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Victor Z. Chen April 12, 2022

YB-1 Post-Transcriptionally Regulates PLXND1 to Mediate Metastasis in Sonic Hedgehog Medulloblastoma

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Abstract

YB-1 Post-Transcriptionally Regulates PLXND1 to Mediate Metastasis

in Sonic Hedgehog Medulloblastoma

By Victor Z. Chen

Medulloblastoma (MB) is one of the most common brain malignancies in children, accounting for nearly 10% of all pediatric brain tumors. The Sonic Hedgehog (SHH) subgroup, which accounts for 30% of MB cases, is characterized by tumor formation via perturbations in SHH signaling during early cerebellar development. Current treatment options are limited to surgical resection, craniospinal irradiation, and chemotherapy. This standard of treatment yields a ~60-70% survival rate with a high-risk of long-term detriments and reduced quality of life. A significant portion of mortalities from MB are caused by metastatic incidence or recurrence. Our study seeks to investigate potential molecular drivers of metastatic progression in SHH MB. Using *in vitro* primary mouse models and cell lines, we identify Y-box-binding protein 1 (YB-1) as a positive modulator of Plexin D1 (PLXND1) expression. Our studies suggest that YB-1 post-transcriptionally regulates PLXND1 to mediate cell migration, invasion, and tumor development. These findings point to this novel YB-1/PLXND1 signaling axis as a potentially relevant target for metastatic inhibition in SHH MB.

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YB-1 Post-Transcriptionally Regulates PLXND1 to Mediate Metastasis in Sonic Hedgehog Medulloblastoma

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Introduction:

Medulloblastoma (MB) is one of the most common malignant brain cancers in children, accounting for nearly 10% of all pediatric brain tumors[1–3]. These tumors, which initially form from the cerebellum, are widely thought to arise from the misregulation of genes and pathways involved in postnatal cerebellar development[4]. MB is heterogeneously classified into four subgroups based on differing molecular and histologic characteristics: Wingless (WNT), Sonic Hedgehog (SHH), Group 3, and Group 4[5]. The SHH subgroup, which accounts for 30% of MB cases, is characterized by tumor development via perturbations in the Sonic Hedgehog (SHH) signaling pathway, a major developmental regulator of cell differentiation and proliferation[4,6]. During postnatal cerebellar development, secretion of SHH morphogenic factor by Purkinje cells sustains the proliferation of select early neuronal progenitor cells[7–9]. These progenitors, also known as cerebellar granule neural precursors (CGNP), are the putative cells-of-origin for SHH MB[10,11]. Under normal developmental conditions, the maturation of CGNPs results in the formation of the external granular layer of the cerebellar cortex. However, the misregulation of SHH signaling in these cells can lead to aberrant CGNP development and, subsequently, tumor formation[12].

The current treatment regimen for MB typically includes surgical resection followed by a combination of craniospinal irradiation and adjuvant chemotherapies[13–15]. With this standard of treatment, the 5-year event-free survival rate is ~60-70%[16,17]. However, these intensive treatments pose a high risk of long-term detriments for pediatric patients and can greatly reduce the quality of life[18,19]. Furthermore, tumor recurrence is almost universally fatal[20]. A significant portion of MB mortality results from metastatic dissemination of the primary tumor via leptomeningeal spread[21]. The two main anatomical routes for metastasis in MB include (1) passive diffusion through the cerebrospinal fluid followed by distal leptomeningeal re-implantation and (2) hematogenous extra-neural circulation followed by re-entry into the leptomeningeal space[22]. Molecular analysis studies have suggested roles for RAS/MAPK, PI3K/AKT, NOTCH, and HGF/cMET signaling in MB metastasis[23–26]. However, because the majority of research on MB therapeutic strategies thus far has focused on targeting of the primary tumor, the molecular mechanisms driving MB metastasis are not well understood. Therefore, there is a need to identify and elucidate molecular drivers of MB metastatic progression.

A potential target that our group has previously identified as a driver of MB oncogenesis is Y-box-binding protein 1 (YB-1), a DNA- and RNA-binding protein implicated in many cancer-associated cellular processes including cell proliferation, cell cycle control, genomic stabilization, angiogenesis, metabolic regulation, and metastatic progression[27]. In the past, we have reported YB-1 elevation across all subgroups of MB. Furthermore, we have shown it to be a downstream target of SHH signaling and demonstrated its induction as a requirement for sustaining CGNP and medulloblastoma cell (MBC) proliferation[28]. Studies performed in bladder, breast, carcinoma, and prostate cancer models have shown a reduction of invasive properties

upon silencing YB-1 expression, and the overexpression of YB-1 has been reported to promote metastasis[29–31]. The roles of YB-1 in regulating epithelial-mesenchymal transition (EMT) and maintaining stemness in other models suggest possible mechanisms by which it promotes metastatic progression[32]. However, the mediation of metastasis by YB-1 has yet to be studied in a MB model.

Due to its ability to bind RNA, YB-1 is involved in various post-transcriptional regulatory processes such as pre-mRNA splicing, mRNA processing and stabilization, mRNP packaging, and translational turnover[33]. The dysregulation of RNA-binding proteins (RBP) such as YB-1 may disrupt post-transcriptional control over gene expression, leading to the altered expression and activity observed in many different cancer phenotypes[34]. Given the significance of YB-1 in MB development, we proposed the dysregulation of YB-1 followed by altered post-transcriptional regulation of oncogenic transcripts as a possible pathway for MB formation. Recent preliminary RNA-binding data from our group identified various novel targets of YB-1 post-transcriptional regulation. One of the targets we identified was Plexin D1 (PLXND1), a transmembrane receptor protein that is classically known for guiding developmental programs during axon growth and vascularization[35]. PLXND1 signaling via activation by its canonical ligand Semaphorin 3E (SEMA3E) has been shown to drive invasion and metastatic progression in colon cancer, and PLXND1 knockdown has been reported to reduce metastasis in xenograft models[36]. Furthermore, SEMA3E/PLXND1-mediated EMT has been shown in an ovarian endometrioid cancer model[37]. Although PLXND1 has yet to be studied in the context of MB, its role during early neuronal development and its involvement in promoting metastasis may prove it to be a potential target of interest for SHH MB.

In the present study, we sought to explore the possibility of YB-1/PLXND1-mediated metastasis in SHH MB. We hypothesized that dysregulation of YB-1 leads to aberrant post-transcriptional modulation of PLXND1, thereby facilitating metastatic progression. To demonstrate this, we investigated (1) the effect of YB-1 modulation on PLXND1 expression and (2) the functional significance of PLXND1 expression in metastatic progression (Fig. 1). We elucidated a YB-1/PLXND1 modulatory association by measuring changes in PLXND1 mRNA and protein expression upon silencing YB-1. Following this, we explored PLXND1 expression in an SHH MB model and tested the functional consequences of knocking down PLXND1 expression on cell migration, invasion patterns, and epithelial-mesenchymal transition (EMT) regulation. Our findings suggest that YB-1 positively modulates PLXND1 at the post-transcriptional level to promote tumor migration, invasion, and development in SHH MB.

Materials and Methods:

Animal Studies

NeuroD2-SmoA1 mice were obtained from Jackson Research Laboratories[38,39]. Breeding, maintenance, and tumor harvest were carried out in compliance with guidelines from the Emory University Institutional Animal Care and Use Committee.

Primary MB Cell Culture

Medulloblastoma cells (MBC) were isolated from NeuroD2-SmoA1 mouse tumors. Cells were seeded on Matrigel (Corning) coated plates with Neurobasal medium containing penicillin and streptomycin, 1 mmol/L sodium pyruvate, 1x B27 supplement, and 2 mmol/L L-glutamine. Primary MBCs were cultured for 4 hours with 10% fetal bovine serum (FBS) prior to a media change to 0% FBS, at which point lentivirus was added with an incubation time of 48 hours prior to experiment initiation.

Immunofluorescence

Cells were fixed for 10 minutes in fresh 4% Paraformaldehyde prior to 3x wash with PBS. Cells were permeabilized with 0.3% Triton X-100 and blocked with 5% Bovine Serum Albumin and 3% Normal Goat Serum. Primary antibody was added overnight at 4ºC and secondary was added for 2 hours at room temperature.

Cell Lines

Mouse MB cell line PZp53Med (PZP) was a generous gift from Dr. Matthew Scott (Stanford)[40]. Human MB cell line ONS-76 was obtained from American Type Culture

Collection. PZP and ONS-76 cells were cultured in DMEM/F12 with 10% FBS. Generation of stable knockdown cell lines was performed through lentiviral delivery of short hairpin constructs followed by puromycin selection. ShSCR and ShSBN were used as negative control constructs for knockdown cell line stabilization. YBX1 knockdown was performed using TRCN0000315309 (referred to as ShYB1-09) in human cell lines and TRCN0000333885 (referred to as ShYB1-85) in mouse cell lines. PLXND1 knockdown was performed using TRCN0000061550 (referred to as ShPLXND1-50) and TRCN0000061552 (referred to as ShPLXND1-52) in human cell lines and TRCN0000078775 (referred to as ShPLXND1-75) and TRCN0000078777 (referred to as ShPLXND1-77) in mouse cell lines (Millipore Sigma).

Western Blotting

Cells were homogenized and lysed in RIPA lysis buffer with protease inhibitors cocktail and phosphatase inhibitors (Thermo Scientific). A total of 15µg of each sample was denatured and separated on 6-14% SDS-PAGE gels, then transferred to immobilon-P PVDF membranes (Millipore). Membranes were blocked with 5% milk in tris-buffered saline with 0.1% Tween-20 (TBST). The following primary antibodies were used: YB1 (D299; CST), PLXND1 (NBP1-33634; NOV), β-Tubulin (sc-166729; SCBT), N-Cadherin (D4R1H; CST), β-Catenin (D10A8; CST), Slug (C19G7; CST), Snail (C15D3; CST), Claudin-1 (D5H1D; CST). Anti-Rabbit IgG and Anti-Mouse IgG horseradish peroxidase-conjugated secondary antibodies were used (Jackson ImmunoResearch). Blots were developed with Pierce ECL Western Blotting Substrate and SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific). For quantification purposes, chemiluminescent signals were normalized to β-Tubulin. Densitometry was performed using ImageJ software. Acronyms: CST - Cell Signaling Technology, NOV - Novus Biologicals, SCBT - Santa Cruz Biotechnology.

RT-qPCR

RNA was isolated from tissue or cell samples using 1mL TRIzol followed by RNA purification according to the manufacturer's protocol (Thermo Scientific). Reverse transcription was performed on 500ng-2µg RNA per 20µL RT reaction according to the manufacturer's instructions using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-qPCR was performed using either BioRad 2X SYBR master mix or Applied Biosystems 2X SYBR master mix. 25-200ng of cDNA was utilized per qPCR reaction and primers were validated based on non-template controls and melt-curve analyses. For quantification purposes, C_t values were normalized to β-Actin. Relative expression was calculated using the $\Delta\Delta C_t$ method. The following primer pairs were used:

Species	Target	Forward Primer	Reverse Primer
Human	β-Actin	5’ AGA GCT ACG AGC TGC CTG AC 3’	5’ AGC ACT GTG TTG GCG TAC AG 3’
	PLXND1	(BioRad qHsaCID0007287)	(BioRad qHsaCID0007287)
	YBX1	5’ CCC CAG GAA GTA CCT TCG C 3’	5’ GTT CCT TCC TCG GAT GGT CAG 3’
Mouse	β-Actin	5’ CCA GTT GGT AAC AAT GCC ATG 3’	5’ GGC TGT ATT CCC CTC CAT CG 3’
	PLXND1	5’ CGC AAC CGT AGC CTA GAA GAC 3’	5’ GGT TAA GGT CGA AGG TGA AGA G 3’
	YBX1	5’ CAG ACC GTA ACC ATT ATA GAC GC 3’	5’ ATC CCT CGT TCT TTT CCC CAC 3’

Cell Migration and Invasion Assay

Cell migration was measured using *in vitro* wound-healing assays. Cells were seeded with 2.5% FBS and grown to ~70-80% confluence before scratching with a sterile P1000 pipette tip. Wound closure was photographed at 0, 16, 24, and 48 hours post-scratch. Wound closure area was measured using ImageJ software. Cell invasion was measured using 3D colony formation assays in Matrigel (Corning). Cells were embedded in 50% Matrigel and incubated with media replacement every 3 to 4 days. Colony formation was photographed at 14 days post-plating.

Statistical Analysis

Statistical comparisons were performed using GraphPad Prism. One-way analysis of variance (ANOVA) was used for western blotting densitometry, and two-way ANOVA was used for wound-healing analyses. Paired t-tests were used for RT-qPCR analyses. All error bars represent data range. A significance threshold of $p < 0.05$ was used in all analyses.

Results:

YB-1 post-transcriptionally modulates PLXND1 expression

The identification of novel targets for YB-1 post-transcriptional regulation was accomplished using RNA immunoprecipitation techniques followed by high-throughput RNA sequencing. This analysis demonstrated PLXND1 mRNA to be one of the most differentially enriched transcripts in the entire YB-1 RNA-binding profile (Fig. 2a). We followed up this finding by performing western blot analysis to measure changes in PLXND1 protein expression upon silencing YB-1. Using human-derived SHH MB cell line ONS-76 and mouse-derived SHH MB cell line PZp53Med (PZP), we demonstrated a significant decrease of PLXND1 protein expression in YB-1 knockdown (KD) cells compared to negative control cells (Fig. 2b and 2c). Finally, we paired this immunoblotting data with matched mRNA expression analysis using quantitative reverse transcription PCR (RT-qPCR). In both cell lines, we found no significant changes in PLXND1 RNA expression in YB-1 KD cells relative to the negative control cells (Fig. 2d). These data suggest a positive modulatory effect of YB-1 on PLXND1. Furthermore, they support the idea that this regulation is limited to the post-transcriptional level.

PLXND1 is expressed in SHH MB

PLXND1 expression in SHH MB was measured through microarray gene expression analysis using a profiling array consisting of 763 primary MB patient samples (GEO accession GSE85217). Analysis showed higher levels of PLXND1 gene expression in SHH tumors compared to WNT-activated, Group 3, and Group 4 tumors (Fig. 3a). We corroborated this finding by performing immunofluorescence (IF) staining in primary SHH medulloblastoma cells (MBCs) derived from NeuroD2-SmoA1 transgenic mice (Fig. 3b). Finally, we compared

PLXND1 protein expression between primary MB tumor tissue and matched non-tumor cerebellar tissue (Fig. 3c and 3d). We observed significant enrichment of PLXND1 in tumor tissue relative to cerebellar tissue. These data show PLXND1 to be robustly expressed in a SHH MB model.

Silencing PLXND1 reduces cell migration

To demonstrate the functional role of PLXND1 in cell migration in a SHH MB model, we performed *in vitro* wound-healing assays using PLXND1 stable knockdown cells in both ONS-76 (ShPLXND1-50 & ShPLXND1-52) and PZP (ShPLXND1-75 & ShPLXND1-77) cell lines (Fig. 4a). Within timepoints, we observed markedly reduced wound closure in PLXND1 KD cells compared to negative control cells for both cell lines (Fig. 4b). In ONS-76 cells, knockdown of PLXND1 in the ShPLXND1-50 and ShPLXND1-52 stable cell lines reduced wound closure by an average of 37.4% and 36.6% respectively by the 24-hour timepoint. In PZP cells, wound closure was reduced by 27.9% and 27.8% by the 16-hour timepoint respectively for ShPLXND1-75 and ShPLXND1-77 cells. These findings strongly suggest a role of PLXND1 in promoting cell migration in the context of SHH MB.

Silencing PLXND1 reduces cell invasion

To demonstrate the functional role of PLXND1 in tumor cell invasion in a SHH MB model, we performed 3D colony formation assays and assessed morphological differences between ONS-76 negative control and PLXND1 KD cells (Fig. 5a). Upon imaging 14 days post-plating, we observed considerably reduced branching morphology in PLXND1 KD cells relative to negative control cells. From the total cell count, we calculated 19.9% invasion in the knockdown cells versus 61.2% invasion in the control cells (Fig. 5b). This data supports the role

of PLXND1 in mediating metastatic progression in a SHH MB model by promoting aggressive tumor cell invasion.

PLXND1 drives select EMT marker expression

To demonstrate how PLXND1 may be driving tumor development, we explored the effect of PLXND1 knockdown on the regulation of epithelial-mesenchymal transition (EMT), a set of developmental mechanisms activated during embryogenesis and organ development[41]. The deregulation of these developmental mechanisms is seen in many different tumor pathologies as it gives rise to enhanced migratory and invasive capacities[42]. Upon silencing PLXND1 in ONS-76 and PZP cell lines, we measured changes in protein expression for various biomarkers of EMT (Fig. 6a & 6b). In both cell lines, we observed a reduction in mesenchymal markers N-cadherin and β-catenin, two protein regulators of cell-to-cell adhesion[43,44]. In ONS-76, we also observed decreases in EMT-promoting transcription factors Slug and Snail as well as an increase in Claudin-1, an epithelial tight junction complex protein[45,46]. While these findings were consistent with our hypothesis of PLXND1 driving a pro-migratory phenotype, changes in Slug, Snail, and Claudin-1 were not recapitulated from cell line to cell line. For PLXND1 KD cells in PZP, we observed increases in Slug and Snail as well as little to no change in Claudin-1. Together, these findings suggest that PLXND1 drives EMT marker expression, and the extent of this regulation may be cell line-dependent.

Discussion:

In this study, we extend previous findings of YB-1 as a driver of tumor development for MB. Our results demonstrate the role of YB-1 as an additional promoter of metastatic progression, a finding that has been reported elsewhere in other cancer models but only now validated in SHH MB[29–31]. Through the elucidation of a positive modulatory association between YB-1 and PLXND1, we establish a regulatory mechanism by which YB-1 regulates oncogenic gene expression. We show that silencing YB-1 elicits significant changes in PLXND1 protein expression with non-significant changes in PLXND1 RNA expression. This pinpoints YB-1 regulation on PLXND1 to the post-transcriptional level, emphasizing the significance of YB-1 RNA-binding functions in driving oncogenic phenotypes.

Our findings identify the various functional roles that PLXND1 plays in mediating metastasis in a SHH MB model. We show PLXND1 to be robustly expressed in SHH MB and highly enriched in MB tumor tissue. We recapitulate previously documented phenotypic effects of PLXND1 on metastatic progression by finding significant reductions in migratory and invasive capacity to be functional consequences of silencing PLXND1 activity[35,36]. Additionally, we demonstrate changes in EMT biomarker expression that are consistent with previous reports of PLXND1 facilitation of mesenchymal marker acquisition and epithelial marker loss in an ovarian cancer model[37]. However, changes in EMT marker expression for select biomarkers were ambiguous as they were not replicated between ONS-76 and PZP cell lines. While this ambiguity may be the result of exploring EMT regulation in two different systems–one in a human, p53 wildtype cell line and another in a mouse, PTCH receptor-deficient, p53 mutant cell line–, it is noteworthy to point out that EMT analysis was performed using whole-cell lysates. In the case of markers that serve as EMT transcription factors such as Slug and Snail, it may be

more informative to instead study changes in nuclear translocation of these markers upon PLXND1 KD[47].

While our findings have established a link between YB-1 and PLXND1, further studies are warranted to better understand the detailed mechanism by which YB-1 induces PLXND1 expression. A potential future direction to further refine a regulatory mechanism could be to perform a polysome extraction and profiling experiment to explore the effect of YB-1 on the translational status of PLXND1[48]. One major limitation of our current study is the lack of *in vivo* validation for our functional experiments. A beneficial future study might involve the generation of xenograft mouse models through a knockdown approach of infected MBC implantation followed by observation of metastatic lesions and spread. Additionally, our studies were confined to a single subgroup model for MB. Given previous reports of YB-1 elevation across all 4 subgroups of MB, it might be interesting to extend our studies to other MB subgroups[28]. This may be especially relevant for Group 3 and Group 4 MB, both of which have higher rates of metastatic incidence and recurrence than SHH-group tumors[49]. Finally, the exploration of PLXND1 binding partners with known therapeutic options may prove to be critical for the targetability of the YB-1/PLXND1 signaling axis for metastatic inhibition[50]. While there are currently no viable options for targeting PLXND1 directly, identifying PLXND1 binding partners and elucidating differentially activated downstream mechanisms may offer alternative and more feasible therapeutic strategy[51]. Taken together, our findings point to YB-1/PLXND1-mediated metastasis as a novel and therapeutically relevant area of study for metastatic inhibition in SHH MB.

Figures:

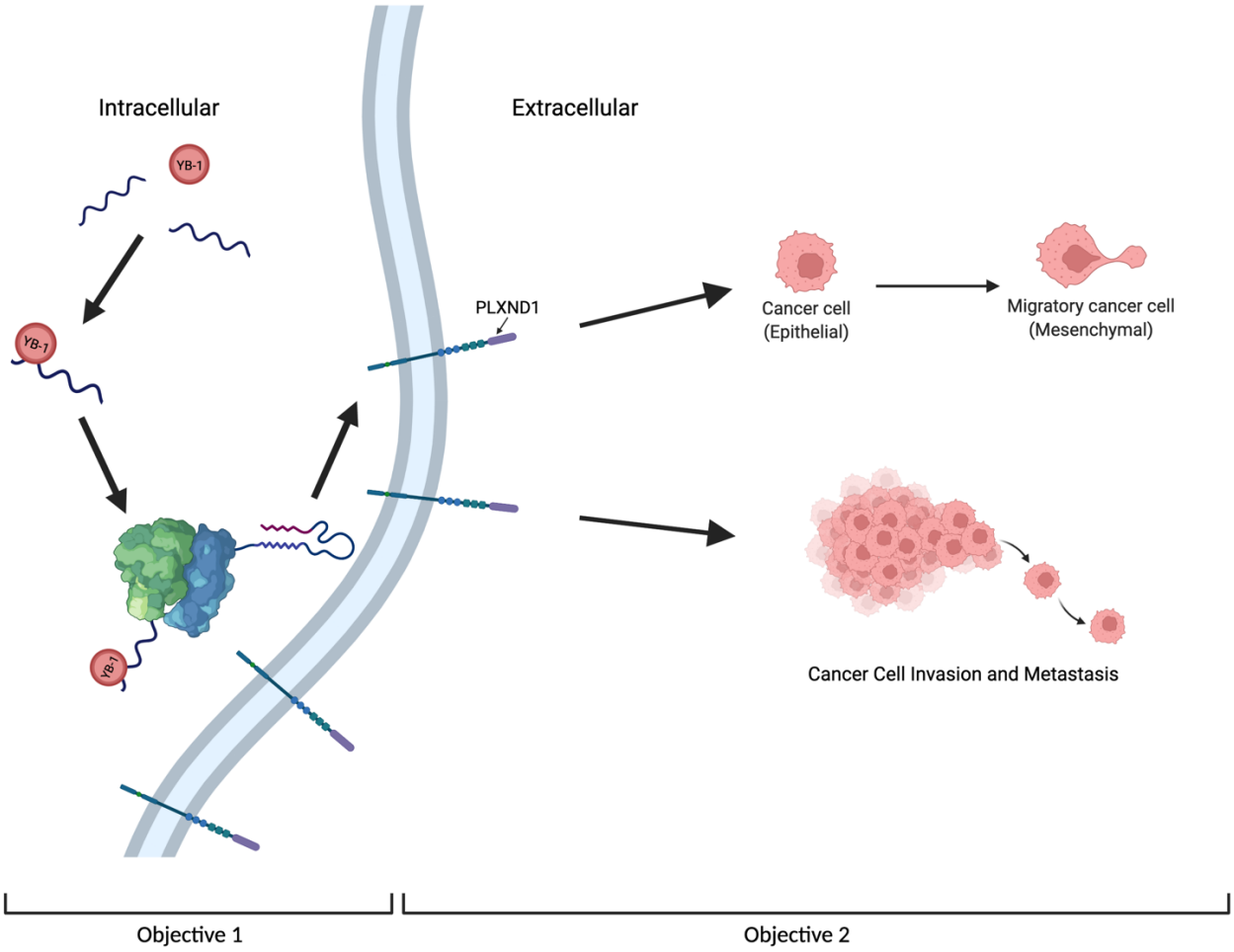

Figure 1. Schematic depiction of YB-1/PLXND1-mediated metastasis. We hypothesized that YB-1 post-transcriptionally regulates PLXND1 to mediate metastatic progression in SHH MB. For Objective 1, we elucidated the effects of YB-1 modulation on PLXND1 expression. For Objective 2, we demonstrated the functional roles of PLXND1 in promoting metastatic phenotypes.

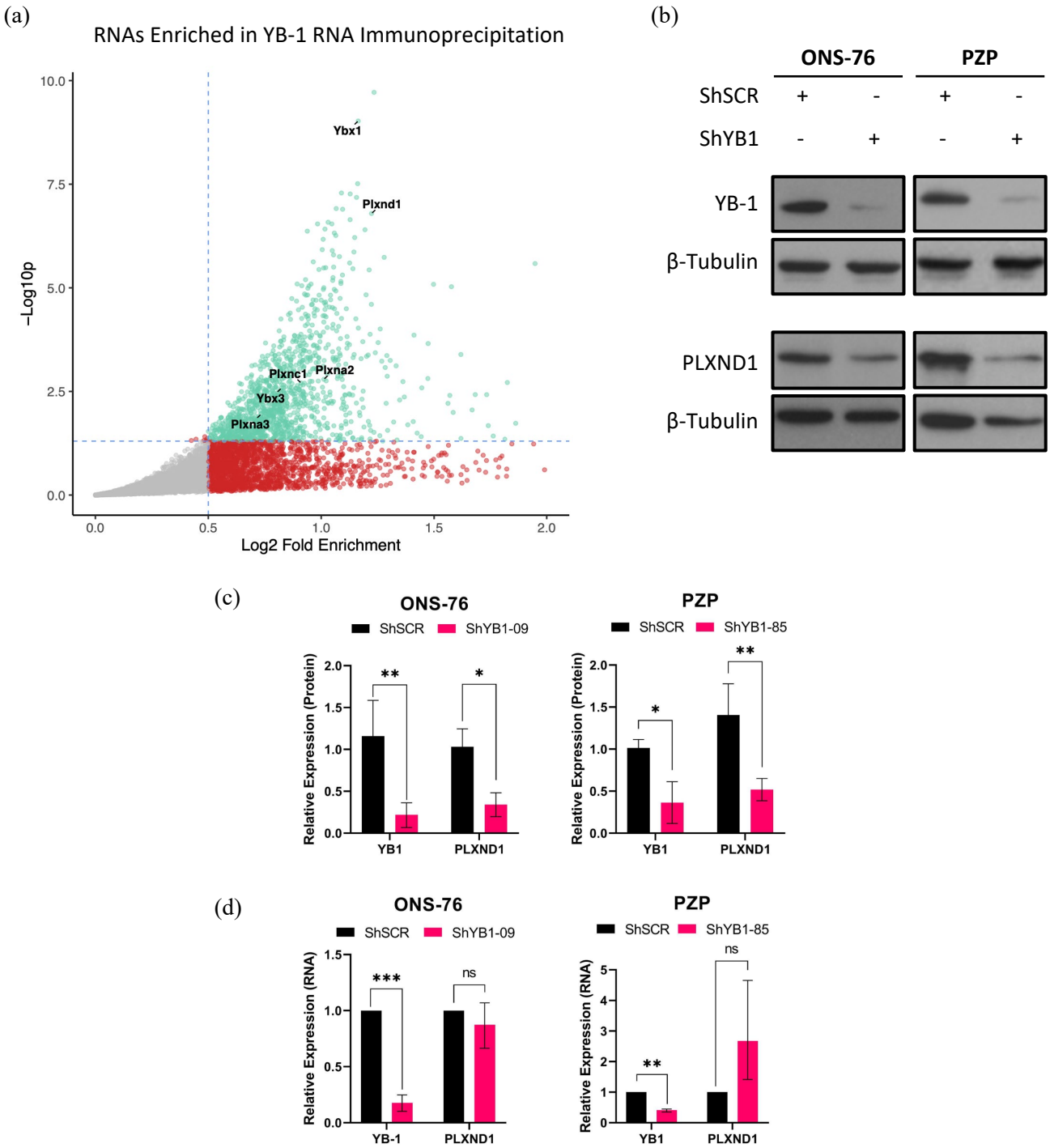

Figure 2. YB-1 positively modulates PLXND1 expression at the post-transcriptional level. (A) Volcano plot of RNA sequencing counts from YB-1 RNA-immunoprecipitation (courtesy of Leon McSwain). YBX1 and YBX3 were used as positive control targets for sequencing analysis. **(B)** Western blot results demonstrating reduced PLXND1 protein expression upon YB-1 knockdown in ONS-76 and PZP cell lines. ShSCR was used as a negative control for knockdown generation. **(C)** Western blot densitometry analysis of YB-1 knockdown in ONS-76 (YB-1, p=0.0034; PLXND1, p=0.0150) and PZP (YB-1, p=0.0308; PLXND1, p=0.0076). Data are normalized to β-Tubulin, mean ± range (n=3). One-way ANOVA (post hoc, Šidák's multiple comparisons test), *p≤0.05, **p≤0.01. **(D)** Quantitative reverse transcription PCR (RT-qPCR) analysis demonstrating non-significant changes in PLXND1 RNA expression upon YB-1 knockdown in ONS-76 (YB-1, p=0.0026; PLXND1, p=0.3967) and PZP (YB-1, p=0.0028; PLXND1, p=0.2364). Relative expression was calculated as $2^{-\Delta\Delta Ct}$ fold change, mean ± range (n=3). Paired t-test, *p≤0.05, **p≤0.01, ***p≤0.001.

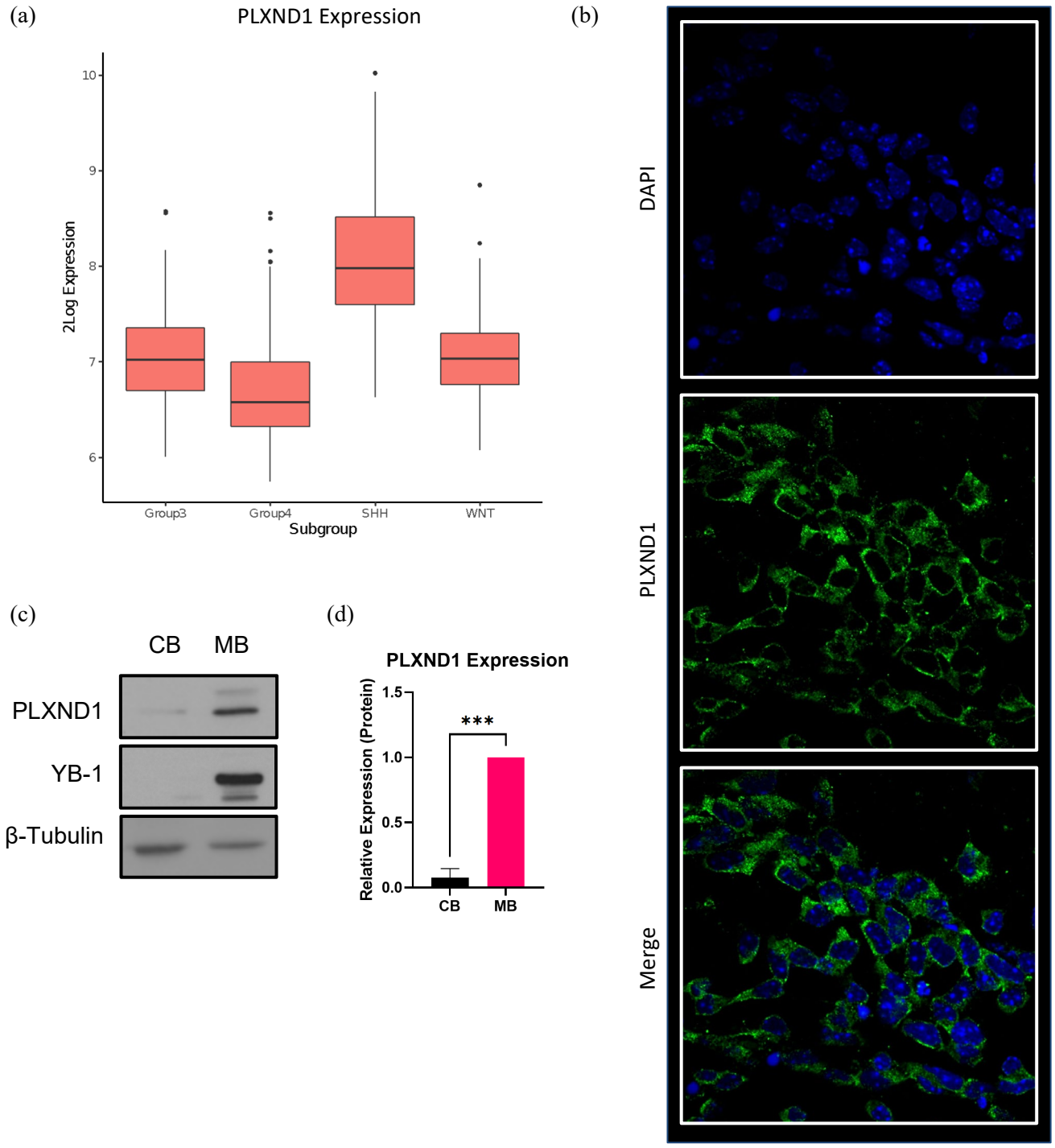

Figure 3. PLXND1 is expressed in SHH MB. (A) Gene expression analysis of PLXND1 in WNT, SHH, Group 3, and Group 4 MB (courtesy of Leon McSwain). Expression data was derived from a previously published microarray profiling set consisting of 763 patient-derived primary MB samples (GEO accession GSE85217). **(B)** Immunofluorescence staining of primary MBCs derived from NeuroD2-SmoA1 transgenic tumor mice. **(C)** Western blot results from MB tumor and non-tumor cerebellar tissue from NeuroD2-SmoA1 transgenic tumor mice. **(D)** Western blot densitometry analysis demonstrating enrichment of PLXND1 in tumor tissue compared to non-tumor tissue ($p=0.0001$). Data are normalized to β-Tubulin, mean ± range ($n=4$). Paired t-test, *$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$.

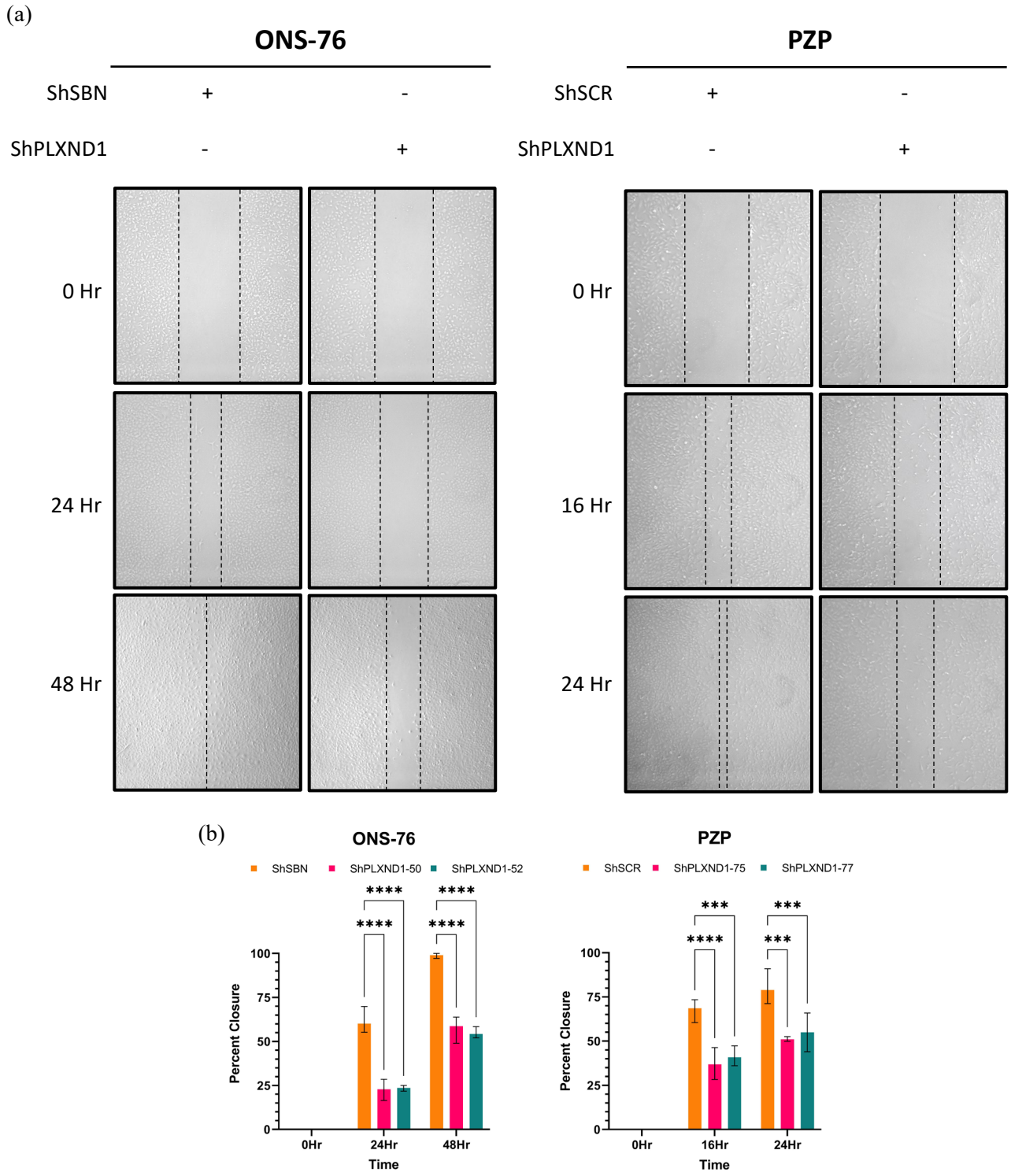

Figure 4. Silencing PLXND1 reduces tumor cell migration. (A) *In vitro* wound healing assays for PLXND1 knockdown in ONS-76 (showing ShPLXND1-50) and PZP (showing ShPLXND1-77) stable cell lines. ShSBN and ShSCR were used as a negative control for knockdown generation. Wounds were imaged at 0, 16, 24, and 48 hours post-scratch. **(B)** Wound closure analysis demonstrating reduced closure in ShPLXND1-50 (24-Hr, $p<0.0001$; 48-Hr, $p<0.0001$), ShPLXND1-52 (24-Hr, $p<0.0001$; 48-Hr, $p<0.0001$), ShPLXND1-75 (16-Hr, $p<0.0001$; 24-Hr, $p=0.0001$), and ShPLXND1-77 (16-Hr, $p=0.0001$; 24-Hr, $p=0.0007$) knockdown cell lines. Percent closure was calculated based on the following formula: 100-100*(Wound Area / 0-Hr Wound Area), mean ± range (n=3). Two-way ANOVA (post hoc, Šidák's multiple comparisons test), *$p\leq0.05$, **$p\leq0.01$, ***$p\leq0.001$, ****$p\leq0.0001$.

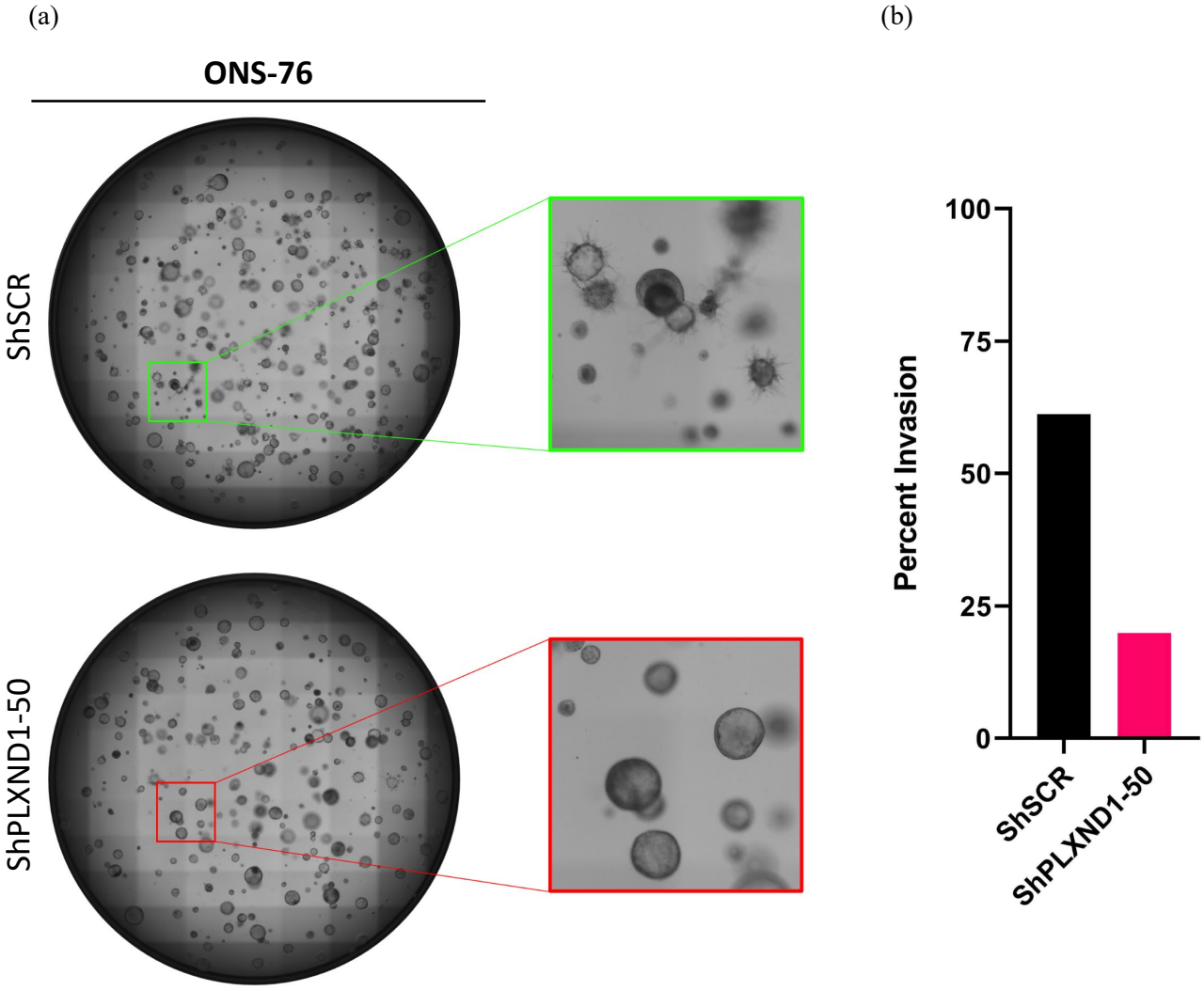

Figure 5. Silencing PLXND1 reduces tumor cell invasion. (A) 3D colony formation assays for PLXND1 knockdown in ONS-76 cell line. ShSCR was used as a negative control for knockdown generation. Colony formation was imaged 14 days post-plating. **(B)** Colony morphology analysis demonstrating reduced tumor cell invasion in ShPLXND1-50 knockdown cell line (19.9%) compared to ShSCR negative control cell line (61.2%). Percent invasion was calculated based on the following formula: 100*(Branched Colony Count / Total Colony Count), (n=1).

(a) (b)

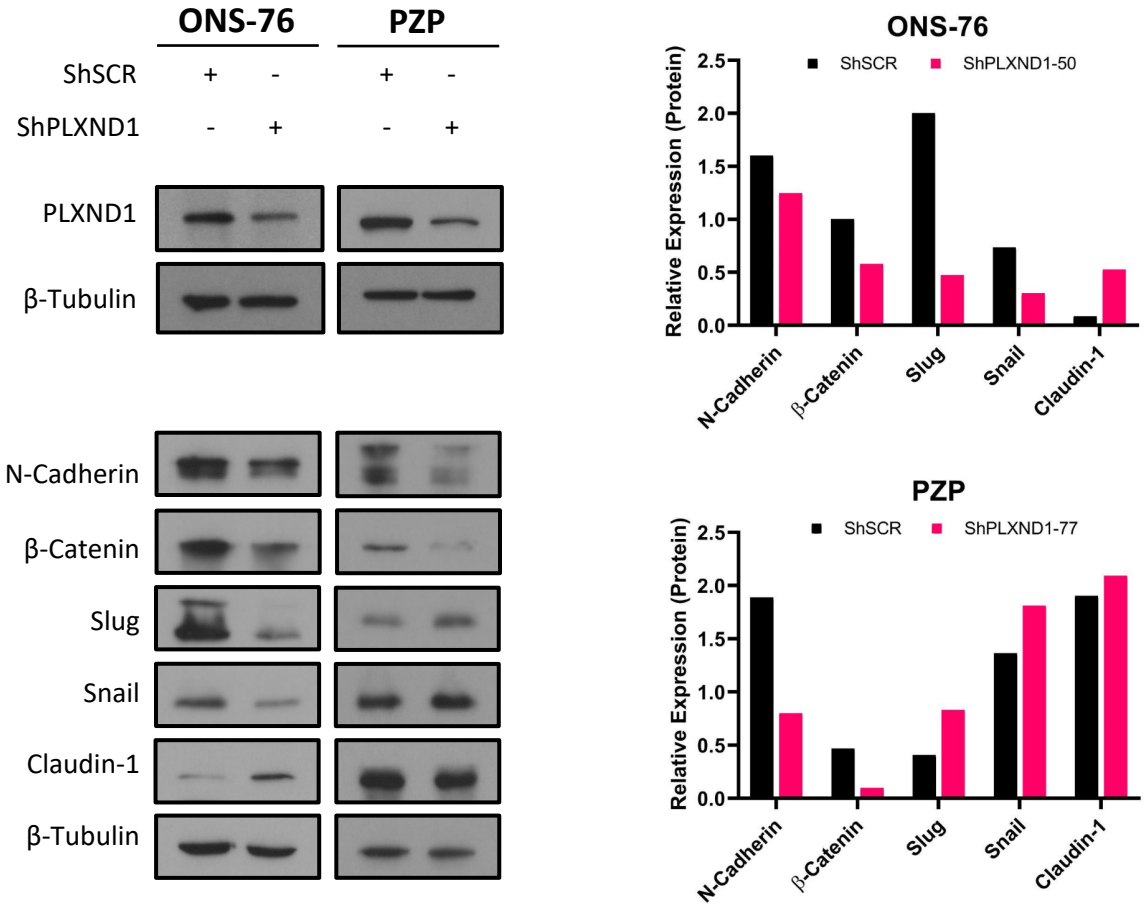

Figure 6. PLXND1 drives select EMT marker expression. (A) Western blot results demonstrating cell line-dependent changes in select epithelial-mesenchymal transition marker expression upon PLXND1 knockdown. ShSCR was used as a negative control for knockdown generation. Mesenchymal markers N-Cadherin, β-Catenin, Slug, and Snail and epithelial marker Claudin-1 were probed in ONS-76 and PZP cell lines. **(B)** Western blot densitometry analysis of EMT biomarkers in ShPLXND1-50 and ShPLXND1-77 knockdown cell lines. Data are normalized to β-Tubulin, (n=1).

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