected two PIM substrates, the nuclear factor of activated T cells 1 (NFATc1) and the chemokine (C-X-C) motif receptor 4 (CXCR4), to the PIM-promoted metastatic behavior of human prostate cancer cells (5-6, 10), but additional PIM effectors affecting cell motility are likely to exist.

One putative candidate for a PIM effector is the glycogen synthase kinase 3β (GSK3B), the phosphorylation of which has been linked to enhanced prostate cancer cell migration as well as to induction of epithelial mesenchymal transition (11). GSK3B has been suggested to be targeted by both AKT (v-akt murine thymoma viral oncogene

homolog) and PIM kinases (12), but so far there has been no direct evidence on GSK3B being a PIM substrate. Phosphorylation of GSK3B at Ser^9 inhibits its activity, while this inhibition is relieved in the presence of the PI3K (phosphatidylinositol 3-kinase) inhibitor LY294002 (11, 13). Interestingly, this inhibitor is not selective to the PI3K/AKT pathway, leaving the possibility that at least part of its effects on GSK3B are mediated via its observed ability to inhibit also PIM activity (14).

Another potential candidate to mediate the effects of PIM kinases is the forkhead box P3 (FOXP3) transcription factor, which is a biomarker for regulatory T cells, but which also acts as a tumour suppressor e.g. in prostate cells (15-16). In human regulatory T cells, FOXP3 has been shown to control PIM expression levels (17), while there are recent reports indicating that its own activity there is differentially regulated by phosphorylation by PIM family members or other kinases (18-20).

Both GSK3B and FOXP3 have been reported to regulate migration of gastric cancer cells as well as expression of the prostaglandin-endoperoxide synthase 2 (PTGS2), also known as cyclooxygenase 2 (COX2) (21-22). PTGS2 is an important inflammatory factor, but is also involved in prostate carcinogenesis (23-25). PTGS2 catalyzes the synthesis of prostaglandin E (PGE2), which in turn influences cancer cell adhesion, migration, invasion and even angiogenesis (26). PTGS2 stimulates integrin-mediated endothelial cell migration and angiogenesis as well as expression of the integrin receptor subunits such as α2 (ITGA2/CD49b), α5 (ITGA5/CD49e) and β1 (ITGB1/CD29/GPIIA) (27-30), which are the major factors regulating the adhesion of prostate cancer cells (31-32).

In this study, we wanted to identify novel PIM effectors to promote cell motility. Therefore we analysed the effects of wild-type GSK3B and FOXP3 proteins as well as corresponding phosphorylation-deficient mutants on motility of PC-3 prostate cancer cells. We also measured expression levels for PTGS2 and assayed the adhesive properties of

the cells. Our data reveal that PIM1 directly interacts with both GSK3B and FOXP3, phosphorylates them and thereby inhibits their tumour-suppressive activities. Interestingly, enhanced PIM activity together with the phosphorylation and inactivation of GSK3B influence cell motility through the regulation of PTGS2 expression. In addition, inhibition of PIM activity reduces integrin-mediated cell adhesion. Altogether, these data provide increasing evidence for the importance of targeted cancer therapy against PIM kinases.

MATERIALS AND METHODS

DNA constructs, subcloning and mutagenesis

The pGEX-6P-1-based bacterial expression vectors and the eukaryotic V5- or RFP-tagged expression vectors for human PIM1 will be described elsewhere (33). The pBJ5-GSK3B-HA vector was a kind gift from C. Beals (Stanford University, CA, USA). The human cDNA for GSK3B was transferred from there into pGEX-6P-3 (GE Healthcare Life Sciences, Little Chalfont, UK) and pEGFP-C3 vectors (Clontech Laboratories, Mountain View, CA, USA) (Table S1). The cDNA for mouse FoxP3 was cut out from the MigR1-AVI-FoxP3 construct (34) and transferred into pGEX-6P-1 (GE Healthcare Life Sciences) and pFlag-CMV-2 (Sigma-Aldrich, St. Louis, MO, USA) (Table S1). The mutagenesis of human GSK3B Ser^{9} and mouse FoxP3 Ser^{418} to alanine residues was performed by the QuikChange site-directed mutagenesis kit (Stratagene, Agilent Technologies, Santa Clara, CA, USA) (Table S1). Human flag-tagged FOXP3 and its phosphomutants S418A, S418D, S422A and S422D as well as GFP-tagged FOXP3 have been previously described (19, 35).

Chemical compounds

PIM activity was inhibited by 10 µM DHPCC-9 (1,10-dihydropyrrolo[2,3-a]carbazole-3-carbaldehyde) (36), PTGS2/COX2 activity by 25 µM Celecoxib (4-[5-(4-Methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]-benzenesulfonamide, Celebrex, SC-58635, Pfizer, New

York, NY, USA) and PTGS1/COX1 activity by 50 µM Indomethacin (1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid, I7378, Sigma-Aldrich). All compounds were diluted into dimethylsulfoxide (DMSO), the maximal cellular concentration of which was kept at 0.1%.

Cell culture, transfections and viability assays

The human androgen-independent prostate epithelial adenocarcinoma cell line PC-3 (CRL1435™, American Type Culture Collection) was cultured and transiently transfected as previously described (5). For silencing of PIM expression, cells were grown on 6-well plates and transfected with Oligofectamine™ and 200 nM *PIM1* or non-targeting (Dharmacon, Lafayette, CO, USA) or *PIM2, PIM3* or non-targeting (Sigma-Aldrich) siRNAs (33). Cell viability was analysed by Trypan blue exclusion assay as previously described (5).

Western blotting

Cells grown on 24-well plates were directly lysed into 50 µl of hot 2x LSB and heated at 95°C degrees for 5 minutes. Protein aliquots were separated in 8-12 % SDS-PAGE gels, immobilized onto PVDF-membrane (EMD Millipore, Merck KGaA, Darmstadt, Germany) and stained with specific primary antibodies (Table S2). Secondary antibodies were used as previously described (6). Equal sample loading was controlled by β-actin staining. Signal intensities were analysed from X-ray films by the ChemiDoc™ MP Imaging System with Image Lab software Version 4.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) or from scanned images after color inversion using ImageJ software (1.48s, Fiji, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

GST-fusion protein production and *in vitro* kinase assays

The *in vitro* kinase assays were carried out as previously described (6, 37). Briefly, GST-fusion proteins were produced in bacteria, after which the GST-tags were cleaved away. Purified protein aliquots containing 0.5 µg of PIM1, 1 µg of GSK3B or 1 µg of FoxP3 were mixed together and incubated for 10 min with DMSO or DHPCC-9. Thereafter kinase assays were performed for 30 min with radioactively labelled γ-^{32}P-ATP or non-radioactive ATP. Proteins were separated by SDS-PAGE. Radioactive gels were stained by Page Blue solution (Thermo Fischer Scientific, Waltham, MA, USA) and analysed by autoradiography. Western blotting was used for analysis of non-radioactive samples. Signal levels were normalized according to protein loading and signal intensities were analysed by the ChemiDoc^{TM} MP Imaging System (Bio-Rad Laboratories, Inc.).

Wound healing and adhesion assays

Wound healing assays were performed as previously described (5-6). Briefly, the cells were plated on 24-well plates (70 000- 150 000 cells/ well), transfected and treated with different compounds. Wounds were scratched by 10 µl pipette tips, after which samples were imaged by light microscopy.

For adhesion assays, PC-3 cells were pretreated with DMSO-diluted compounds. For this purpose, cells were trypsinised, treated with trypsin inhibitor (T9128, Sigma-Aldrich), washed, and diluted in serum-free media. 96-well plates (E-plates, Roche Life Science, Indianapolis, IN, USA) were coated with 0,1 mg/ml of lyophilized Poly-L-lysine (P9155, Sigma-Aldrich), 5 µg/cm^2 of Collagen I (PureCol®, Advanced BioMatrix, Carlsbad, CA, USA) or 3 µg/cm^2 of human fibronectin (F0895, Sigma-Aldrich), and blocked with 1% BSA prior to addition of the cells (15 000 cells/ well). As a control, BSA-treated wells were used. Cells were plated in serum-free media and their real-time adhesion was followed by xCelligence (Roche Life Science). For statistical analysis of adhesion indices, the BSA

control values were in each case subtracted from the original values, after which the highest control (DMSO) value was set as 1.0.

Immunofluorescence and confocal microscopy

PC-3 cells were plated on coverslips and transfected with FOXP3-GFP or GSK3B-GFP expression vectors along with either PIM1-RFP or empty control vectors. After 48 hours, samples were fixed and mounted with Mowiol. Colocalization was imaged by Leica TCS SP5 spectral confocal microscope with Leica Confocal Software by sequential scanning (Leica Microsystems GmbH, Wetzlar, Germany). Physical interactions were measured and analysed by fluorescence-lifetime imaging microscopy (33, 38).

Flow cytometry

For the analysis of integrin subunit α2 surface expression levels and subunit β1 activation state, PC-3 cells were pretreated with DMSO-diluted compounds, 1mM $MnCl_2$ (for integrin activation) or 5 mM EDTA (for integrin inactivation) for 5 min. Cells were trypsinised, washed and suspended in blocking buffer (1 % FCS in PBS). Cells were incubated for 1 h with primary antibody (Table S2) and for 1 h with anti-mouse (A16167, Life Technologies, Carlsbad, CA, USA) or anti-rat (81-9511, Invitrogen, Carlsbad, CA, USA) secondary antibody (60 µg/ml). As controls, unlabeled or only FITC-labeled samples were used. Cells were washed and suspended in PBS, and integrin levels were measured with FACSCalibur (BD Biosciences, Franklin lakes, NJ, USA). Graphs were drawn with Flowing Software 2 (Turku Centre for Biotechnology, Turku, Finland).

Statistical analyses and figure preparation

Bar graphs were produced by Microsoft Excel 2014 (Microsoft Corporation, Redmond, WA, USA), while student's T-test was used for statistical analyses. $P>0.05$ was interpreted as significant (*). Error bars represent SD values in each graph. Figures were prepared by

Corel Draw X5 (Corel, Ottawa, Canada) or Adobe Illustrator CS4 (Adobe Systems, San Jose, CA, USA).

RESULTS

GKS3B is a direct substrate for PIM1 affecting PC-3 cell motility

Since GSK3B has been suggested to be a PIM substrate (12), we wanted to test whether PIM1 can directly phosphorylate it. For this purpose, we produced wild-type and kinase-deficient human PIM1 as well as human GSK3B in bacteria as fusion proteins tagged with glutathione S-transferase (GST), cleaved the tags away and performed radioactive *in vitro* kinase assays. Strongly phosphorylated bands co-migrating with GSK3B and PIM1 proteins were observed only in the presence of wild-type, but not mutant PIM1 (Figure 1a, left panel). In addition, the GST control protein remained unphosphorylated. These data indicate that GSK3B indeed is a direct PIM1 substrate and that PIM1, but not GSK3B can autophosphorylate itself under these conditions.

Since the amino acid sequence around Ser^9 (-RPRTTSF-) in GKS3B shares some complementarity with the reported PIM1 consensus site (K/R-K/R-R-K/R-X-S/T-X´) (39-40), we expected Ser^9 to be targeted by PIM1. Therefore, we carried out site-directed mutagenesis to change Ser^9 to an alanine residue that cannot be phosphorylated. The remarkably reduced phosphorylation signal from an *in vitro* kinase assay with the S9A mutant indicated that Ser^9 is the major PIM1 target site in GSK3B (Figure 1a, right panel). This conclusion was confirmed in another non-radioactive kinase assay, where PIM1-induced phosphorylation of wild-type GSK3B, but not the S9A mutant could be detected by Western blotting using a phosphospecific antibody against P-Ser^9 (Figure 1b). Also there, hardly any autophosphorylation of Ser^9 in GSK3B was observed in the absence of PIM1.

To determine whether the cellular phosphorylation of GSK3B on Ser^{9} is dependent on PIM activity, we investigated the effects of our PIM-selective inhibitor DHPCC-9 in PC-3 prostate cancer cells that endogenously express both GSK3A and B. For this purpose, we analysed the phosphorylation status of GSK3 proteins by Western blotting with the phosphospecific antibody that also recognises P-Ser^{21} in GSK3A. As a result, a clear decrease was observed in the phosphorylation of Ser^{9} in GSK3B in cells treated with 10 µM DHPCC-9 as compared to the DMSO-treated control cells (Figure 1c, left lanes). Similar results with DHPCC-9 were obtained when GSK3B was transiently overexpressed in PC-3 cells (Figure 1c, middle lanes). As expected, the corresponding S9A mutant remained unphosphorylated also in the DMSO-treated control samples (Figure 1c, right lanes). Very little phosphorylation of Ser^{21} in GSK3A was observed in either case, probably due to some negative feedback mechanism downregulating GSK3A expression in cells overexpressing GSK3B.

Even though DHPCC-9 has shown high selectivity for PIM kinases under *in vitro* conditions (36), we wanted to exclude the possibility that its cellular effects were mediated via inhibition of AKT, which can also target Ser^{9} in GSK3B (12). Western blotting analyses with antibodies against AKT1/2/3 or their active forms phosphorylated on Ser^{473} confirmed that neither expression nor activity of AKT kinases was affected by DHPCC-9 treatment as compared to the DMSO-treated control samples (Figure 1d).

Since the phosphorylation of GSK3B on Ser^{9} inhibits its catalytic activity (13), the S9A mutation is expected to render the kinase constitutively active. To analyse the role of GSK3B phosphorylation in prostate cancer cell motility, either wild-type GSK3B or the S9A mutant was transiently overexpressed in PC-3 cells. After 24 h, cells were treated with DMSO or the PIM inhibitor DHPCC-9, after which cell migration was followed up by wound healing assays during a 24 h time period. Overexpressed GSK3B proteins significantly

slowed down the wound healing process as compared to the mock-transfected control samples, but the non-phosphorylatable S9A mutant was slightly more effective than the wild-type protein there (Figure 1e, f). However, even stronger anti-migratory effects were obtained when PIM activity was inhibited by DHPCC-9. These results suggest that phosphorylation of GSK3B is essential, but not alone sufficient for PIM kinases to promote PC-3 cell migration.

FOXP3 also regulates PC-3 cell motility in a phosphorylation-dependent manner

Murine FoxP3 and human PIM1 were produced as GST-fusion proteins in order to analyse whether also FoxP3 is a Pim substrate. Results from radioactive *in vitro* kinase assays with cleaved proteins indicated that FoxP3 is phosphorylated in the presence of wild-type, but not mutant PIM1 (Figure 2a). Furthermore, this phosphorylation is abrogated by preincubation of the kinase with the PIM-selective inhibitor DHPCC-9. Since Ser^{418} in human FOXP3 has been shown to be a functionally important phosphorylation site (18) and since its well conserved surrounding sequence (-RKKRSQ-) resembles the reported PIM1 consensus sequence (39-40), we decided to mutate Ser^{418} in the murine FoxP3 to an alanine residue. However, *in vitro* kinase assays revealed that the S418A mutation only partially inhibited PIM1-dependent phosphorylation (Figure 2b), suggesting that also other sites in FoxP3 may be targeted. When wound healing assays were carried out in PC-3 cells overexpressing either wild-type or mutant FoxP3 protein with equivalent levels, the wild-type protein clearly reduced cell migration, while the mutant had no inhibitory effects (Figure 2c-d), confirming the importance of this site for the inhibitory effects of FoxP3.

While our work was in progress, PIM1 was reported to phosphorylate human FOXP3 on Ser^{422} and thereby inhibit its activity (19). Interestingly, this site is conserved between human and rat, but not mouse (40), so we could not mutate it from murine FoxP3. Since we were mostly interested in the crosstalk of FOXP3 and PIM kinases in human

prostate cancer, we decided to continue our work with human wild-type FOXP3 or corresponding mutants, where either Ser^{418} or Ser^{422} had been replaced with alanine (A) to obtain phosphorylation-deficient proteins or aspartic acid (D) to get phosphomimicking proteins (19).

When these proteins were transiently transfected into PC-3 cells, all of them were expressed at similar physiological levels as the endogenous FOXP3, which migrated slightly slower in protein gels than its Flag-tagged counterparts (Figure 3a). Results from wound healing assays revealed that all FOXP3 proteins except for the S418A mutant reduced cell migration (Figure 3b, c), as was expected based on our data with the murine protein (Figure 2). However, wild-type human FOXP3 was not as potent as the murine protein in inhibiting cell motility, possibly due to the differential ability of PIM kinases to phosphorylate them. Accordingly, loss of Ser^{422} phosphorylation by the S422A mutation or the S418D phosphomimicking mutation rendered FOXP3 more active, resulting in stronger suppression of cell migration as compared to the wild type (Figure 3b, c). As in the case of GSK3B overexpression (Figure 1), strongest anti-migratory effects were observed in the presence of the PIM inhibitor DHPCC-9 (Figure 3b, c). By contrast, overexpression of the S422D phosphomimicking mutant only slightly decreased cell migration and even partially rescued the effects of PIM inhibition by DHPCC-9, with similar cell migration efficiency as observed in mock-transfected control cells. These results suggest that phosphorylation of FOXP3 at Ser^{422} is essential, but not alone sufficient to mediate the pro-migratory effects of PIM kinases in human prostate cancer cells.

PIM1 counteracts the anti-migratory effects of GSK3B and FOXP3

We have previously shown that PC-3-derived stable cell lines overexpressing PIM family members are more migratory and invasive than parental or mock-transfected PC-3 control cells, leading to lymph node and lung metastases in orthotopically xenografted mice (6).

Now we wanted to determine, whether the regulation of GSK3B and/or FOXP3 contribute to the observed motility difference between these cell lines. When the wild-type or mutant forms of GSK3B or FOXP3 were transiently overexpressed in either control or PIM1-overexpressing cells, their expression levels were comparable to each other (Figure S1a-c). Analyses from wound healing assays revealed that stably overexpressed PIM1 was able to antagonize the anti-migratory effects of both wild-type GSK3B and FOXP3, but not those of the corresponding S9A and S422A phosphodeficient mutants, while the phosphomimicking S422D mutant of FOXP3 behaved similarly to the wild-type protein (Figure 4a-d). To confirm that differences in cell migration were not due to differences in cell number, we also counted the numbers of viable cells from the wound healing samples, but observed no major differences during the 48 h follow-up period (Figure 4e, f).

GSK3B and FOXP3 colocalize and interact with PIM1 in PC-3 cells

While FoxP3 and PIM1 have recently been shown to interact within regulatory T cells (19), there is no such data from cancer cells or on GSK3B and PIM1. Therefore, we used both confocal and fluorescence-lifetime imaging microscopy (FLIM) to analyse the subcellular localization and putative interactions between PIM1 and either GSK3B or FOXP3 in PC-3 cells. With the FLIM method, physical interaction of two proteins can be detected by measuring the fluorescence resonance energy transfer between two closely located fluorophores. In the proximity of an acceptor (e.g. RFP), this leads to a decrease in the lifetime of the donor fluorophore (e.g. GFP) (38). For imaging purposes, human proteins fluorescently tagged with GFP or RFP were transiently overexpressed in different combinations, using empty vectors as controls (Figure 5a). Being a transcriptional repressor, FOXP3 was expectedly found in the nucleus, while the GSK3B kinase was detected also in the cytoplasmic compartment (Figure 5b). According to the acquired imaging data, both GFP-tagged GSK3B and FOXP3 colocalized (Figure 5b) and interacted

(Figure 5c, d) with RFP-tagged PIM1 within the nuclei of PC-3 cells. Thus, coexpression with PIM1 did not change the localization on these proteins.

PIM kinases support PTGS2 expression to promote cell motility

Since both GSK3B and FOXP3 have been connected to regulation of PTGS2 expression (21-22), we next wanted to determine whether PTGS2 activity is essential for the ability of PIM kinases to promote prostate cancer cell motility. Therefore, we compared the effects of DMSO, the PIM inhibitor DHPCC-9 and the PTGS2 inhibitor Celecoxib in wound healing assays, using either mock- or PIM1-transfected PC-3 cells. While transient overexpression of PIM1 significantly increased cell migration as compared to the mock-transfected control cells, its pro-migratory effects were efficiently blocked by either DHPCC-9 or Celecoxib (Figure 6a, b), suggesting that PTGS2 activity is required for PIM-dependent cell migration. Western blotting data from the cell samples confirmed that Celecoxib did not affect PTGS2 expression levels, while DHPCC-9 decreased and PIM1 overexpression increased them (Figure 6b, S2a). A similar decrease in PTGS2 levels was also detected, when PIM expression was inhibited by PIM isoform-specific RNA interference reagents (Figure 6c). Silencing of *PIM2* inhibited PTGS2 expression more efficiently than silencing of *PIM1* or *PIM3*. However, as demonstrated by Western blotting with PIM isoform-specific antibodies, the higher efficiency of the *PIM2*-targeting siRNA was most likely due to its unexpected ability to reduce also PIM1 and PIM3 expression levels (Figure 6c).

To determine the relative contribution of GSK3B and FOXP3 on the PTGS2-dependent pro-migratory pathway, we analysed their effects on PTGS2 expression. As a control, part of the samples was treated with the PIM inhibitor DHPCC-9 to decrease GSK3B phosphorylation and thereby increase its activity. According to Western blotting results from transiently transfected PC-3 cells, both the wild-type GSK3B and the phosphorylation-deficient S9A mutant decreased PTGS2 protein levels (Figure 6d, S2b),

while the wild-type or mutant FOXP3 proteins did not have any significant effects (Figure 6e, S2c). As an additional control, we treated PC-3 cells with Indomethacin, which inhibits PTGS1 (prostaglandin-endoperoxide synthase 1, also known as COX1, cyclooxygenase 1), but this treatment had no major effects on cell motility (Figure S3a, b). Altogether, these results suggest that in PC-3 cells, phosphorylation of GSK3B rather than FOXP3 mediates the observed effects of PIM kinases on the PTGS2-promoted cell migration.

PIM activity is essential for integrin-dependent adhesion of PC-3 cells

To examine how important PIM and PTGS2 activities are for integrin-dependent cell adhesion, PC-3 cells were treated with DMSO, DHPCC-9 or Celecoxib for either 1 or 24 h prior to initiating adhesion assays. In these assays, integrin-dependent spreading of serum-deprived cells was followed up to five hours on plates coated with either collagen I or fibronectin using xCelligence technology. Cell spreading on collagen I is mainly dependent on integrin α2 subunits, whereas α5 subunits mediate spreading on fibronectin (41). As an integrin-independent control, another group of cells was allowed to spread on plates coated with poly-L-lysine.

Interestingly, already the 1 h pretreatment with the PIM inhibitor DHPCC-9 reduced adhesion on plates coated with collagen I or fibronectin, but not on the control surface coated with poly-L-lysine (Figure 7a). By contrast, the effects of the PTGS2 inhibitor Celecoxib did not differ from those of DMSO (Figure S4a). After the longer 24 h pretreatment, both DHPCC-9 and Celecoxib inhibited cell adhesion, but while the effects of DHPCC-9 remained integrin-dependent (Figure 7b), Celecoxib also affected spreading of cells on the control plates coated with poly-L-lysine (Figure S4b). However, the effects of DHPCC-9 on plates coated with collagen I were more transient than those of Celecoxib. In accordance, we did not detect changes in the cell surface expression levels of the integrin α2 subunits (Figure S5). Thus, the inhibited cell adhesion could not be explained by

reduced number of the adhesion molecule on the cell surface. We also analysed the activation state of the integrin β1 subunit to see whether DHPCC-9 treatment causes conformational inactivation of the β1 subunit, but according to flow cytometry analyses, this was not the case (Figure S6a-c). While Celecoxib inactivates PTGS2 more completely than DHPCC-9 that rather affects PTGS2 expression, also the faster cellular response to DHPCC-9 suggests that in addition to PTGS2 activity, other PIM effectors may also influence integrin-dependent adhesion.

DISCUSSION

PIM kinases are often overexpressed in malignant and metastatic tumours (3-4, 8-9), but their prognostic roles in prostate cancer have remained controversial, most likely due to their highly heterogenous expression patterns (3-4, 42-45). Yet in mouse models for human prostate cancer, PIM overexpression has clearly been connected to increased tumour growth and metastasis formation (6, 46). In addition, we have previously observed that the phosphorylation of some PIM effectors such as NFATc1 and CXCR4 stimulates their tumour-promoting activities and results in increased migration and invasion of prostate cancer cells (5-6).

Here we have identified GSK3B and FOXP3 as novel PIM interaction partners regulating prostate cancer cell motility. However, unlike the other above-mentioned PIM targets, active GSK3B and FOXP3 have tumour-suppressive and anti-migratory activities that are abrogated by PIM-dependent phosphorylation of Ser^{9} in GSK3B or Ser^{422} in FOXP3. Indeed, these phosphorylation events are essential for the ability of PIM kinases to promote prostate cancer cell migration, since phosphorylation-deficient mutants of GSK3B or FOXP3 can efficiently counteract the pro-migratory effects of PIM kinases. By contrast, the phosphomimicking S422D mutant of FOXP3 only slightly decreases cell migration and can even partially rescue the negative effects of the PIM-selective inhibitor

DHPCC-9. Thus, the phosphorylation of FOXP3 Ser^{422} by PIM1 is likely to inhibit FOXP3 activity and thereby support prostate cancer progression. This is well in line with previous results connecting decreased FOXP3 activity to prostate and breast cancer progression (16, 47-48).

Interestingly, the Ser^{422} residue is not conserved between human FOXP3 and mouse FoxP3, explaining the stronger anti-migratory effects observed for the mouse protein. The close-by Ser^{418} residue on the contrary is conserved, and its phosphorylation is required for proper DNA binding and tumour-suppressive activities of both human and mouse proteins (18), as also indicated by our wound healing data. Due to the observed phosphorylation of FOXP3 by PIM1 at both Ser^{418} and Ser^{422} (19), a feedback regulatory loop may exist in human cells, where PIM kinases partly inhibit FOXP3 activity by phosphorylation of Ser^{422} and partly induce it by phosphorylation of Ser^{418}. However, it is possible that PIM kinases target also additional sites in mouse FoxP3 or human FOXP3. This is supported by the recent report on PIM2 inhibiting regulatory T cell functions via N-terminal phosphorylation of FoxP3 at Ser^{33} which is present in both mouse and human proteins (20, 40).

GSK3B has previously been reported to have either positive or negative effects on prostate cancer progression and cell motility (11, 49-52), likely due to differential cellular contexts. Our results suggest that the presence of active PIM kinases may modulate the outcome by the phosphorylation-induced inactivation of GSK3B, leading to changes in prostate cancer cell migration. Interestingly, GSK3B has tumour suppressor activity also in squamocellular carcinoma cells (53), the motility of which we have previously shown to be stimulated by PIM kinases similarly to prostate cancer cells (5). Thus, the phosphorylation-induced inactivation of GSK3B is probably essential for the pro-migratory effects of PIM kinases also there.

According to our data, PIM kinases also upregulate PTGS2 levels. This is of interest, since PTGS2 stimulates integrin-mediated cell motility (27-30), while integrins in turn influence cell attachment to extracellular matrix (41). PTGS2 expression and activity are essential for the pro-migratory effects of PIM kinases, since the PTGS2 inhibitor Celecoxib, but not the PTGS1 inhibitor Indomethacin, decreases prostate cancer cell migration almost similarly to the PIM inhibitor DHPCC-9, which reduces expression levels for PTGS2. Reduction of PIM expression by RNA interference also interferes with PTGS2 expression.

Active GSK3B counteracts the PIM-dependent upregulation of PTGS2 expression levels, while either wild-type or mutant FOXP3 have no major effects there. Since GSK3B can be inactivated by Ser^9-targeted phosphorylation by both PIM and AKT kinases, and since the inhibition of the PI3K/AKT signaling pathway by LY294002 has been shown to negatively regulate PTGS2 expression (22), it might have been possible that our PIM inhibitor DHPCC-9 had indirectly reduced phosphorylation of GSK3B via targeting also AKT kinases. However, according to our data, DHPCC-9 does not affect either the expression or the activity of AKT kinases, indicating that at least in PC-3 cells, PIM family members bear the major responsibility for the negative regulation of GSK3B activity, and thereby also for the PTGS2-mediated enhancement of cell motility. It should also be noted that among the PIM substrates, GSK3B, FOXP3 as well as NFATc1 can all influence or interact with each other, making the regulatory network even more complex (54-57).

Integrins are involved in cell motility and in forming adhesion sites at the proceeding edges of cells. Moreover, integrin recycling allows cells to lose contacts at their rear ends, and to bring new molecules to cell protrusions. Therefore it is of interest to notice that the inhibition of PIM kinase activity by DHPCC-9 fairly similarly reduces cell spreading that is mediated by either integrin subunit α2 or α5. The fast effects of DHPCC-9

on adhesion but not cell surface integrin expression levels suggest a more direct influence on integrins than via transcriptional regulation. Recycling and internalization of integrins from the cell surface or altered integrin–cytoskeleton interactions could explain the reduced cell spreading, even though we did not see any changes in the overall activation of the β1 subunit after DHPCC-9 treatment.

PTGS2 inhibition has been reported to reduce integrin-mediated cell spreading on vitronectin, but not on fibronectin, while PTGS2 stimulates expression of the integrin α2 and α5 subunits (27-30). According to our data, the effects of PTGS2 inhibition on cell adhesion are integrin-independent but rather slow as compared to PIM inhibition.

The novel findings described here provide important insights on the therapeutic potential of PIM-regulated pathways promoting cell motility and metastatic behavior. Furthermore, the observed effects on PTGS2 suggest that PIM kinases might be suitable targets also for anti-inflammatory therapies.

CONCLUSIONS

Based on the data presented above, the PIM1 kinase promotes prostate cancer cell migration by multiple signalling pathways, including phosphorylation-dependent inactivation of the tumour-suppressive and anti-migratory activities of GSK3B and FOXP3. More indirectly, PIM1 upregulates PTGS2 levels, while inhibition of its kinase activity negatively affects integrin-dependent cell adhesion. Altogether, our data suggest that PIM kinases are attractive targets for anti-metastatic and possibly also for anti-inflammatory therapies.

COMPETING INTERESTS

Authors declare no competing interests.

AUTHOR'S CONTRIBUTIONS

NS, MS, HA and SE performed the research and analysed data. NS, JH, ER and PK coordinated the study. NS, MS and PK wrote the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

We are grateful for obtaining research materials from F. Anizon and P. Moreau (Clermont Université, Université Blaise Pascal and CNRS, France), A. Ristimäki (University of Helsinki, Finland), A. Uri (University of Tartu, Estonia), C. Beals (Stanford University, CA, USA), R. Mentlein (University Medical Center Schleswig-Holstein UKSH, Kiel, Germany), B. Li (Chinese Academy of Sciences, Shanghai, China) and A. Rudensky (Sloan-Kettering Institute, New York, USA). Microscopy was carried out in the facilities of the Cell Imaging Core of Turku Centre for Biotechnology, Turku, Finland. For funding, we acknowledge the Academy of Finland (grants 121533 and 287040 to PJK, 259769 to JH); Drug Research Doctoral Programme, Emil Aaltonen Foundation, Cancer Organizations for the Western Finland, Orion-Farmos Research Foundation, Finnish Cultural Foundation, Cancer Society of Finland and Turku University Foundation (NMS); The National Doctoral Programme in Informational and Structural Biology, and Finnish Cultural Foundation in Southwestern Finland (MS).

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FIGURE LEGENDS

Figure 1 GSK3B is phosphorylated at Ser^{9} by PIM1 to promote PC-3 cell migration

(a) Human GST-fusion proteins were produced in bacteria and the GST-tags were cleaved away. Wild-type (WT) GSK3B or the S9A mutant was incubated with wild-type or kinase-deficient (KD) PIM1 in the presence of radioactive ATP. Shown above are the phosphorylated proteins detected by autoradiography and below the amounts of proteins measured by Page Blue staining. **(b)** The kinase assay was repeated in the presence of non-radioactive ATP, after which Western blotting was used to detect either phosphorylated Ser^{9} or total levels of GSK3B protein. **(c)** PC-3 cells were treated for 24 hours with either DMSO (-) or 10 μM DHPCC-9 (+), after which phosphorylation and expression levels of endogenously (C) or ectopically expressed GSK3B proteins were analysed by Western blotting. Note that the phosphospecific antibody also recognizes Ser^{21} in GSK3A. β-actin staining was used as a loading control, against which the relative signal intensities were calculated **(d)** Phosphorylated Ser^{473} or total levels of AKT family proteins were also analysed from PC-3 cells treated with DMSO or DHPCC-9. **(e-f)** Migration of PC-3 was analysed by wound healing assays. Wounds were scratched across cultures of mock-transfected control (C) cells or cells transfected with wild-type (WT) GSK3B or the S9A phosphomutant, after which cells were treated with DMSO or DHPCC-9 and their migration followed up for 24 hours. All *in vitro* or cellular assays were performed at least three times, except for Western blotting for AKT staining, which was repeated twice.

Figure 2 Phosphorylation of mouse FoxP3 on Ser^{418} is essential for its ability to inhibit PC-3 cell migration

(a) GST-fusion proteins were produced in bacteria and the GST-tags were cleaved away. Wild-type (WT) or kinase-deficient (KD) PIM1 aliquots were pretreated with DMSO or 10

μM DHPCC-9, and then incubated with wild-type (WT) FoxP3 in the presence of radioactive ATP *in vitro*. Shown above are the phosphorylated proteins detected by autoradiography and below the amounts of proteins measured by Page Blue staining. **(b)** Radioactive *in vitro* kinase assays were also performed with the S418A mutant of mouse FoxP3. **(c-d)** PC-3 cells were transiently transfected with an empty vector (C) or either wild-type or mutant FoxP3, after which cell migration was followed up for 24 hours by wound healing assays**.** Expression levels for indicated proteins were analysed by Western blotting. At least three highly similar experiments were performed in each case and shown are average results and representative images from one experiment.

Figure 3 Phosphorylation at the PIM target sites Ser^{418} or Ser^{422} differentially regulates the ability of human FOXP3 to inhibit PC-3 cell migration

PC-3 cells were transiently transfected with flag-tagged vectors expressing human wild-type (WT) FOXP3 or phosphorylation-deficient (SA) or phosphomimicking mutants (SD), where either Ser^{418} or Ser^{422} had been mutated. An empty vector (C) was used as a control. **(a)** Endogenous and ectopic FOXP3 expression levels were analysed by Western blotting with FOXP3 or flag antibodies. β-actin staining was used as a loading control. **(b-c)** Migration of transfected cells treated with DMSO or 10 μM DHPCC-9 was followed up for 24 hours by wound healing assays. Experiments were performed at least three times and shown are averages from independent experiments along with representative images.

Figure 4 PIM overexpression rescues the anti-migratory effects of wild-type but not phosphodeficient GSK3B and FOXP3

(a-d) Mock- or PIM1-transfected stable PC-3-derived cell lines were transiently transfected with empty vectors (C) or vectors expressing wild-type GSK3B or FOXP3 or their corresponding phosphomutants (SA and SD), and their migration was analysed by wound healing assays. **(e-f)** Cell viability was measured by Trypan blue exclusion assays. Two

independent experiments were performed and shown are results from one experiment. Western blotting data is included as supplementary information (Figure S1).

Figure 5 PIM1 colocalizes and interacts with GSK3B and FOXP3 in PC-3 cells

PC-3 cells were plated on coverslips and transfected with GFP-tagged control (C), GSK3B or FOXP3 vectors along with RFP-tagged control (C) or PIM1 vectors. **(a)** Expression of tagged proteins of correct size was confirmed by Western blotting with antibodies against GFP or PIM1. **(b)** Subcellular localization of the fluorescent proteins was analysed by sequential scanning with a confocal microscope. Shown are representative examples for nuclear colocalization (yellow areas). **(c-d)** Protein-protein interactions were analysed by fluorescence-lifetime imaging microscopy (FLIM). Shown are lifetimes of GFP-tagged proteins and representative interaction images (green and blue dots). The numbers of analysed cells are displayed inside the bars.

Figure 6 PIM kinase activity is needed for proper PTGS2 expression

(a-b) PC-3 cells transiently transfected with PIM1 (P1) or an empty control (C) vector were treated with DMSO, 10 µM DHPCC-9 or 25 µM Celecoxib, after which they were subjected to wound healing assays for 24 h followed by Western blotting. Similar data were obtained from two independent experiments. **(c)** PC-3 cells were transfected with non-targeting (NT) control siRNAs or siRNAs targeting *PIM1* (P1), *PIM2* (P2) or *PIM3* (P3), which had been purchased from Dharmacon (^{D}) or from Sigma-Aldrich (^{S}). At 48 h after transfection, part of the NT^{D}-transfected cells was treated with 10 µM DHPCC-9. At 24 h later, expression levels for indicated proteins including PTGS2 were analysed by Western blotting, and signal intensities were measured relative to the β-actin levels. **(d-e)** Mock-transfected PC-3 cells (C) or cells transiently transfected with wild-type (WT) human GSK3B or FOXP3 or their corresponding phosphomutants (SA or SD) were treated with DMSO or 10 µM

DHPCC-9 for 24 h, after which Western blotting analyses were performed. Additional statistical data on them are included in supplementary information (Figure S2).

Figure 7 Inhibition of PIM activity decreases integrin-dependent cell adhesion

PC-3 cells were preincubated for 1 (**a**) or 24 h (**b**) with DMSO or 10 µM DHPCC-9, after which cells were plated on wells coated with poly-L-lysine, collagen I or fibronectin. Real-time cell adhesion was followed up for 5 h. Three independent experiments were performed and shown are results from one representative experiment.

Figure 1

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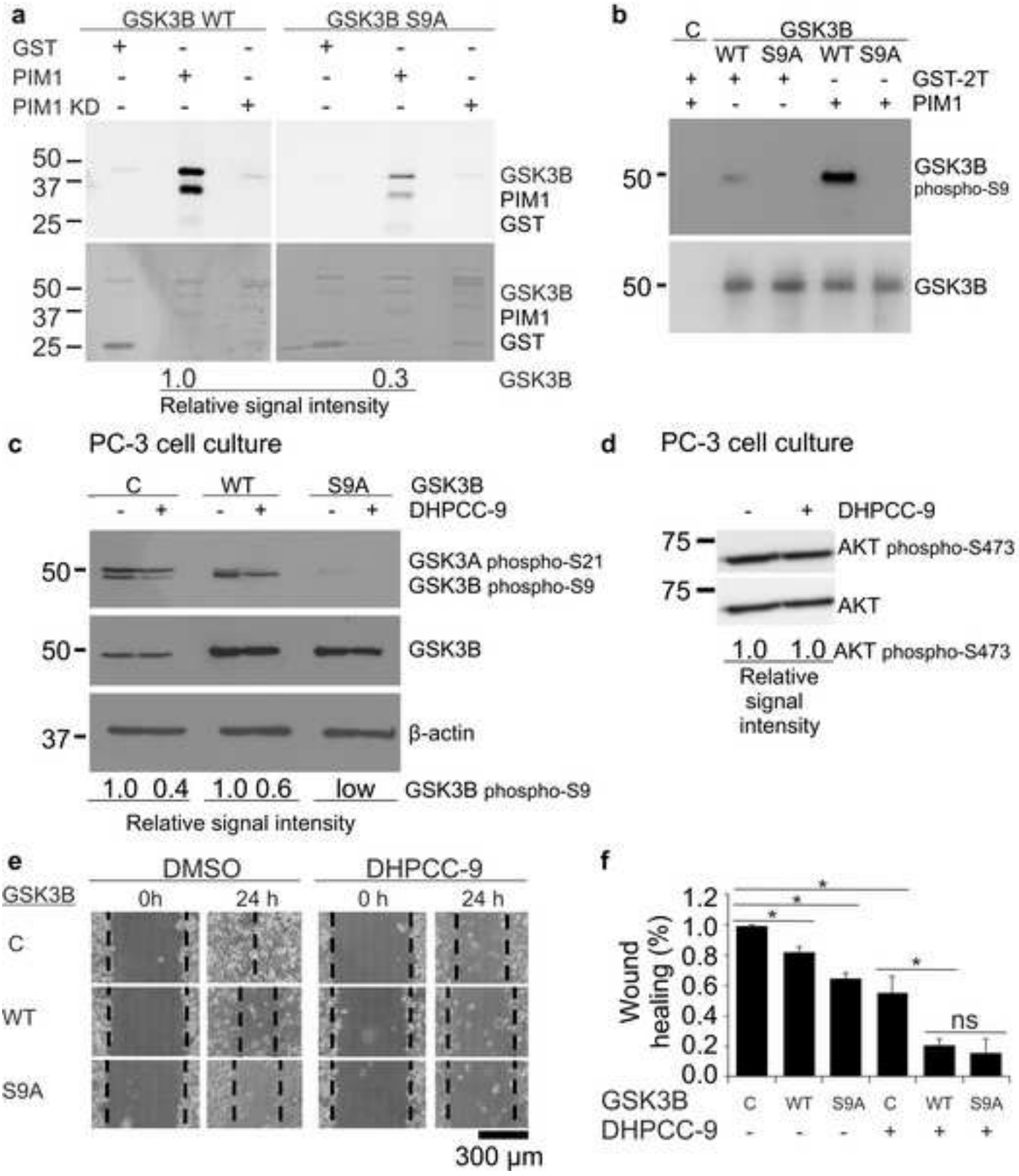

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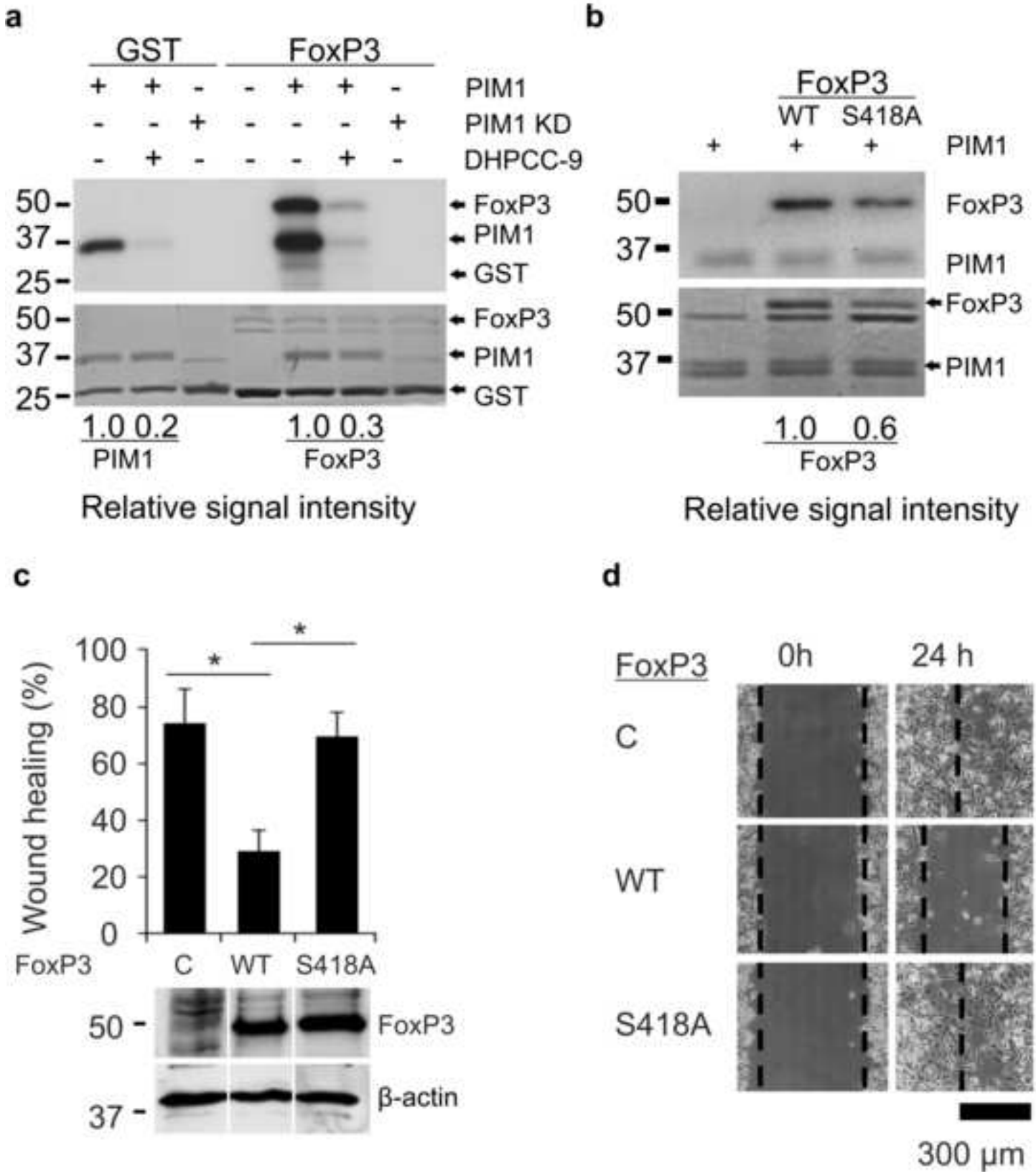

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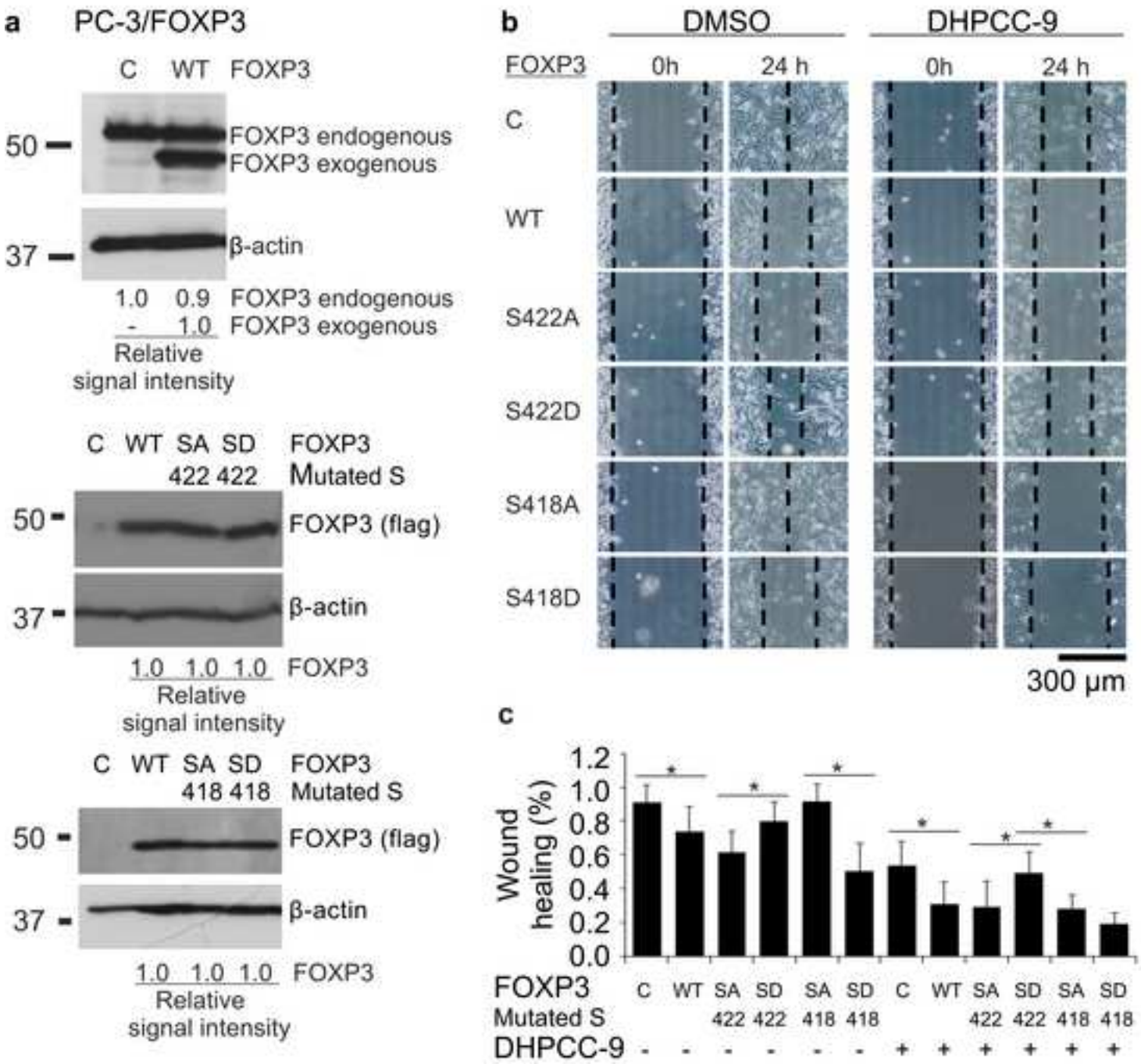

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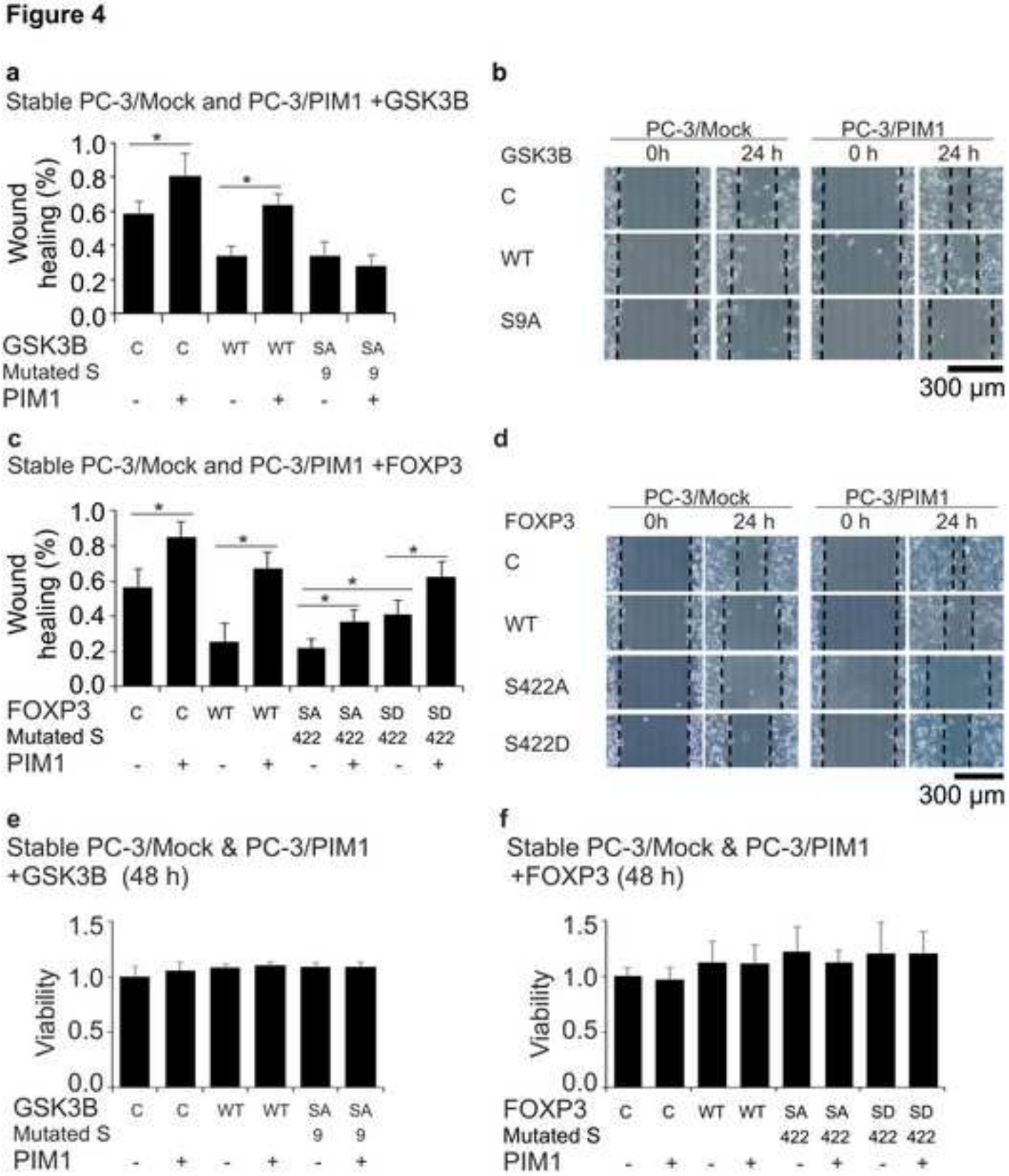

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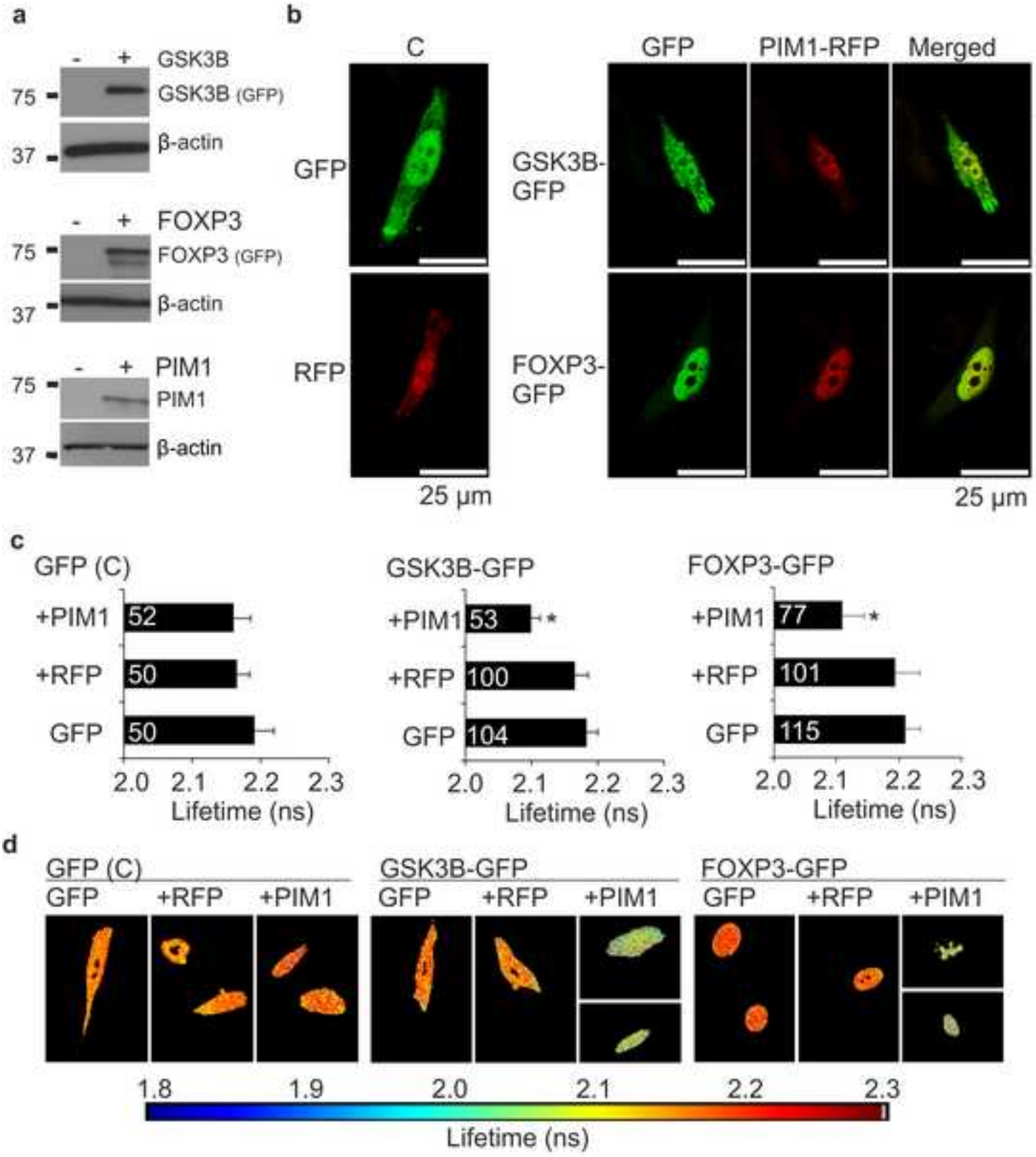

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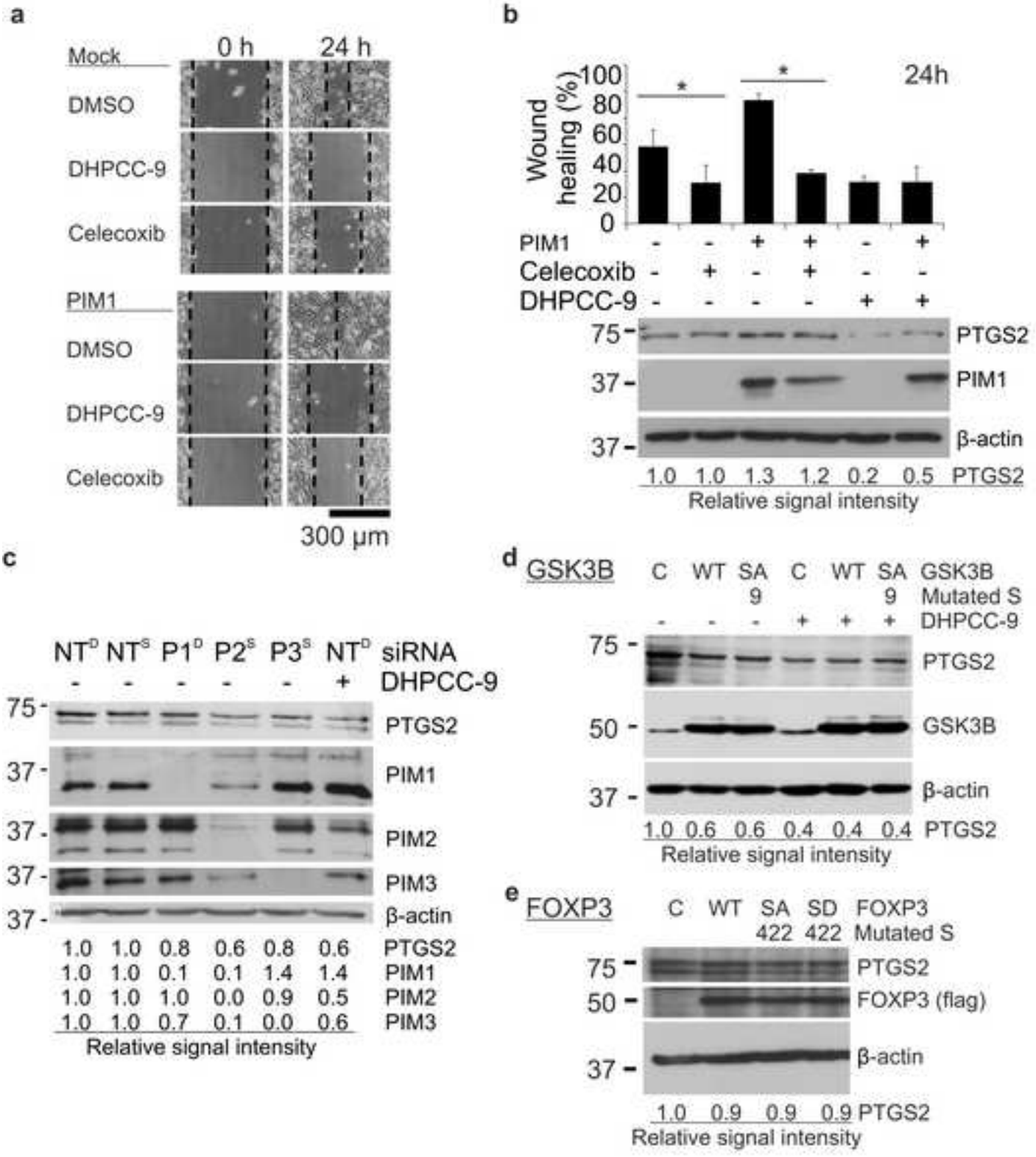

Figure 7

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