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BRIEF REPORT

ZEB1 transcription factor induces tumor cell PD-L1 expression in melanoma

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Abstract
Tumor cells can evade antitumor immune response by expressing the PD-L1 ligand, leading to the inhibition of PD-1-expressing T lymphocytes. The mechanisms that regulate PD-L1 expression in cancer cells are imperfectly characterized. The transcription factor ZEB1, a major regulator of phenotype switching in melanoma cells, was shown to promote immune escape in melanoma by repressing T cell infiltration. Using inducible models of phenotype switching and ZEB1 gain/loss-of-function melanoma, we show that ZEB1 binds to the *CD274* (PD-L1) promoter, directly enhancing PD-L1 mRNA transcription and its expression at the cell membrane. Furthermore, using single-cell spatial analyses on human primary melanoma samples, we demonstrate the correlation of ZEB1 and PD-L1 expression in tumor cells. Overall, these data identify ZEB1-mediated regulation of PD-L1 tumor expression as a mechanism that could contribute to immune escape in melanoma.

Keywords Melanoma · ZEB1 · PD-L1 · Phenotype switching · Pseudo-EMT

Introduction

Immune checkpoint blockade (ICB) using anti-programmed cell death 1 (anti-PD-1) antibodies has revolutionized the management of metastatic melanoma. Tumor cells can evade antitumor immune response by expressing the PD-L1 ligand, which inhibits PD-1-expressing T lymphocytes (TLs) within the tumor microenvironment. Blocking the PD-1/PD-L1 axis allows the restoration of antitumor immunity, achieving a clinical benefit in approximately 40% of cases [1]. However, most patients still present primary or acquired resistance to anti-PD-1 therapy. PD-L1 expression has been associated with a significantly lower overall survival [2, 3], and correlates with response to PD-1 blockade [4]. Thereby, defining the mechanisms that regulate PD-L1 expression in melanoma is crucial.

PD-L1 expression in tumor cells is regulated at several levels, including genomic alterations, epigenetic modification, transcriptional regulation, and posttranscriptional and posttranslational modifications [5]. The tumor microenvironment, especially IFN-γ produced by TLs [6], is a crucial regulator of PD-L1 expression. Additionally, TNFα induces PD-L1 expression by activating the nuclear factor κB (NF-κB) pathway in prostate and colon cancer cells [7] or by promoting an epithelial-to-mesenchymal transition in hepatic cancer [8]. Among tumor-intrinsic mechanisms, MYC and oncogenic RAS were shown to regulate its transcription and RNA stability [9], whereas the eIF4F-STAT1 axis controls its translation [10], and CMTM4 and CMTM6 regulate its localization at the cell membrane [11].

In melanoma, a pseudo-epithelial-to-mesenchymal transition (pseudo-EMT) allows the reversible transition from a proliferative/melanocytic state to an invasive cell state

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with neural crest-like stem cell (NCSC) features [12–14]. Melanoma cell phenotype switching is mainly controlled by the microphthalmia transcription factor (MITF) [12, 15]. In addition, we previously showed that the EMT-inducing transcription factor, zinc finger E-box binding 1 (ZEB1), negatively regulates MITF-dependent proliferative/melanocytic programs and promotes AP-1-driven invasive and stem-like programs [16, 17]. ZEB1 expression is associated with a significant decrease in the expression of differentiation antigens, including MLANA, tyrosinase and MC1R, while simultaneously enhancing nerve growth factor receptor (NGFR) expression, a NCSC marker [16–18]. Melanoma cell plasticity promotes tumor progression, distant migration, resistance to targeted therapy, and more recently escape from the immune system [19, 20]. Indeed, the $NGFR^{high}$ NCSC state was associated with immune exclusion and resistance to ICB [21, 22], and we recently demonstrated that ZEB1 expression in melanoma cells is associated with decreased $CD8^+$ T cell infiltration by repressing the expression of the T cells attracting chemokine CXCL10, thus promoting resistance to anti-PD-1 treatment [23]. In lung and colorectal carcinomas, ZEB1 regulates PD-L1 tumor expression [24–26], suggesting that ZEB1-mediated phenotype switch in melanoma might regulate the expression of immune checkpoint ligands promoting immune escape.

Herein, using inducible models of phenotype switching and ZEB1 gain/loss of function, we investigated the role of ZEB1 in the regulation of tumor cell PD-L1 expression, and further analyzed their concomitant expression using single-cell spatial analyses on human primary melanoma samples. Our results demonstrate that ZEB1 binds to the *CD274* (PD-L1) promoter, directly enhances PD-L1 mRNA transcription and its expression at the cell membrane.

Materials and methods

Culture cell lines

A375 human melanoma cell line was purchased from ATCC and was cultured in DMEM complemented with 10% FBS (Cambrex) and 100 U/ml penicillin–streptomycin (Invitrogen). Previously described patient-derived short-term cultures, GLO and C-09.10, established from BRAFV600 metastatic melanomas [16], were grown in RPMI complemented with respectively 20 and 7% FBS and 100 U/ml penicillin–streptomycin. TNFα (100ng/mL) and TGFβ (10ng/mL) (PeproTech) were replaced in the culture medium every 3 days. Generation of ZEB1-overexpressing C-09.10 cells using retroviral infection and HA-Zeb1 in a pBabe-puro vector was described previously [18]. Generation of Zeb1 knocked-out A375 cells was also described previously [16].

Immunoblot analyses

Cells were washed twice with phosphate-buffered saline (PBS) containing $CaCl_2$ and then lysed in a 100 mM NaCl, 1% NP40, 0.1% SDS, 50 mM Tris pH 8.0 RIPA buffer supplemented with a complete protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitors (Sigma-Aldrich). Loading was controlled using anti-GAPDH or anti-actin. Horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibodies (Glostrup) were used as secondary antibodies. Western blot detections were conducted using the Luminol reagent (Santa Cruz). Western blot digital imaging was performed with ChemiDoc™ MP Imager (Bio-Rad). Primary antibodies used: anti-ZEB1 (1/500, Sigma #HPA027524, rabbit); anti-NGFR (1/1000, Cell Signaling #8238S, rabbit); anti-MITF (1/500, Millipore #MAB3747, mouse); and anti-GAPDH (1/20000, Sigma-Aldrich, Cat# ABS16, rabbit).

RT-qPCR

Total RNA was isolated using RNeasy Kit (QIAGEN) and reverse transcribed using a high cDNA capacity reverse transcription kit following the manufacturer's instructions (Fisher Scientific). Quantitative PCR was done using the Azure Cielo (Azure Biosystem). Human GAPDH was used for normalization. The following primers were used:

ZEB1_Forward GAGGTAAAGCGTTTATAGCCTCTATCA, and ZEB1_Reverse AGGGCACACCAGAAGCCAG, CD274_Forward AGGGAGAATGATGGATGTGAAA and CD274_Reverse TGCTGGATTACGTCTCCTCC.

Flow cytometry analyses

To analyze the expression of cell surface markers, about 500.000 cells per condition were incubated with anti-PD-L1/CD274 (PE, 1/100, 29e.2a3 from BioLegend) and anti-NGFR/CD271 (Alexa Fluor 647, 1/100, from BD Pharmingen) antibodies for 1h in the dark before being counted on a BD LSRFortessa™ flow cytometer (BD Biosciences-IN) on the flow cytometry core facility (CYLE, CRCL, Lyon). DAPI was used to evaluate cell viability. Data were analyzed using the FlowJo 10.8.1 software.

Chromatin immunoprecipitation

The ChIP assay was carried out according to the protocol from the iDeal ChIP-seq kit for transcription factors (Diagenode, Denville, NJ, USA) as previously described [16]. Briefly, cells from one 15-cm dish were cross-linked with 1% formaldehyde at RT for 10 min and quenched in 125 mM glycine for 5 min. The cross-linked chromatin was

isolated and sonicated to generate DNA fragments averaging 200–500 bp in length by Bioruptor plus sonication device (Diagenode). Chromatin fragments were immunoprecipitated with antibodies directed against ZEB1 (1 µg, Genetex, GTX105278, RRID:AB_11162905), or IgG (1 µg, Bio-Rad, PRABP01, RRID:AB_321631) as negative control. qPCR primers on promoters used for quantification: CD274_Forward CTCGCTGGGCACTTTAGGAC and Reverse TACTGCCCCCTAGACCATCG; MITF_Forward CTGAAGATCCCAGCGGGTTG and Reverse GAGGTGACTCCAAGCGAACT.

ZEB1 ChIP-seq data generated in A375 and GLO cells [16] are publicly available on the Gene Expression Omnibus (GEO) database under accession number GSE246672.

Human tumor samples

Retrospective melanoma tumor samples were obtained through the Biological Resource Centre of the Hospital Lyon Sud, Hospices Civils de Lyon. Human tumor samples were used with the patient's written informed consent. This study was approved by the local ethics committee (Comité Scientifique et Éthique des Hospices Civils de LYON CSE-HCL–IRB 00013204, approval n° 22_680) and the French data protection authority (Commission nationale de l'informatique et des libertés, approval n° 22_5680) for the collection of associated clinical data. A cohort of n = 22 cutaneous primary melanoma patient lesions was used for multi-immunofluorescence analyses. Associated clinical data are listed in Supplementary Table 1. Four tumor lesions were excluded because of ripped tissue. Eighteen samples were included in the final analysis.

Seven-color immunofluorescence multiplex analyses and immunohistochemical staining

A 3-µm tissue sections were cut from formalin-fixed paraffin-embedded human melanoma specimens. The sections underwent immunofluorescence staining using the OPAL™ technology (Akoya Biosciences) on a Leica Bond RX, on the Research pathology platform East (ANAPATH RECHERCHE, CRCL, Lyon). DAPI was used for nuclei detection. Sections were digitized with a Vectra Polaris scanner (Perkin Elmer, USA). An autofluorescence treatment of images was carried out using the Inform software (PerkinElmer). Quantification of the number and percentage of the staining was carried out using the Inform software. Antibodies used were as followed: anti-SOX10 (OPAL-480, Santa Cruz #sc-365692, 1/1000, 30 min incubation, mouse); 1/40, 30 min incubation, rabbit); anti-ZEB1 (OPAL-570, Sigma #HPA027524, 1/100, 30 min incubation, rabbit); anti-PD-L1 (OPAL-620, Diagomics #1-PR292, 1/100, 60 min incubation, rabbit); anti-CD68 (OPAL-690, Abcam #EPR20545, 1/8000, 30 min incubation, rabbit). The cutoff value to define tumor cells based on SOX10 signal intensity was set up at 2. Cutoff values to define positivity were 3 for ZEB1, 1.3 for PD-L1 and 4 for CD68.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, Inc., San Diego, USA). Data are presented as mean ± SEM. To determine significant differences between two groups, parametric data were analyzed using Student's t-test. All statistical tests were two-tailed. The p-values obtained were considered significant when < 0.05.

Melanoma public datasets analysis

ZEB1 ChIP-seq data were retrieved from the Gene Expression Omnibus (GEO) database under accession number GSE246672. The analysis was performed as previously described in Durand et al. [16].

Single-cell RNA-seq data of melanoma cell lines from Wouters et al. [27] were downloaded from GEO (GSE134432). Single-cell RNA-seq data were analyzed and visualized using Seurat (4.3.0).

Results

Phenotype switching toward ZEB1^{high}/NGFR^{high} invasive/neural crest-like state is associated with increased PD-L1 expression at the cell membrane

Besides ZEB1 overexpression, TNFα combined or not with TGFβ treatment is a potent inducer of phenotype switching toward invasive/neural crest-like states in melanocytic patient-derived short-term cultures, as assessed by increased ZEB1 and NGFR expression, and decreased MITF expression (Fig. 1A, Supp. Figure 1A). Flow cytometry analyses of NGFR membrane expression further showed that TNFα ± TGFβ treatment significantly increases the proportion of NGFR^{high} cells in C-09.10 following 7 or 14 days of treatment, to similar levels compared to ZEB1-overexpressing cells (Fig. 1B, Supp. Figure 1B). We therefore used these models of phenotype switching toward ZEB1^{high}/NGFR^{high} invasive/stem-like states to investigate the expression of PD-L1 at the cell membrane by flow cytometry. We observed a significant increase in PD-L1 membrane expression in both GLO and C-09.10 cell lines upon TNFα ± TGFβ treatment and in ZEB1-overexpressing C-09.10 cells (Fig. 1C). Interestingly, cells expressing PD-L1 concomitantly expressed NGFR, as evidenced by the significant increase in double

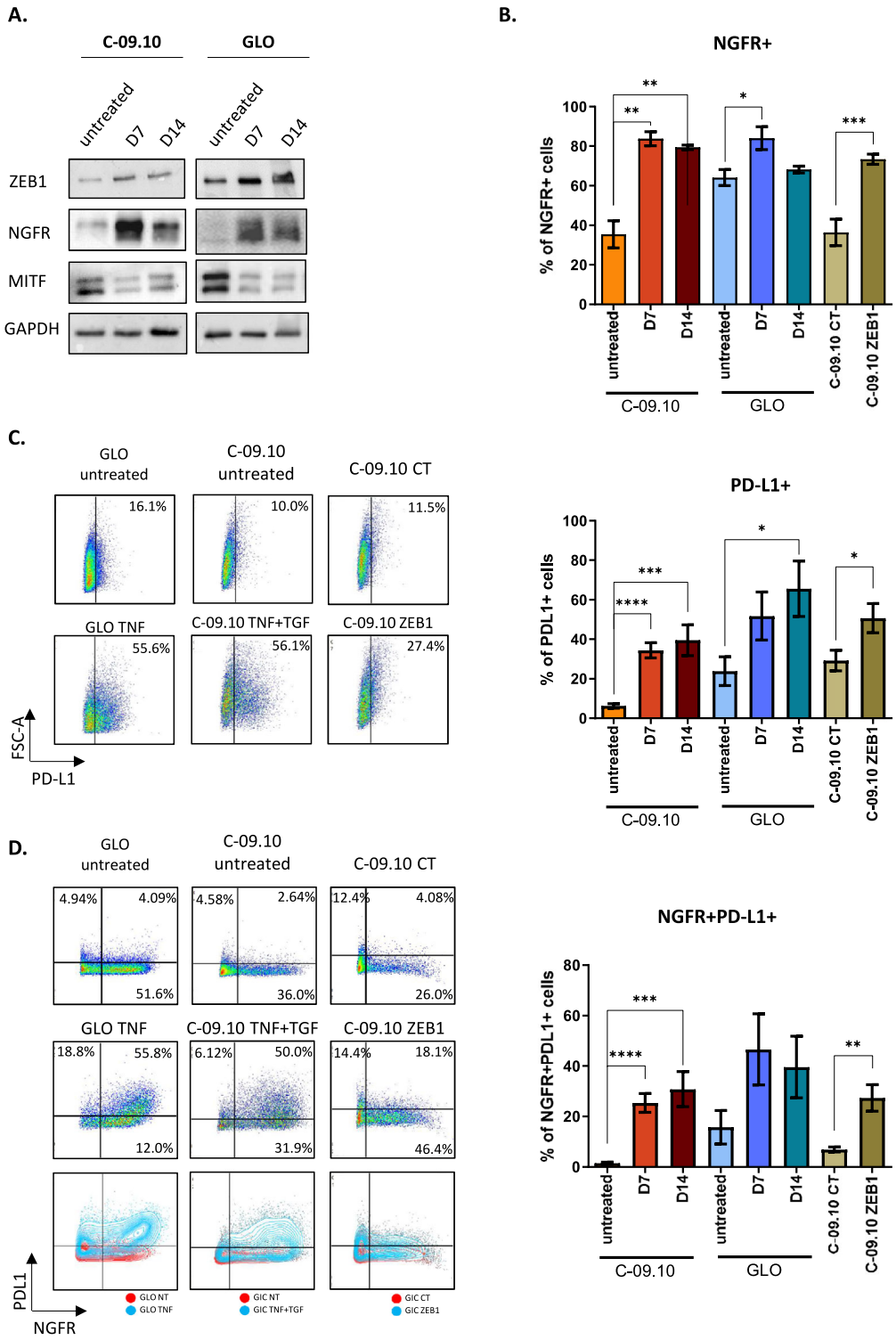

Fig. 1 Phenotype switching toward ZEB1^{high}/NGFR^{high} dedifferentiated state is associated with increased PD-L1 expression at the cell membrane. C-09.10 cells were treated for 7 (D7) and 14 days (D14) with TNFα and TGFβ, and GLO cells were treated with TNFα for 7 (D7) and 14 days (D14). **A** Western blot analyses of ZEB1, NGFR, and MITF expression. GAPDH was used as loading control. **B** NGFR expression measured by flow cytometry (mean ± SEM) in C-09.10 and GLO cells (n = 3), in C-09.10 control (CT) or overexpressing ZEB1 (ZEB1) (n = 5). **C** PD-L1 expression measured by flow cytometry (left), the percentage of the PD-L1^{+} population is indicated for each condition (right) (mean ± SEM) (n = 3). **D** NGFR and PD-L1 expression measured by flow cytometry (left), the percentage of the NGFR^{+}/PD-L1.$^{+}$ population is indicated (mean ± SEM) (n = 3). P values were determined by a two-tailed unpaired Student's t-test (B-D). Differences were considered statistically significant at *P ≤ 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001

positive PD-L1^{high}/NGFR^{high} cells in the C-09.10 cellular models (Fig. 1D). Moreover, analysis of single-cell RNA-seq public datasets of melanoma cell lines with well-characterized phenotypes [27] showed that both PD-L1 and ZEB1 display an increased expression in mesenchymal-like melanoma cells (Supp. Figure 1C).

ZEB1 is recruited on the *CD274* promoter and induces its transcription

We then investigated how ZEB1 may promote PD-L1 expression upon melanoma cells dedifferentiation. In our recent ZEB1 chromatin immunoprecipitation sequencing (ChIP-Seq) dataset [16], we observed that ZEB1 is bound to the transcription start site (TSS) of the *CD274* (*PD-L1*) promoter in ZEB1^{high} A375 cells, as well as in differentiated GLO cells upon TNFα-induced phenotype switching (Fig. 2A). We next validated by ChIP-QPCR the binding of ZEB1 on the *PD-L1* promoter in A375 cells (Fig. 2B) and in GLO cells treated with TNFα compared to the control condition (Fig. 2C). Enrichment levels were similar to those on the *MITF* promoter, which served as a positive control.

Our results therefore suggest that ZEB1 might promote *PD-L1* transcriptional activation by binding to its promoter. Consistently, increased PD-L1 mRNA expression was observed upon TNFα treatment in both C-09.10 and GLO cells (Fig. 2D). To confirm a specific role of ZEB1 in this induction, we quantified the expression of PD-L1 mRNA in ZEB1 gain/loss-of-function models (Fig. 2E). In C-09.10 cells overexpressing ZEB1 (C-09.10 ZEB1), we observed a trend toward an increased PD-L1 mRNA expression (Fig. 2F). Accordingly, in A375 cells knocked-out for ZEB1 (A375 AZ1) [16], PD-L1 mRNA expression exhibited a 40% decrease, indicating that ZEB1 participates in PD-L1 expression regulation (Fig. 2F). Altogether, our results demonstrate that ZEB1 binds to *PD-L1* promoter and directly induces its transcription, leading to a significant increase in PD-L1 expression at the cell membrane.

ZEB1 is quantitatively and spatially associated with PD-L1 in human primary melanoma samples

Next, we wanted to further study the correlation between ZEB1 and PD-L1 tumor-intrinsic expression in human samples. Bulk RNA-seq analyses on tumor samples are severely biased since PD-L1 is also expressed by immune cells (notably macrophages) and ZEB1 by stromal and immune cells [23], thus precluding robust analysis of cell type-specific expression. We therefore designed a multiplex immunofluorescence panel to precisely investigate tumor cell-specific correlation between ZEB1 and PD-L1 expression in human primary melanoma samples, using SOX10 to distinguish the tumor cells from the stromal tissue, as well as CD68 to identify macrophages. We reconstructed the whole-slide images using R.

In human tumors, PD-L1 is not only expressed by tumor cells, but also by infiltrating immune cells (mainly CD68$^+$ macrophages and DC) [28]. We therefore excluded PD-L1$^+$ CD68$^+$ cells from the analysis to focus on PD-L1 expression in the tumor cells compartment. The analysis of PD-L1 expression in SOX10$^+$ melanoma cells showed a frequent expression at the periphery of tumor nodules or closely associated with CD68$^+$ cells infiltrating the tumor (Fig. 3A and B). Importantly, we observed that PD-L1 expression was mainly found in tumor cells expressing a high level of ZEB1. Fifteen percent of ZEB1 positive cells in all tumors were positive for PD-L1 (min 1.62%, max 44.3%, mean 15.0%), compared to 4.21% in ZEB1 negative cells (min. 0.35%, max 15.6%, mean 4.21%) ($p = 0.0030$, Fig. 3C). By segregating tumors in ZEB1^{high} and ZEB1^{low} tumors, we further showed that PD-L1 tumor cell expression was significantly higher in ZEB1^{high} compared to ZEB1^{low} tumors (Fig. 3D). Moreover, we observed a significant correlation between ZEB1 expression and PD-L1 expression in tumor cells ($r^2 = 0.4368$, $p = 0.002823$, Fig. 3E). Altogether, these results confirm that ZEB1 is quantitatively and spatially associated with PD-L1 expression in human primary melanoma samples.

Discussion

The expression of ligands of inhibitory immune checkpoints by tumor cells is a major mechanism of escape from immune surveillance. Tumor cell plasticity is also increasingly recognized as a tumor-intrinsic mechanism of immune escape [20]. Previous experiments in syngeneic mouse models demonstrated that ZEB1, a major regulator of melanoma plasticity in melanoma, driving cells toward a dedifferentiated state, promotes immune escape and tumor growth in a CD8$^+$ TLs-dependent manner [23]. Here, we demonstrated that ZEB1 directly binds to the *CD274* promoter, induces PD-L1 mRNA transcription, and increases its expression at the cell membrane. This work provides additional mechanistic insight into how ZEB1 may promote immune escape by directly increasing tumor cell PD-L1 expression potentially inhibiting PD-1$^+$ CD8$^+$ TLs.

A link between EMT and PD-L1 expression was previously reported in epithelial tumors [25, 29]. In their study, Mak et al. observed that a pan-cancer EMT signature correlated with high expression of multiple immune checkpoints, including PD-L1. In NSCLC, miR-200 is a negative regulator of both ZEB1 and PD-L1, and ZEB1 represses miR-200 expression leading to an indirect increase of PD-L1 [24]. In melanoma cells, in which ZEB1 expression is not regulated by miR-200 [17], we rather demonstrate by ChIP assay that ZEB1 directly binds to the *CD274* promoter, to efficiently

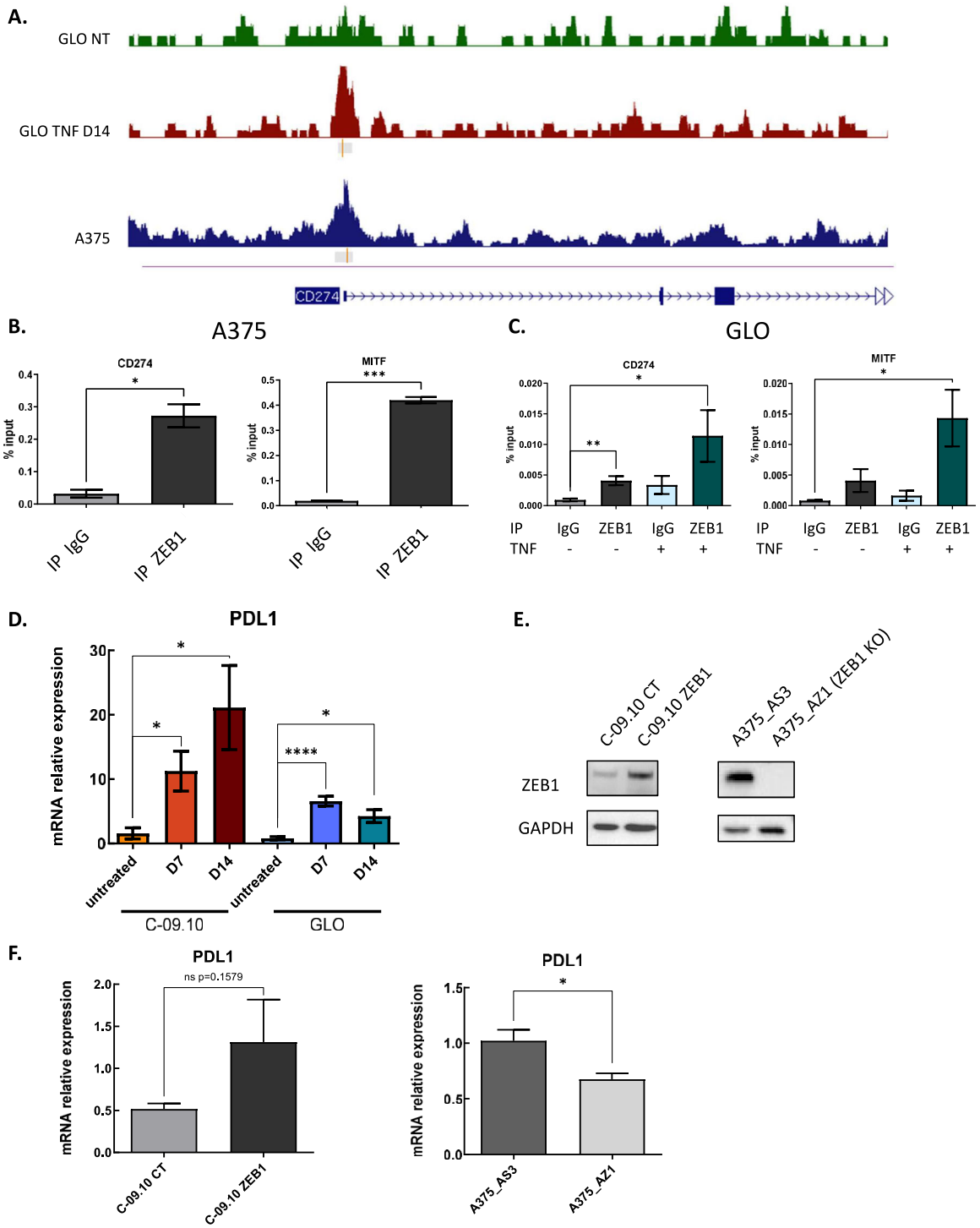

Fig. 2 ZEB1 is recruited on the *CD274* promoter and induces its transcription. **A** Geneviewer captures of ZEB1 chromatin immunoprecipitation sequencing in untreated (GLO NT, green), or TNFα-treated GLO cells for 14 days (GLO TNF D14, red) and in A375 (blue). **B-C** ZEB1 ChIP-qPCR on *CD274* promoter in A375 cells (AS3 control) (n = 2) (B) and in GLO NT and GLO TNF D14 (n = 4) (C). *MITF* promoter was used as a positive control. **D** PD-L1 mRNA relative expression measured by qPCR (mean ± SEM) in C-09.10 untreated or treated 7 and 14 days with TNFα and TGFβ (respectively D7 and D14) (n = 5), in GLO untreated and treated 7 and 14 days with TNFα (respectively D7 and D14) (n = 5). **E** Western blot analyses of ZEB1 expression in C-09.10 cells overexpressing ZEB1 and in A375 control (AS3) or knocked-out for ZEB1 (AZ1). GAPDH was used as loading control. **F** PD-L1 mRNA relative expression measured by qPCR (mean ± SEM) in C-09.10 control (CT) or overexpressing ZEB1 (n = 5) and in A375 control (AS3) and A375 knocked-out for ZEB1 (AZ1) (n = 5). P values were determined by a two-tailed unpaired Student's t-test (B-D, F). Differences were considered statistically significant at *P ≤ 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001

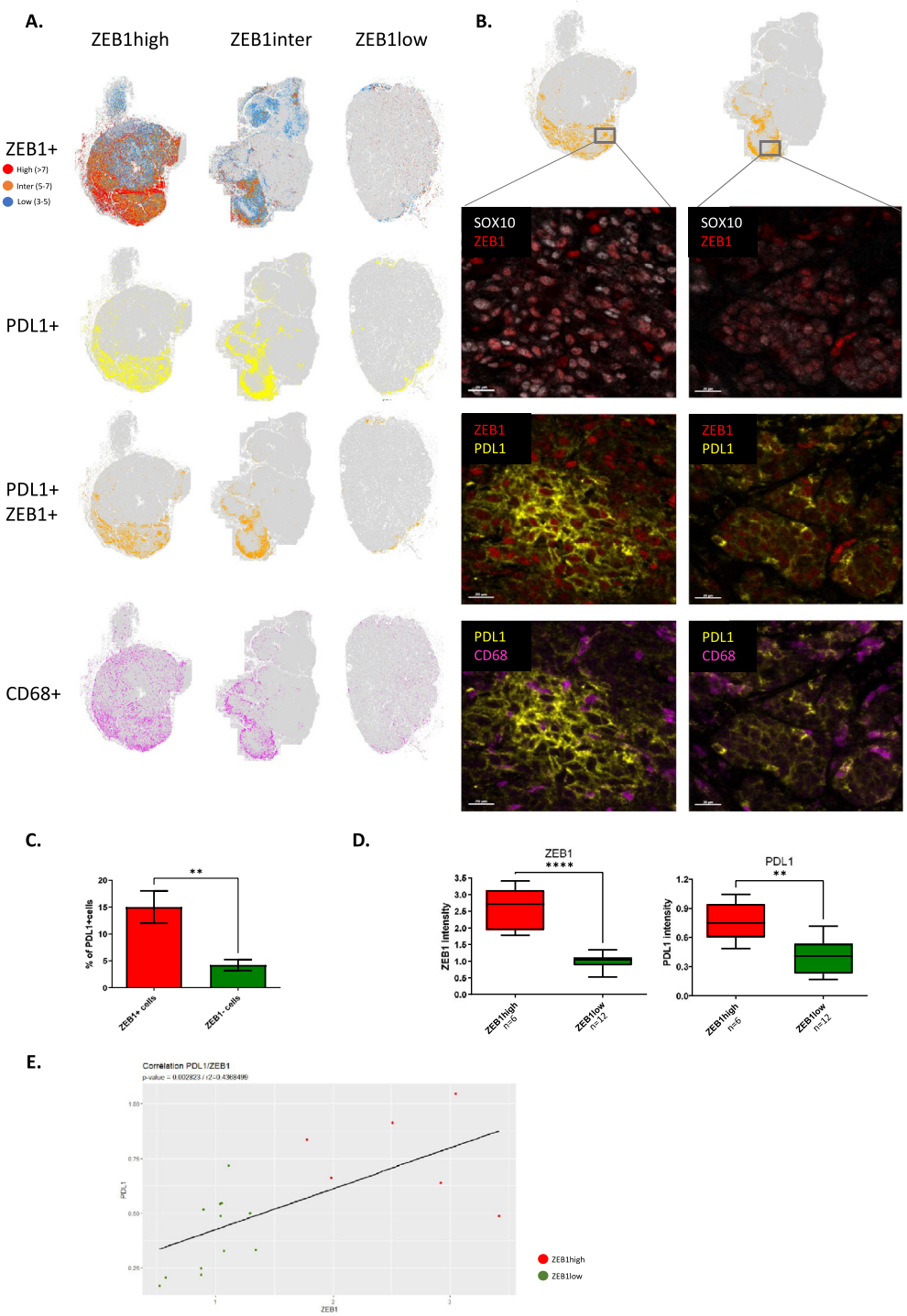

Fig. 3 Quantitative and spatial correlation of ZEB1 and PD-L1 expression in human primary melanoma samples. **A** Reconstitution of a ZEB1 high, a ZEB1 intermediary (ZEB1inter), and a ZEB1 low tumor according to the intensity of ZEB1 in tumor cells (3–5: blue; 5–7: orange; > 7: red); the positivity for PD-L1 alone (yellow), or ZEB1 and PD-L1 (orange) and CD68 (magenta) is indicated. For ZEB1, PD-L1, and ZEB1 and PD-L1 markers, only tumor cells with a SOX10 intensity superior to 2 were analyzed. **B** Representative picture of multi-immunofluorescence OPAL staining for SOX10 (white), ZEB1 (red), PD-L1 (yellow), CD68 (magenta) visualized on Phenochart software (scale bar = 20µm). **C** Percentage of PD-L1 positive tumor cells (SOX10 > 2, PD-L1 > 1.3) in ZEB1 positive cells (ZEB1 > 3) and ZEB1 negative cells (right). **D** ZEB1 intensity (left) measured by multiplex immunofluorescence (mean ± SEM) in ZEB1^{high} tumors (n = 6, ZEB1^{+} cells > 10%) and in ZEB1^{low} tumors (n = 12). PD-L1 intensity (right) measured by multiplex immunofluorescence (mean ± SEM) in ZEB1^{high} (n = 6) and ZEB1^{low} (n = 12) tumors. **E** Spearman correlation between ZEB1 intensity and PD-L1 intensity in all tumors. ZEB1^{high} tumors are shown in red and ZEB1.^{low} tumors in green. P values were determined by a two-tailed unpaired Student's t-test (C, E). Differences were considered statistically significant at *P ≤ 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001

induce PD-L1 transcription. Accordingly, PD-L1 mRNA expression was upregulated upon ZEB1-overexpression. Altogether, we demonstrate that ZEB1 is a direct positive regulator of PD-L1 expression in melanoma tumor cells.

Besides tumor-intrinsic/cell autonomous regulation, PD-L1 tumor expression might be further increased in the immune tumor microenvironment by the presence of TNFα ± TGFβ, favoring ZEB1 expression and phenotype switching. Interestingly, our ChIP experiments showed a significant increase in ZEB1 recruitment on the *CD274* gene promoter upon TNFα ± TGFβ treatment, suggesting that the integration of external stimuli might amplify tumor-intrinsic regulation of PD-L1 expression. TNFα expression is associated with resistance to immunotherapy. Consistently, TNF blockade increases the $CD8^+$ TLs tumor infiltration and decreases PD-L1 expression in melanoma mouse models [30]. This is consistent with the recently proposed model from the Kumar-Jolly laboratory, suggesting that intra-tumor heterogeneity in PD-L1 expression is controlled by a cooperation between IFNγ signaling and tumor-intrinsic TF activity [31].

Although PD-L1 was found positively associated with response to immunotherapy in melanoma patients, its expression alone is not a sufficient predictive marker. In addition, there is currently no standardized methodology to measure PD-L1 expression, and its evaluation can differ between assays, mainly due to the high variability of immunohistochemistry analyses across distinct centers [32]. In the present study, we used multiplex immunofluorescence to precisely investigate the spatial localization of $PD\text{-}L1^+$ tumor cells and their co-localization with ZEB1 expression, as a surrogate of dedifferentiated melanoma cells, and observed a preferential association between PD-L1 and ZEB1 expression in primary tumor samples from advanced melanoma patients. Overall, our results identify ZEB1-mediated regulation of PD-L1 tumor expression as a mechanism that could contribute to immune escape in melanoma lesions. It will be interesting to further investigate in the future whether ZEB1/PD-L1 melanoma cell heterogeneity associates with immunotherapy response in patients.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00262-025-03978-5.

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Author contributions CW performed and analyzed most experiments, prepared figures, and wrote the manuscript. SD performed experiments and statistical analyses, prepared figures, and wrote the manuscript. FB, MP, VB, and LB performed and analyzed experiments. MG performed multi-IF staining. GT, BB, and OH performed pathological examination. AE and SDa provided human samples and clinical data and wrote the manuscript. JL co-supervised the project and wrote the manuscript. JC conceived and supervised the project and wrote the manuscript.

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Data availability No datasets were generated or analyzed during the current study.

Declarations

Conflict of interest The authors declare that there are no competing interests.

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