

The proto-oncogene Bcl3 induces immune checkpoint PD-L1 expression, mediating proliferation of ovarian cancer cells

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The proto-oncogene Bcl3 induces survival and proliferation in cancer cells; however, its function and regulation in ovarian cancer (OC) remain unknown. Here, we show that Bcl3 expression is increased in human OC tissues. Surprisingly, however, we found that in addition to promoting survival, proliferation, and migration of OC cells, Bcl3 promotes both constitutive and interferon- γ (IFN)-induced expression of the immune checkpoint molecule PD-L1. The Bcl3 expression in OC cells is further increased by IFN, resulting in increased PD-L1 transcription. The mechanism consists of an IFN-induced, Bcl3- and p300-dependent PD-L1 promoter occupancy by Lys-314/315 acetylated p65 NF- κ B. Blocking PD-L1 by neutralizing antibody reduces proliferation of OC cells overexpressing Bcl3, suggesting that the pro-proliferative effect of Bcl3 in OC cells is partly mediated by PD-L1. Together, this work identifies PD-L1 as a novel target of Bcl3, and links Bcl3 to IFN γ signaling and PD-L1-mediated immune escape.

The proto-oncogene Bcl3 is a member of I κ B family that was first identified in patients with chronic lymphocytic leukemia (1, 2). However, unlike other I κ B proteins in cancer cells, Bcl3 is a predominantly nuclear protein, which contains a transactivation domain, and can be recruited to NF- κ B-responsive promoters, resulting in transcriptional activation or repression, depending on the subunit composition of NF- κ B complexes, and other transcription factors and regulators present in the transcription complexes (3–9). High expression of Bcl3 has been reported in a number of hematological malignancies (10–16), as well as in several solid tumors, including breast cancer, nasopharyngeal carcinoma, and colorectal and cervical cancer (17–23). Consistent with its oncogenic function, Bcl3 can transform cells and induce their proliferation and tumor growth (24). Recent studies have shown that miR-125b, which targets Bcl3, is down-regulated in ovarian cancer (OC)² tissues (25, 26), suggesting an increased Bcl3 expres-

sion in ovarian cancer. However, the Bcl3 expression in OC has not been investigated, and its function in OC cells remains unknown.

Epithelial ovarian cancer (EOC) is the most common gynecological cancer in women, with poor survival and high mortality rates. As many other types of cancer, EOC is characterized by an increased activity of the transcription factor NF- κ B (27–29), which promotes expression of anti-apoptotic and pro-angiogenic genes. However, recent studies have shown that in addition to inducing expression of anti-apoptotic and pro-inflammatory genes, NF- κ B induces transcription of the immune checkpoint molecule, programmed death ligand 1 (PD-L1; B7-H1, CD274) (30–35). PD-L1 expression on tumor cells is induced by interferon- γ (IFN). By binding to programmed cell death-1 (PD-1) expressed on cytotoxic T cells, PD-L1 then induces T cell apoptosis and tolerance, thus inhibiting the anti-tumor immunity. However, tumor PD-L1 has also tumor-intrinsic effects that include increased cancer cell survival and proliferation, regulation of tumor glucose utilization, and inhibition of autophagy (36–38). PD-L1 is expressed on the surface of OC cells, and its increased expression correlates with poor prognosis in OC patients (38–41); however, the mechanisms that regulate the PD-L1 expression in OC cells are not known.

Here, we show that Bcl3 expression is increased in human EOC tissues, and Bcl3 overexpression promotes survival, proliferation, and migration of OC cells. Remarkably, however, our results show that in addition to promoting survival and proliferation, Bcl3 induces both constitutive and IFN-induced PD-L1 expression in OC cells. The mechanism consists of Bcl3- and p300-mediated recruitment of Lys-314/315 acetylated p65 NF- κ B to the PD-L1 promoter in IFN-treated cells. In OC cells overexpressing Bcl3, neutralization of the induced PD-L1 decreases cell proliferation, indicating that the pro-proliferative effect of Bcl3 is partly mediated by PD-L1. These data identify PD-L1 as a novel target of Bcl3, and suggest that in addition to promoting cell proliferation, Bcl3 regulates immune escape in cancer cells.

Results

Bcl3 expression is increased in human OC tissues, and promotes survival, proliferation, and migration of OC cells

Expression of miR-125b, which targets Bcl3, is down-regulated in OC tissues (25, 26). However, it is not known whether Bcl3 gene expression is increased in ovarian cancer. To evaluate

transferase; CBP, cAMP-response element-binding protein.

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² The abbreviations used are: OC, ovarian cancer; EOC, epithelial ovarian cancer; WCE, whole cell extract; PD-L1, programmed death ligand 1; IFN, interferon- γ ; IL, interleukin; TNF, tumor necrosis factor; TSS, transcription start site; Lys-314/315 ac-p65, Lys-314/315 acetylated p65; HAT, histone acetyl-

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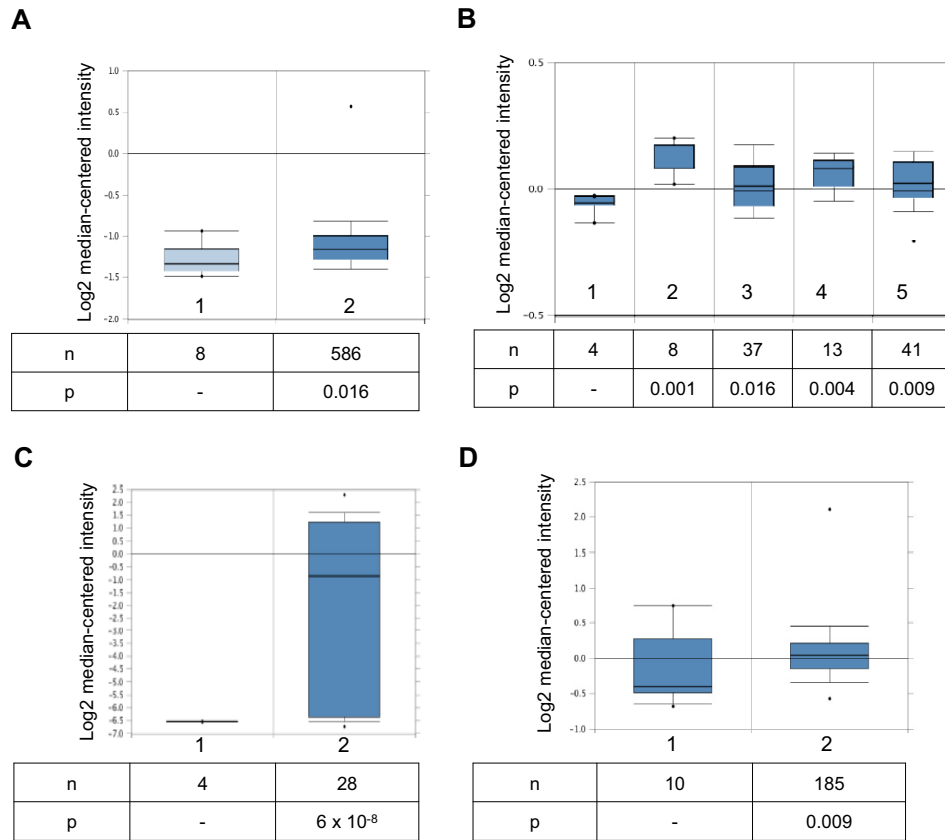


Figure 1. Bcl3 gene expression is increased in human OC tissues. A, expression of *Bcl3* mRNA in normal ovary tissues (column 1) and ovarian serous cystadenocarcinoma (column 2). Data were retrieved from The Cancer Genome Atlas (TCGA) Oncomine data set. B, *Bcl3* mRNA levels in normal ovary tissues (column 1), ovarian clear cell adenocarcinoma (column 2), ovarian endometrioid adenocarcinoma (column 3), ovarian mucinous adenocarcinoma (column 4), and ovarian serous adenocarcinoma (column 5). Data were retrieved from the Hendrix Ovarian Statistics (42) dataset. C, *Bcl3* expression in normal ovary tissues (column 1) and ovarian serous surface papillary carcinoma tissues (column 2) retrieved from the Welsh dataset (43). D, *Bcl3* expression in normal ovary tissues (column 1) and in ovarian carcinoma (column 2) retrieved from the Bonome dataset (44).

the *Bcl3* expression in OC tissues, we analyzed *Bcl3* levels using the Oncomine database (<https://www.oncomine.org/resource/login.html>).³ Analysis of the TCGA dataset containing 586 ovarian serous cystadenocarcinoma, the most common type of EOC, revealed a significantly (fold-change = 1.131, $p = 0.016$) increased *Bcl3* expression compared with normal ovary tissues (Fig. 1A). In addition, analysis of Hendrix dataset (42) showed a significantly increased *Bcl3* expression in ovarian clear cell adenocarcinoma ($n = 8$, fold-change = 1.123, $p = 0.001$), ovarian endometrioid adenocarcinoma ($n = 37$, fold-change = 1.060, $p = 0.016$), ovarian mucinous adenocarcinoma ($n = 13$, fold-change = 1.095, $p = 0.004$), and ovarian serous adenocarcinoma ($n = 41$, fold-change = 1.565, $p = 0.009$), compared with normal ovary tissues (Fig. 1B). Interestingly, there was a dramatic increase in the *Bcl3* expression in ovarian serous surface papillary carcinoma ($n = 28$, fold-change = 23.955, $p = 6 \times 10^{-8}$) in the Welsh (43) dataset (Fig. 1C). In addition, *Bcl3* was increased in ovarian carcinoma in the Bonome ($n = 185$, fold-change = 1.361, $p = 0.009$; Fig. 1D) (44) dataset. Together, analysis of four different public datasets containing 26 control ovarian tissues and 898 OC samples has shown a statistically increased *Bcl3* gene expression in OC tissues ($p \leq 0.016$).

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To explore the functional significance of *Bcl3* in OC cells, we first examined the effect of *Bcl3* suppression on OC cell apoptosis, proliferation, and migration. Suppression of *Bcl3* by siRNA decreased *Bcl3* mRNA (Fig. 2A) and protein (Fig. 2, B and C) levels in SKOV3 and OVCAR3 cells by about 50% compared with control scramble siRNA. Of note, in whole cell extracts (WCE) of OC cells, *Bcl3* runs as a doublet of an approximate 50 kDa on SDS gels (Fig. 2B), consistent with previous reports demonstrating *Bcl3* phosphorylation (24, 45–47). Importantly, *Bcl3* suppression significantly increased apoptosis, evaluated by nucleosome release into the cytoplasm (Fig. 2D) (48) and by caspase-3 assay (Fig. 2E), and decreased proliferation of SKOV3 (Fig. 2F) and OVCAR3 (Fig. 2G) cells. Furthermore, *Bcl3* suppression by siRNA significantly reduced migration of OC cells (Fig. 3).

To validate the above data, we suppressed and overexpressed *Bcl3* in SKOV3 cells using CRISPR knockout and activation plasmids. Suppression of *Bcl3* by CRISPR/Cas9 reduced both *Bcl3* mRNA (Fig. 4A) and protein levels (Fig. 4, B and C). Importantly, *Bcl3* suppression significantly increased apoptosis (Fig. 4D) and decreased proliferation (Fig. 4E) in SKOV3 cells. Conversely, *Bcl3* overexpression decreased apoptosis (Fig. 4D) and increased cell proliferation (Fig. 4F). To confirm these data, we generated SKOV3 cells stably transfected with *Bcl3* shRNA. Compared with the control SKOV3 cell line transfected with

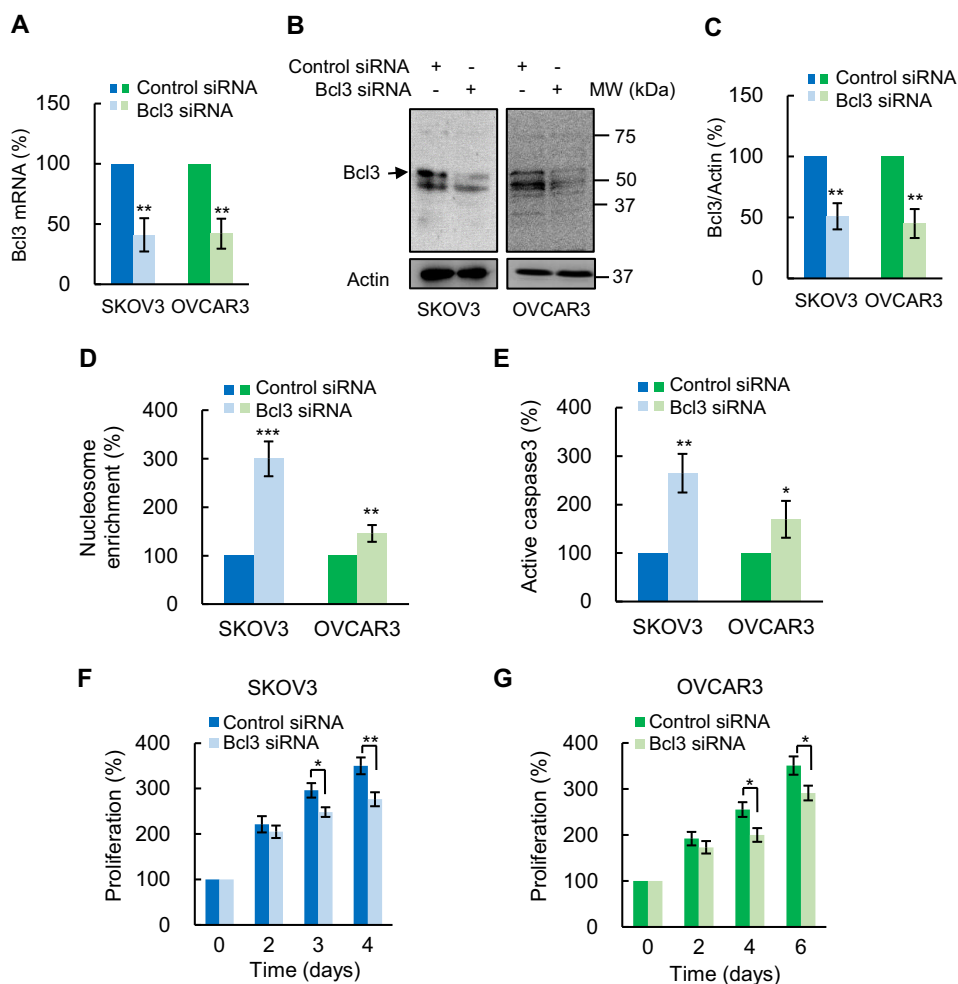


Figure 2. Bcl3 suppression induces apoptosis and reduces proliferation of OC cells. A, *Bcl3* mRNA analyzed by quantitative RT-PCR in SKOV3 and OVCAR3 cells transfected with control and Bcl3 siRNA ($n = 4$). B, Bcl3 Western blotting in WCE prepared from SKOV3 and OVCAR3 cells transfected with control and Bcl3 siRNA. C, densitometric evaluation of Bcl3 protein levels shown in B. The Bcl3 densities were normalized to actin. Apoptosis measured by cytoplasmic nucleosome enrichment assay (D), and caspase-3 activity (E) in SKOV3 and OVCAR3 cells transfected with control or Bcl3 siRNA. Cell proliferation was measured by CellTiter 96 One Solution cell proliferation assay in SKOV3 (F) and OVCAR3 (G) cells transfected with control or Bcl3 siRNA. The values represent the mean \pm S.E. of four experiments; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with cells transfected with the corresponding control siRNA.

empty expression vector, SKOV3 cells stably transfected with Bcl3 shRNA express significantly decreased Bcl3 mRNA (Fig. 4A) and protein (Fig. 4, B and C) levels. Importantly, these cells exhibit increased apoptosis (Fig. 4D) and reduced proliferation (Fig. 4G) compared with control SKOV3 cells. These data demonstrate that Bcl3 promotes OC cell survival, migration, and proliferation.

Bcl3 mediates constitutive PD-L1 expression in OC cells

Because Bcl3 regulates NF- κ B-dependent transcription, we analyzed expression of NF- κ B-dependent genes *cIAP1*, *BclxL*, *TGF β 1*, and *I κ B α* in SKOV3 and OVCAR3 cells transfected with Bcl3 siRNA. In addition, because PD-L1 promotes OC growth and cell proliferation (38, 49), and is regulated by NF- κ B (30–35), we wondered whether Bcl3 might regulate PD-L1 expression in OC cells. Remarkably, whereas Bcl3 suppression by siRNA did not have a substantial effect on *cIAP1*, *BclxL*, *TGF β 1*, and *I κ B α* mRNA levels, it significantly reduced PD-L1 expression in SKOV3 and OVCAR3 cells (Fig. 5A). In addition, Bcl3 suppression by siRNA signifi-

cantly decreased the intracellular PD-L1 protein levels in both cell types (Fig. 5, B and C).

To confirm these results, we analyzed PD-L1 expression in SKOV3 cells transfected with Bcl3 CRISPR knockout and activation plasmids. Although Bcl3 suppression by CRISPR knockout did not have a significant effect on *cIAP1*, *BclxL*, *TGF β 1*, and *I κ B α* mRNA levels, it significantly reduced PD-L1 mRNA (Fig. 5D). Conversely, Bcl3 overexpression increased PD-L1 mRNA in SKOV3 cells, but not expression of *cIAP1*, *BclxL*, *TGF β 1*, or *I κ B α* (Fig. 5D). Bcl3 suppression by CRISPR knockout also significantly decreased the intracellular PD-L1 levels, whereas Bcl3 overexpression increased the PD-L1 protein expression in SKOV3 cells (Fig. 5, E and F). Importantly, SKOV3 cells stably transfected with Bcl3 shRNA express significantly reduced PD-L1 protein levels (Fig. 5, E and F). Together, these results indicate that Bcl3 promotes constitutive PD-L1 expression in OC cells.

IFN induces Bcl3 expression in OC cells

PD-L1 expression on tumor cells, including OC cells, is induced by IFN produced by CD8 T cells (50–52). Because our

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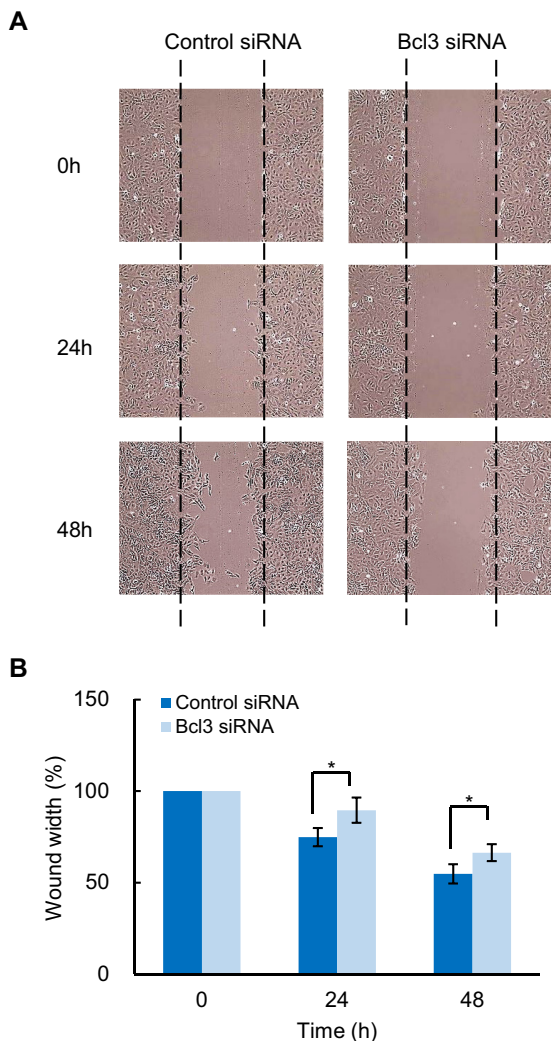


Figure 3. Bcl3 suppression inhibits migration of OC cells. *A*, SKOV3 cells transfected with control or Bcl3 siRNA were subjected to wound healing assay. Representative photographs at the indicated times from three independent experiments performed in triplicates are shown. Magnification: $\times 10$. *B*, the wound width was measured in five random fields using ImageJ software by normalizing the average wound width at 24 and 48 h to the average wound width at 0 h. The samples were measured in triplicates and expressed as mean \pm S.E.

data showed that Bcl3 promotes PD-L1 expression in OC cells (Fig. 5), we asked whether IFN might regulate the Bcl3 expression. In this regard, Bcl3 expression was reported to be up-regulated by pro-inflammatory cytokines including TNF α , IL-1 β , and IL-6 (20). However, to our knowledge, there is no available evidence showing that IFN induces Bcl3 expression. Indeed, human recombinant IFN significantly increased *Bcl3* mRNA levels in SKOV3 and OVCAR3 cells (Fig. 6A). In addition, in agreement with a previous study demonstrating increased surface expression of PD-L1 in IFN-treated OC cells (52), IFN significantly increased *PD-L1* mRNA levels in both OC cell lines (Fig. 6B). Importantly, IFN also increased intracellular Bcl3 and PD-L1 protein levels in SKOV3 and OVCAR3 cells (Fig. 6, C and D). Together, these data demonstrated that IFN induces Bcl3 expression in OC cells, and suggested a link between IFN, Bcl3, and PD-L1 signaling.

Bcl3 mediates IFN-induced PD-L1 expression in OC cells

Having shown that Bcl3 promotes the basal PD-L1 expression in OC cells (Fig. 5), and that IFN increases the intracellular levels of Bcl3 and PD-L1 (Fig. 6, A–D), we wanted to determine whether Bcl3 mediates also the IFN-induced PD-L1 expression in OC cells. To this end, we analyzed PD-L1 expression in IFN-treated SKOV3 and OVCAR3 cells transfected with control and Bcl3 siRNA. Bcl3 suppression (Fig. 6E) significantly attenuated the IFN-induced *PD-L1* expression in SKOV3 and OVCAR3 cells (Fig. 6F), indicating that Bcl3 mediates the IFN-induced PD-L1 expression in OC cells.

IFN induces PD-L1 promoter occupancy by p65, Lys-314/315 acetylated p65, and p300

Because recent studies have shown that PD-L1 expression is regulated by p65 NF- κ B (30–35), we wanted to determine whether the Bcl3-mediated PD-L1 expression in IFN-treated OC cells is associated with an increased p65 promoter occupancy. Furthermore, because Lys-314/315 acetylation of p65 regulates its transcriptional activity in OC cells (53), we analyzed the *PD-L1* promoter occupancy by Lys-314/315 ac-p65. The human *PD-L1* promoter contains several putative NF- κ B-binding sites: κ B1 site (GGAAAGTCCA) (30) located at position –65 upstream from the transcription start site (TSS), κ B2 site (GGGGGACGCC) (34) located –358 from TSS, κ B3 site (GGGAAGTTCT) located –600 from TSS (30), and κ B4/ κ B5 sites containing an identical putative NF- κ B binding sequence (GGGAAGTCAC) located –1256 and –1283 from TSS (Fig. 7A). So far, p65 recruitment to the κ B2 site has been demonstrated in non-small cell lung cancer and triple negative breast cancer cells (34, 35), and p65 was also recruited to the κ B3 site in lipopolysaccharide-stimulated macrophages (30). However, it is not known whether NF- κ B binds to the κ B1 and/or κ B4/ κ B5 sites, or whether NF- κ B occupies the *PD-L1* promoter in OC cells.

Thus, we first analyzed using chromatin immunoprecipitation (ChIP) whether p65 and Lys-314/315 ac-p65 are recruited to the *PD-L1* κ B1, κ B2, κ B3, and κ B4/ κ B5 sites (Fig. 7A) in IFN-treated SKOV3 cells. Compared with ChIP using control IgG, we detected a statistically significant recruitment of p65 to κ B1 (Fig. 7B) and κ B2 (Fig. 7C) sites in 6-h IFN-treated cells, even though this occupancy was relatively low. We did not observe any significant recruitment of p65 to κ B3 (Fig. 7D) and κ B4/ κ B5 (Fig. 7E) sites. Intriguingly, however, IFN induced a robust recruitment of Lys-314/315 ac-p65, which regulates the specificity of NF- κ B-dependent transcription (54, 55), to all κ B sites in the *PD-L1* promoter (Fig. 7, B–E).

To determine whether the increased *PD-L1* promoter occupancy by Lys-314/315 ac-p65 is associated with an increased occupancy of a histone acetyltransferase (HAT), we analyzed recruitment of the HATs cAMP-response element-binding protein (CBP) and p300, known to acetylate p65 (56). Although CBP was not significantly recruited, p300 was heavily recruited to all κ B sites in the *PD-L1* promoter, and this recruitment was further enhanced by IFN treatment (Fig. 7, B–E). In addition, we tested whether the *PD-L1* promoter is occupied by Bcl3;

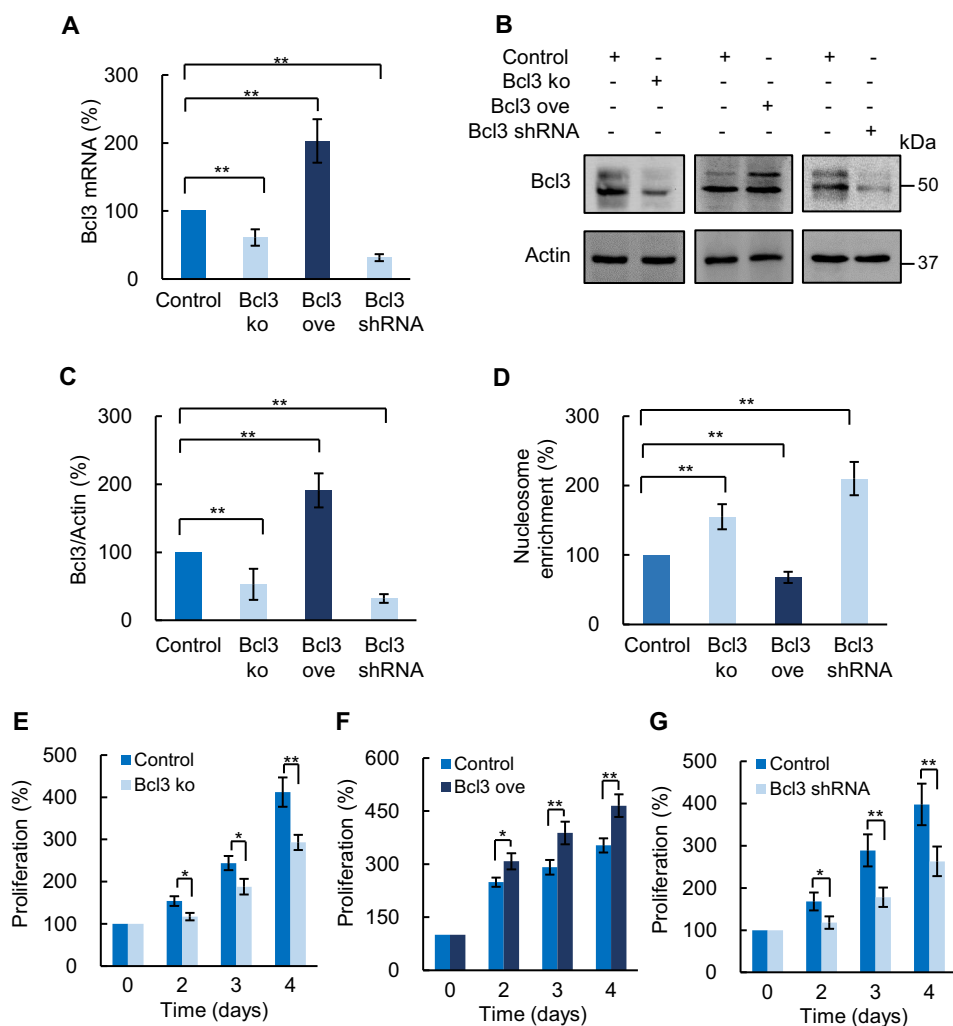


Figure 4. Bcl3 overexpression induces survival and proliferation of OC cells. *A*, RT-PCR of *Bcl3* mRNA in SKOV3 cells transfected with CRISPR knockout (KO), overexpression (ove) plasmid, or stably transfected with Bcl3 shRNA. *B*, Western blotting of Bcl3 in WCE prepared from SKOV3 cells transfected with control, Bcl3 KO, Bcl3 ove plasmids, or stably transfected with Bcl3 shRNA. *C*, densitometric evaluation of Bcl3 protein levels shown in *B*; the Bcl3 densities were normalized to actin, and expressed as % compared with cells transfected with control plasmid. *D*, apoptosis measured by nucleosome enrichment assay in SKOV3 cells transfected with control, Bcl3 KO, Bcl3 ove plasmid, and stably transfected with Bcl3 shRNA. Cell proliferation was measured by CellTiter 96 One Solution cell proliferation assay in SKOV3 cells transfected with (*E*) Bcl3 KO, (*F*) Bcl3 ove plasmid, or (*G*) stably transfected with Bcl3 shRNA. The values represent the mean \pm S.E. of three experiments; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with cells transfected with the corresponding control plasmid.

however, we did not observe any significant recruitment (Fig. 7, *B–E*).

Bcl3 and p300 mediate IFN-induced Lys-314/315 ac-p65 recruitment to PD-L1 promoter

Even though Bcl3 was not directly recruited to *PD-L1* promoter, we wanted to test whether it might mediate the IFN-induced Lys-314/315 ac-p65, p65, and p300 occupancy. In addition, because p300 was recruited to the *PD-L1* promoter, we analyzed whether it might facilitate the Lys-314/315 ac-p65 promoter occupancy. To this end, we measured Lys-314/315 ac-p65, p65, and p300 recruitment to *PD-L1* promoter in SKOV3 cells transfected with control, Bcl3, and p300 siRNA and treated with IFN (0 and 50 ng/ml) for 6 h. Interestingly, both Bcl3 and p300 silencing significantly suppressed the IFN-induced *PD-L1* promoter occupancy by Lys-314/315 ac-p65 (Fig. 8*A*). In contrast, p65 recruitment to *PD-L1* promoter was not Bcl3- or p300-dependent (Fig. 8*B*); however, given the relatively low occupancy of p65 at the *PD-L1* promoter, it was

difficult to accurately assess the role of Bcl3 and p300 in p65 recruitment. The occupancy of p300 at the *PD-L1* promoter in both untreated and IFN-treated cells was also not suppressed by Bcl3 silencing (Fig. 8*C*), indicating that p300 resides on the *PD-L1* promoter even in the absence of Bcl3. Together, these data suggest that the *PD-L1* promoter in OC cells is permanently occupied by p300, and upon IFN stimulation, Bcl3 facilitates Lys-314/315 p65 acetylation and promoter occupancy, resulting in increased *PD-L1* transcription.

PD-L1 mediates Bcl3 pro-proliferative effect in OC cells

Because in addition to suppressing the anti-tumor activity of cytotoxic T cells, tumor PD-L1 has tumor-intrinsic effects (36–38), we asked whether the Bcl3 pro-proliferative effect in OC cells might be mediated by PD-L1. To address this question, we analyzed proliferation of SKOV3 and OVCAR3 cells transfected with Bcl3 overexpression or control plasmids, in the presence of PD-L1 neutralizing antibody, or isotype-matched control IgG. The results demonstrated that compared with

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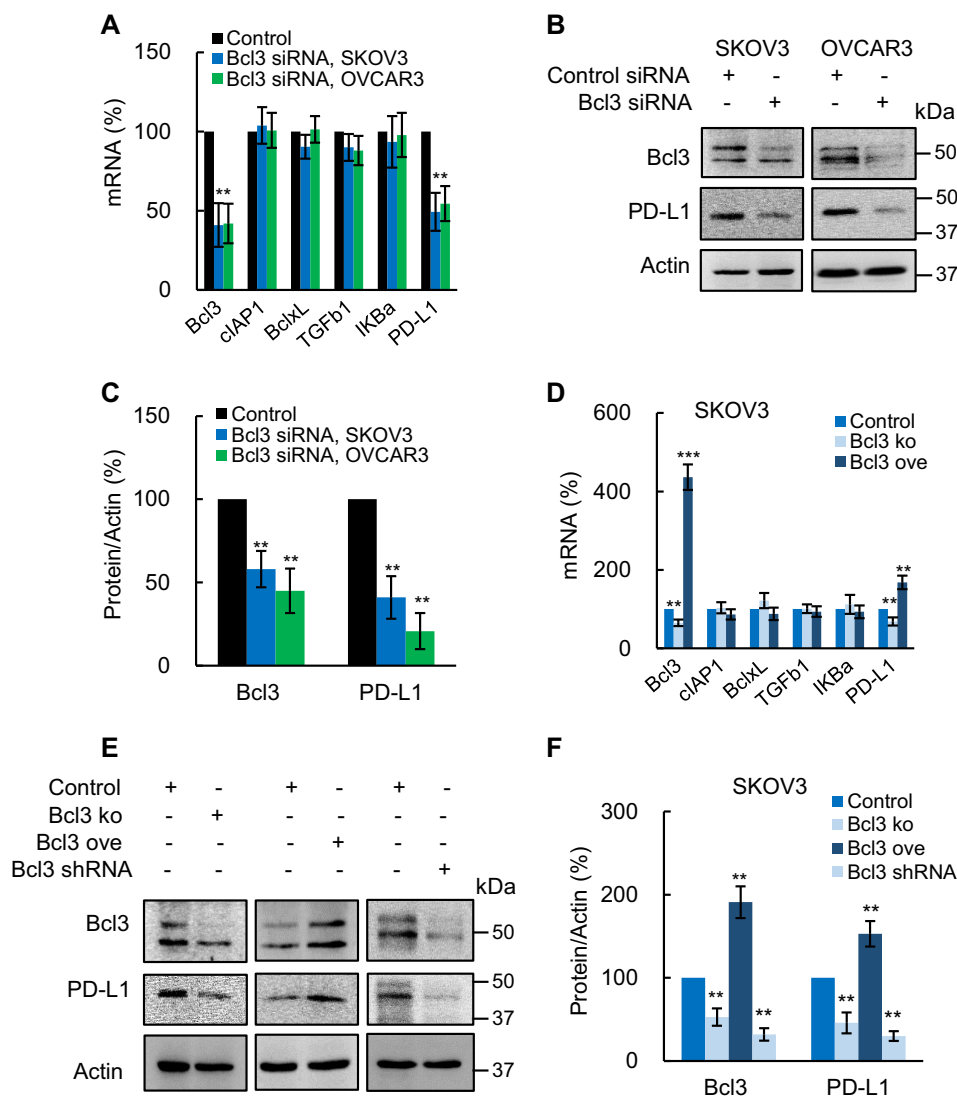


Figure 5. Bcl3 promotes constitutive PD-L1 expression in OC cells. A, expression of NF- κ B-dependent genes measured by RT-PCR in SKOV3 and OVCAR3 cells transfected with control and Bcl3 siRNA. B, Western blotting of Bcl3, PD-L1, and control actin in WCE from SKOV3 and OVCAR3 cells transfected with control and Bcl3 siRNA. C, densitometric evaluation of Bcl3 and PD-L1 protein levels shown in B; Bcl3 and PD-L1 densities were normalized to actin, and expressed as % compared with cells transfected with the control plasmids. D, RT-PCR of NF- κ B-dependent genes in SKOV3 cells transfected with control, Bcl3 KO, or Bcl3 ove plasmids. E, Western blotting of Bcl3 and PD-L1 in WCE from SKOV3 cells transfected with control, Bcl3 KO, Bcl3 ove plasmids, or stably transfected with Bcl3 shRNA. For cells stably transfected with Bcl3 shRNA, the same gel as in Fig. 4B was used and the membrane was re-probed with PD-L1 antibody (the same images for Bcl3 and actin were used as in Fig. 4B). F, densitometric evaluation of Bcl3 and PD-L1 protein levels shown in E; Bcl3 and PD-L1 densities were normalized to actin, and expressed as % compared with cells transfected with control plasmids. The values represent the mean \pm S.E. of three experiments.

control IgG, PD-L1 neutralizing antibody significantly reduced proliferation of SKOV3 cells, both in cells transfected with control plasmid, and in cells transfected with Bcl3 overexpression plasmid (Fig. 9A). Similar results were observed in OVCAR3 cells (Fig. 9B), indicating that the pro-proliferative effect of Bcl3 in OC cells is partly mediated by PD-L1.

Because IFN increases the Bcl3 expression in OC cells (Fig. 6), and promotes OC tumor growth in mice (52), we tested whether OC cell proliferation in IFN-treated cells also depends on PD-L1. Incubation of SKOV3 (Fig. 9C) and OVCAR3 (Fig. 9D) cells with IFN in the presence of control IgG increased cell proliferation, but this effect was observed only during later incubation times. Importantly, the OC cell proliferation in IFN-treated cells was significantly reduced in the presence of PD-L1 neutralizing antibody. These data are consistent with the recent *in vivo* study by Abiko *et al.* (52) demonstrating that the

IFN-induced OC tumor growth is PD-L1 dependent. Together, these results indicate that IFN induces Bcl3 expression, resulting in the increased PD-L1 transcription and OC cell proliferation (Fig. 10).

Discussion

Our study shows, rather surprisingly, that in addition to promoting cell survival and proliferation, the proto-oncogene Bcl3 induces expression of PD-L1 in ovarian cancer cells. In addition, our findings demonstrate that Bcl3 expression is increased in OC tissues, and is induced by IFN in OC cells. The mechanism of how Bcl3 induces PD-L1 transcription in IFN-stimulated cells involves an increased, Bcl3- and p300-dependent recruitment of Lys-314/315 ac-p65 to PD-L1 promoter. Because blocking PD-L1 with neutralizing antibody reduces proliferation of OC cells overexpressing Bcl3 or treated with

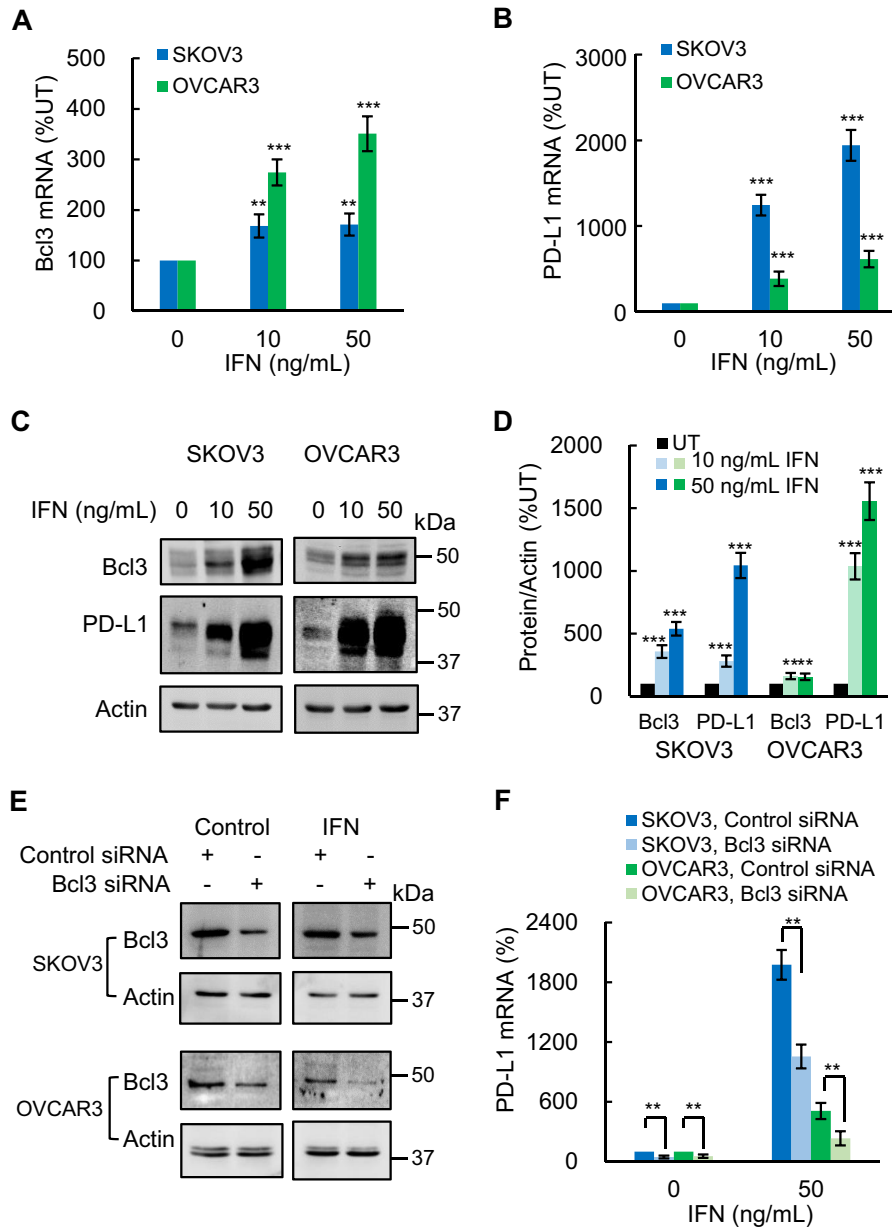


Figure 6. IFN induces Bcl3 expression, resulting in increased PD-L1 expression in OC cells. A and B, RT-PCR of *Bcl3* (A) and *PD-L1* (B) mRNA in SKOV3 and OVCAR3 cells incubated 48 h with increasing IFN. C, Western blotting of Bcl3 and PD-L1 in WCE of SKOV3 and OVCAR3 cells incubated 48 h with IFN. D, densitometric evaluation of Bcl3 and PD-L1 protein levels shown in C; Bcl3 and PD-L1 densities were normalized to actin, and expressed as % compared with untreated (UT) cells. E, Western blotting of Bcl3 in WCE from IFN-treated SKOV3 and OVCAR3 cells transfected with control and Bcl3 siRNA. F, *PD-L1* mRNA in IFN-treated SKOV3 and OVCAR3 cells transfected with control and Bcl3 siRNA. The values represent the mean \pm S.E. of three experiments.

IFN, these results suggest that the pro-proliferative effect of Bcl3 in OC cells is partly mediated by PD-L1. Together, these data link Bcl3 to IFN γ and PD-L1 signaling, and suggest that in addition to mediating cell survival and proliferation, Bcl3 promotes immune escape in cancer cells (Fig. 10).

Bcl-3 was originally identified as a candidate proto-oncogene up-regulated in B-cell chronic lymphocytic leukemia (1, 2); later studies demonstrated its increased expression also in other hematological malignancies, as well as in several types of solid cancer (10–23). The link between Bcl3 overexpression and malignant transformation was suggested to stem from its transcriptional up-regulation of cyclin D1 (57), increased expression of HDM2, the main negative regulator of p53 (58),

and regulation of DNA damage response (59). In addition, recent studies have shown that Bcl3 induces expression of pro-inflammatory cytokines IL-8 and IL-17 in cutaneous T cell lymphoma cells (16), and TGF β signaling in breast cancer (60). Our present findings demonstrate that Bcl3 promotes expression of PD-L1, indicating that in addition to regulating NF- κ B-dependent genes involved in cell survival and proliferation, Bcl3 controls genes involved in immune escape. However, the regulation of NF- κ B-dependent transcription by Bcl3 is gene specific; whereas Bcl3 induces transcription of *PD-L1*, it does not have a significant effect on the expression of NF- κ B-regulated genes *cIAP1*, *BclxL*, *TGF β 1*, or *I κ B α* , in OC cells (Fig. 5).

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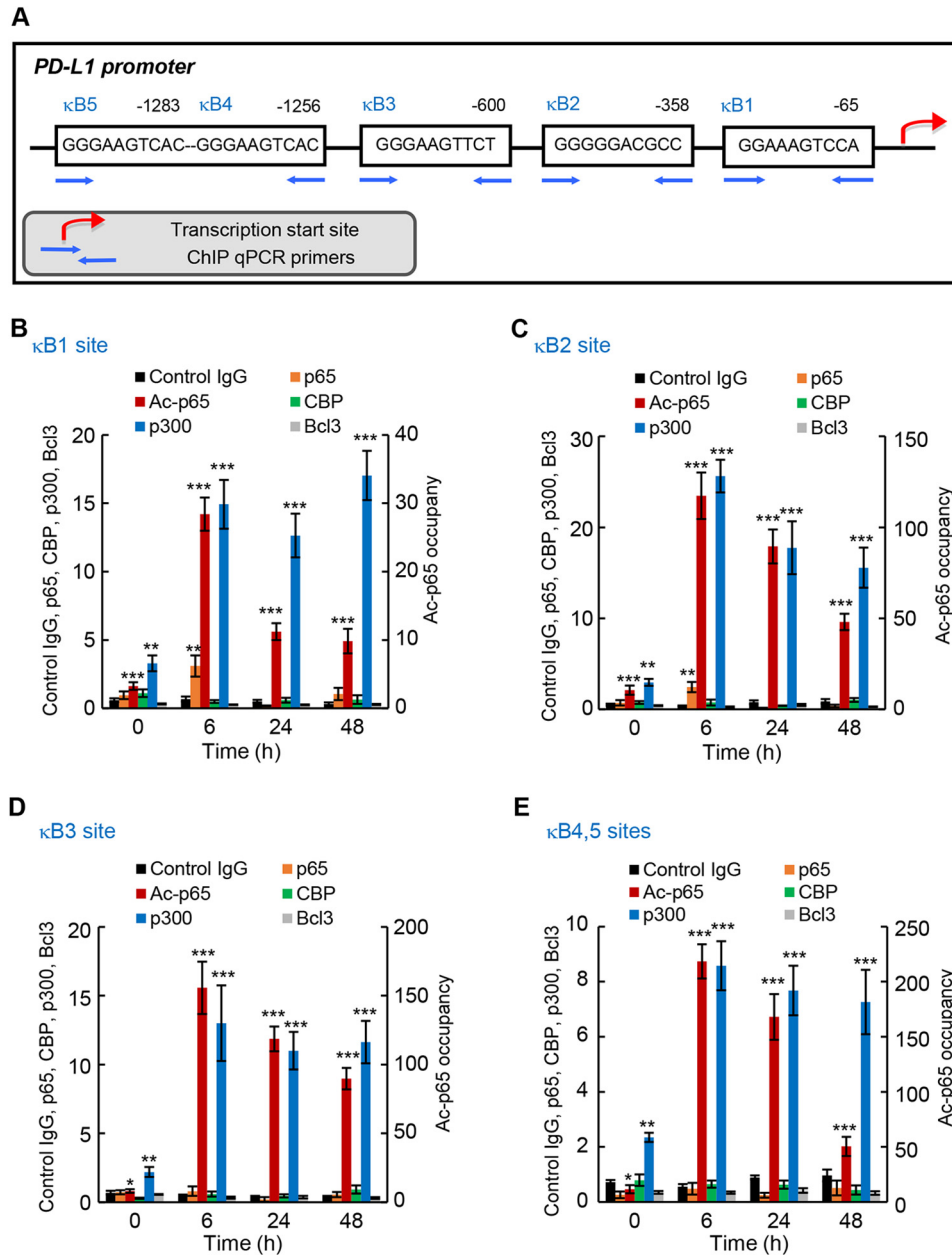


Figure 7. IFN induces PD-L1 promoter occupancy by p65, Lys-314/315 acetylated p65, and p300 in OC cells. A, schematic illustration of NF- κ B-binding sites in human PD-L1 promoter, and ChIP primers used in the ChIP assay. B–E, recruitment of p65, Lys-314/315 ac-p65, CBP, p300, and Bcl3 to PD-L1 κ B1 (B), κ B2 (C), κ B3 (D), and κ B4/ κ B5 sites (E) in IFN (50 ng/ml)-treated SKOV3 cells was analyzed by ChIP and quantified by real-time PCR; ChIP using control IgG is also shown. Each condition (antibody used at each time point) represents $\sim 1.25 \times 10^5$ cells. The data are presented as fold-difference in occupancy of the particular protein at the particular locus compared with the human IGX1A (SA Biosciences) locus, and represent the mean \pm S.E. of three experiments. Asterisks denote a statistically significant (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) change compared with ChIP using control IgG at the corresponding time.

What determines the specificity of the transcriptional regulation by Bcl3? Because Bcl3 contains a transactivation domain, it can modulate transcription depending on the transcription factors and co-regulators present in the transcription complexes (6–9, 16, 61, 62). In this context, Bcl3 was shown to interact with the NF- κ B subunits p50 and p52, the AP-1 transcription factors c-Jun and c-Fos, STAT1, STAT3, PPAR γ , class I histone deacetylases, and the HATs CBP and p300 (6–9, 16, 22, 24, 57, 62–66). Our results demonstrate that even though Bcl3 is not directly recruited to the PD-L1 promoter, it mediates, together with the HAT p300 present at the PD-L1

promoter (Fig. 7), the promoter occupancy by Lys-314/315 acetylated p65 in IFN-treated OC cells (Fig. 8A). Interestingly, previous studies have reported that acetylation of p65 at Lys-314/315 is mediated by p300, and results in a gene-specific regulation of NF- κ B-dependent genes in TNF-stimulated cells (54, 55). Together, our data support a model in which the PD-L1 promoter in OC cells is occupied by p300, and upon IFN stimulation, Bcl3 promotes PD-L1 transcription by facilitating the promoter-specific occupancy by Lys-314/315 ac-p65. Future studies should determine whether IFN induces p65 acetylation on Lys-314/315 by p300, and/or

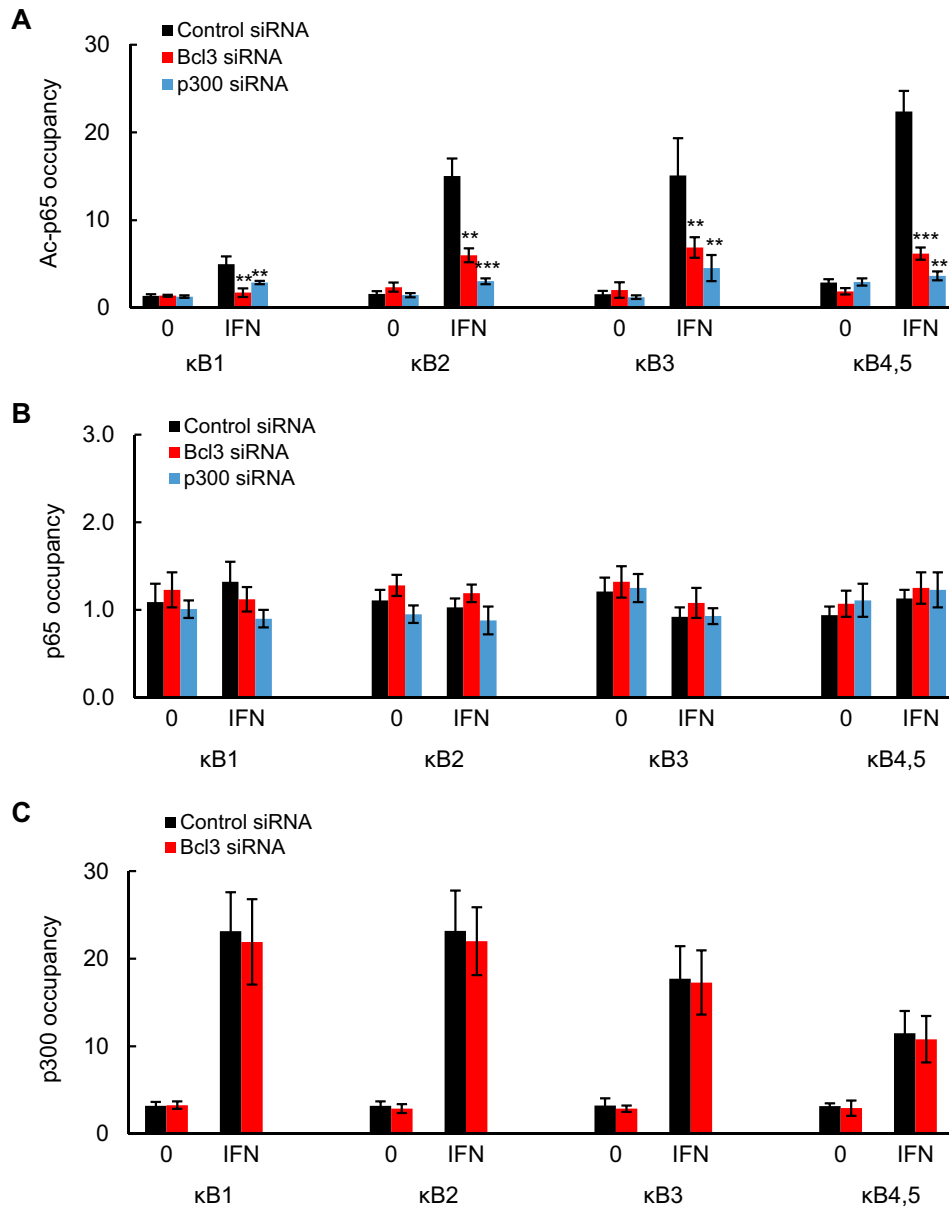


Figure 8. Bcl3 and p300 mediate Lys-314/315 ac-p65 occupancy at PD-L1 promoter in IFN-treated OC cells. A–C, ChIP of PD-L1 promoter occupancy by Lys-314/315 ac-p65 (A), p65 (B), and p300 (C) in SKOV3 cells transfected with control, Bcl3, and p300 siRNA, and treated 6 h with IFN (0 and 50 ng/ml). Each condition represents $\sim 0.25 \times 10^5$ cells. The data are presented as fold-difference in occupancy of the particular protein at the particular locus compared with the human IGX1A (SA Biosciences) locus, and represent the mean \pm S.E. of three experiments. Asterisks denote a statistically significant (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) change compared with cells transfected with control siRNA.

whether it induces histone deacetylases' removal. In addition, it will be interesting to determine whether the high promoter occupancy by Lys-314/315 ac-p65 in IFN-treated OC cells is unique for PD-L1, or whether IFN induces Lys-314/315 ac-p65 recruitment to other NF- κ B-dependent promoters as well.

Little is known about the signaling pathways inducing Bcl3 expression in cancer cells. In line with the reported induction of Bcl3 by p65 NF- κ B (68), Bcl3 expression was shown to be up-regulated by pro-inflammatory cytokines including TNF α , IL-1, and IL-6 (20). Our study is the first to demonstrate that the Bcl3 expression is induced also by IFN γ (Fig. 6). The induction of Bcl3 by IFN is intriguing, especially because our data also show that the IFN-induced Bcl3 expression promotes expres-

sion of PD-L1 in IFN-stimulated cells (Fig. 6), thus linking Bcl3 to IFN and PD-L1 signaling.

Increased PD-L1 expression in OC tissues promotes tumor growth (39–41), but the regulation of PD-L1 expression in OC cells is little understood. Our study demonstrates that the PD-L1 expression in OC cells is regulated by the proto-oncogene Bcl3. Analysis of four different public datasets, together containing 26 control ovarian tissues and 898 OC samples, has revealed that the Bcl3 gene expression is statistically increased in OC tissues ($p \leq 0.016$; Fig. 1). Interestingly, the Bcl3 expression was most significantly increased in ovarian serous surface papillary carcinoma (Fig. 1C) (43). Even though the sample size was relatively small, these data suggest that this type of OC has a significantly higher Bcl3 expression compared with other

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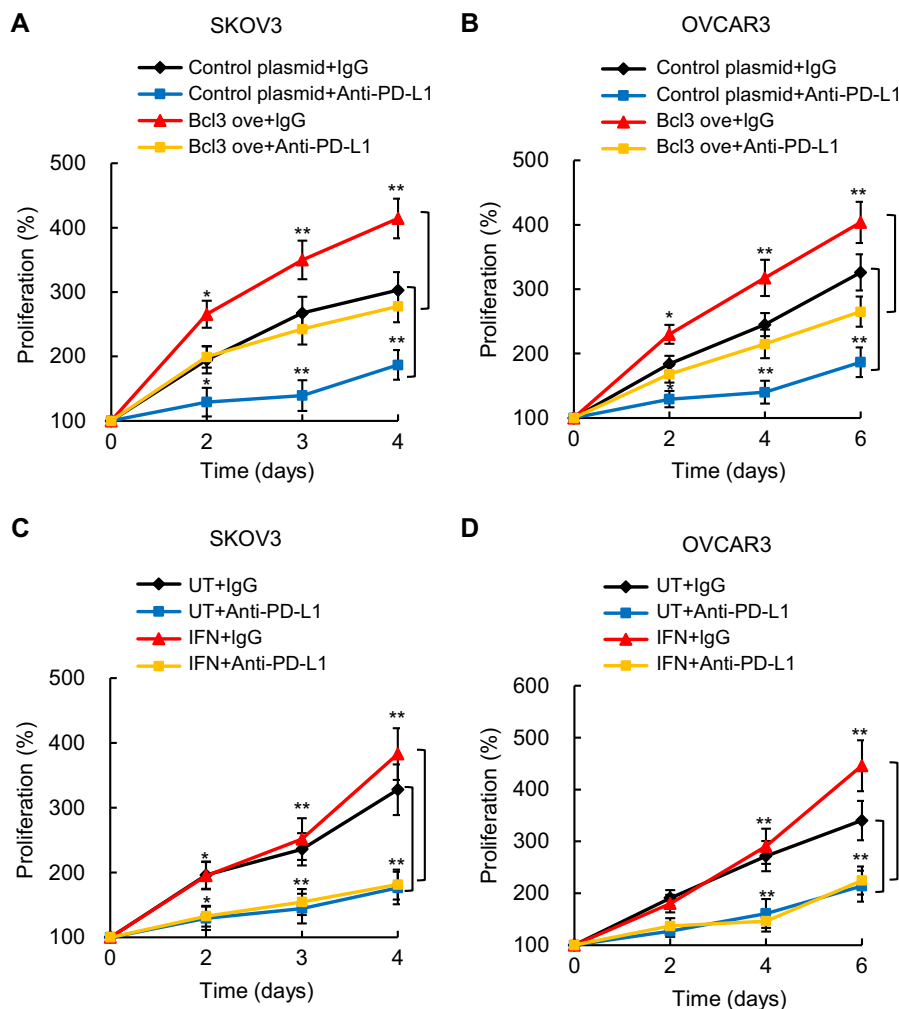


Figure 9. Bcl3-increased proliferation of OC cells is mediated by PD-L1. Cell proliferation of SKOV3 (A) and OVCAR3 (B) cells was measured by CellTiter 96 One Solution cell proliferation assay in cells transfected with control or Bcl3 overexpression plasmids, measured in the presence of PD-L1 neutralizing antibody, or control IgG1. Cell proliferation of IFN-treated (0 and 50 ng/ml) SKOV3 (C) and OVCAR3 (D) cells was measured in the presence of PD-L1 neutralizing antibody or control IgG1. The values represent the mean \pm S.E. of three experiments; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with cells treated with the control IgG1.

types of OC. Alternatively, as the tumor samples are often heterogeneous, a subset of cancer cells may express higher levels of *Bcl3*. It will be important to correlate these data in future with *Bcl3* protein levels in individual cells. In addition, because the *Bcl3* transcriptional activity is regulated by phosphorylation (24, 45–47), future studies should analyze the phosphorylation status of nuclear *Bcl3* in OC tissues.

In addition to inhibiting anti-tumor cytotoxic T cells, the tumor-expressed PD-L1 has tumor-intrinsic effects that include the regulation of cancer cell survival and proliferation, autophagy, and regulation of glucose metabolism and mTOR signaling (36–38). Our results show that *Bcl3* has pro-survival and pro-proliferative effects in OC cells (Figs. 2–4). However, because blocking the *Bcl3*-induced PD-L1 by neutralizing antibody decreases proliferation in *Bcl3*-overexpressing cells (Fig. 9), these data indicate that the *Bcl3* pro-survival effect in OC cells is, at least partly, mediated by the *Bcl3*-up-regulated PD-L1. In summary, our study identifies PD-L1 as a novel target of *Bcl3*, indicating that *Bcl3* regulates not only cancer cell proliferation and survival, but also immune escape.

Experimental procedures

Cell culture

Human ovarian cancer SKOV3 and OVCAR3 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured (5×10^5 cells/ml) in 6-well plates in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) and antibiotics at 37 °C with 5% CO₂ as described (53). For treatment with IFN γ , human recombinant IFN γ (285-IF-100; R&D Systems, Minneapolis, MN) was reconstituted in sterile water. Cell viability was measured by using trypan blue exclusion.

Transfection with siRNA and CRISPR knockout and overexpression plasmids

Human *Bcl3* (sc-29789) and nonsilencing (sc-37007) siRNAs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Prior to transfection, 2×10^5 cells were seeded into a 6-well plate and incubated in a humidified 5% CO₂ atmosphere at 37 °C in antibiotic-free RPMI medium supplement with 10%

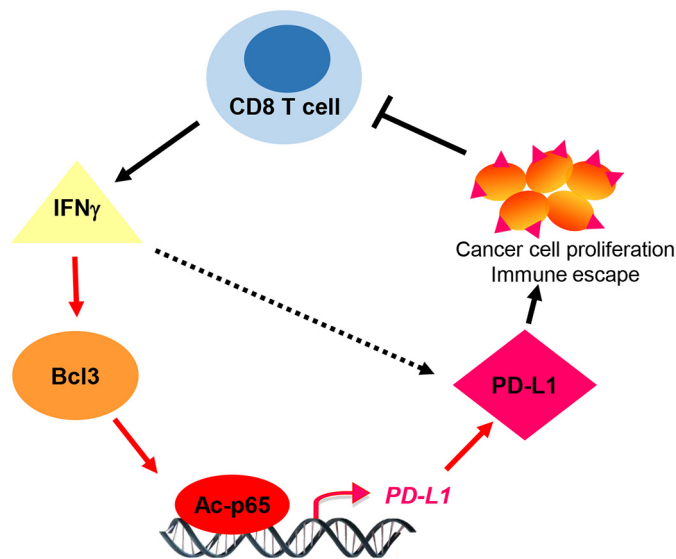


Figure 10. Proposed model of IFN-induced Bcl3 expression, resulting in increased PD-L1 promoter occupancy by Lys-314/315 ac-p65, and increased PD-L1 expression and proliferation in OC cells. Findings from this study are indicated by red arrows.

FBS for 24 h to about 80% confluence. For each transfection, 80 pmol of either nonsilencing siRNA control or Bcl3 siRNA were used. Cells were transfected 7 h in transfection medium with siRNA transfection reagent according to the manufacturer's instructions (Santa Cruz Biotechnology). After transfection, fresh medium with antibiotics was added, and cells were grown for 24 h before treatment.

Bcl3 CRISPR/Cas9 knockout (KO) plasmid (sc-400740), control CRISPR/Cas9 plasmid (sc-418922), Bcl-3 CRISPR activation plasmid (sc-400740-ACT), and control CRISPR activation plasmid (sc-437275) were obtained from Santa Cruz Biotechnology. Prior to transfections, 2×10^5 cells were seeded into a 6-well plate and incubated in antibiotic-free RPMI medium supplement with 10% FBS for 24 h to 80% confluence. For each transfection, 3 μ g of Bcl3 CRISPR/Cas9 KO or activation plasmids, or the corresponding control plasmids were used. Cells were transfected 24 h in plasmid transfection medium according to the manufacturer's instructions (Santa Cruz Biotechnology). After transfection, fresh medium with antibiotics was added, and cells were grown for 24 h before treatment.

For stable transfection, Bcl3 shRNA (sc-29789-SH) and control shRNA (sc-108060) plasmids were obtained from Santa Cruz Biotechnology. For each transfection, 2 μ g of Bcl3 shRNA or control shRNA plasmid were used, and cells were transfected using shRNA plasmid transfection medium (sc-108062) and transfection reagent (sc-108061) according to the manufacturer's instructions (Santa Cruz Biotechnology). Transfected colonies were selected using 3 μ g/ml of puromycin.

Apoptosis, cell proliferation, and PD-L1 neutralization assays

Apoptosis was evaluated using a cell death detection ELISA kit that quantifies release of nucleosomes into the cytoplasm (Cell Death Detection ELISAPLUS, Roche Applied Science) (48), and by measuring caspase 3 activity using a human active caspase 3 ELISA kit (ab181418, Abcam, Cambridge, MA).

Cell proliferation was measured by CellTiter 96 One Solution Cell Proliferation Assay (Promega, Madison, WI). Transfected cells were seeded into 96-well plates at a density of 5000 cells/100 μ l of medium, and incubated at 37 $^{\circ}$ C. At the indicated time points, 20 μ l of CellTiter 96 One Solution Reagent was added to each well, incubated for 4 h at 37 $^{\circ}$ C, and absorbance at 490 nm was measured.

For PD-L1 neutralization experiments, transfected cells were incubated in the presence of 500 nM anti-PD-L1 (CD274) neutralizing IgG1 κ antibody (catalog 71213; BPS Bioscience, San Diego, CA) or isotype control IgG1 κ antibody (catalog 14-4714-82; Thermo Fisher Scientific), and cell proliferation was measured as described above.

Wound healing assay

SKOV3 cells were seeded in 6-well plates (2×10^5 cells/well) and transfected with control or Bcl3 siRNA as described above. Once the cells became confluent, a wound area was created by scraping the cell monolayer with a sterile 200- μ l pipette tip. After washing twice with PBS, RPMI medium without FBS was added to the wells. The scratch area was monitored under a phase-contrast microscope at 0, 24, and 48 h after transfection. The wound width was measured in five random fields using ImageJ software. All samples were tested in triplicates.

Real-time RT-PCR

Total RNA was isolated using RNeasy mini-kit (Qiagen, Valencia, CA). The iScript one-step RT-PCR kit with SYBR Green (Bio-Rad) was used as a Supermix and 20 ng/ μ l of RNA was used as template on a Bio-Rad MyIQ Single Color Real-Time PCR Detection System (Bio-Rad). The primers used for quantification of human Bcl3, PD-L1, cIAP1, BclxL, TGF β 1, I κ B α , p65, and actin mRNA were purchased from SA Biosciences (Frederick, MD). The mRNA values are expressed as a percentage of control or untreated samples, which were arbitrarily set as 100%.

Western blot analysis

WCE were prepared as described previously (48, 53). Denatured proteins were separated on 12% denaturing polyacrylamide gels and transferred to nitrocellulose membrane (Hybond C; Amersham Biosciences). Membranes were blocked with a 5% (w/v) nonfat dried milk solution containing 10 mM Tris-Cl, pH 7.5, 140 mM NaCl, 1.5 mM MgCl $_2$, and 0.1% Tween 20 (TBSTM), and incubated with Bcl3 (23959-1-AP; Proteintech, Rosemont, IL) or PD-L1 (E1L3N; Cell Signaling, Danvers, MA) antibodies diluted in TBSTM. After washing, the membranes were incubated with horseradish peroxidase-labeled secondary antibodies and the labeled proteins were detected using the ECL detection system (Amersham Biosciences). To confirm equivalent amounts of loaded proteins, the membranes were stripped and re-probed with control anti-actin antibody as described (48, 53).

Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed as described (67). Briefly, proteins and DNA were cross-linked by formaldehyde, and cells

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were washed and sonicated. The lysates were centrifuged (15,000 × g, 10 min, 4 °C), and the supernatant extracts were diluted with ChIP dilution buffer and pre-cleared with Protein A/G-agarose (Santa Cruz Biotechnology) for 2 h at 4 °C. Immunoprecipitations were performed overnight at 4 °C, using p65 (MAB3026; Sigma), Lys-314/315 acetylated p65 (HW136; Signalway Antibody, College Park, MD), CBP (sc-7300; Santa Cruz Biotechnology), p300 (sc-585; Santa Cruz Biotechnology), Bcl3 (23959-1-AP; Proteintech), and control IgG (sc-2025) antibodies that were pre-incubated (6 h, 4 °C) with Protein A/G-agarose, and the immune complexes were collected by centrifugation (150 × g, 5 min, 4 °C), washed, and extracted with 1% SDS, 0.1 M NaHCO₃. After reversing the cross-linking, proteins were digested with proteinase K, and the samples were extracted with phenol/chloroform, followed by precipitation with ethanol. Immunoprecipitated DNA was analyzed by real-time PCR (25 μl reaction mixture) using the iQ SYBR Green Supermix and the Bio-Rad MyIQ Single Color Real-Time PCR Detection System (Bio-Rad). Each immunoprecipitation was performed at least three times using different chromatin samples, and the occupancy was calculated by using the human IGX1A negative control primers (SA Biosciences, Frederick, MD), which detect specific genomic ORF-free DNA sequence that does not contain a binding site for any known transcription factors. The results were calculated as fold-difference in occupancy of the particular protein at the particular locus compared with the IGX1A locus.

The PD-L1 primers used for real-time PCR were as follows: PDL1-κB1: forward, 5'-CTTTATTCCTAGGACACCAACACT-3' and reverse, 5'-CAAGGCAGCAAATCCAGTTT-3'; PDL1-κB2: forward, 5'-TGGGTCTGCTGCTGACTTTTAA-3' and reverse, 5'-AGAGGGGTAAGAGCTTAAGGT-TAC-3'; PDL1-κB3: forward, 5'-TTCCGCAGCCTTAATCC-TTA-3' and reverse 5'-ACTTCCTCAAAGTTCCTCGACA-3'; PDL1-κB4/κB5: forward, 5'-TGCCACATAATGTCTATA-TTTTCC-3' and reverse 5'-CCAGCTCAGATGTTCCCT-TCTTT-3'.

Statistical analysis

The results represent at least three independent experiments. Numerical results are presented as mean ± S.E. Data were analyzed by using InStat software package (GraphPad, San Diego, CA). Statistical significance was evaluated by using Mann-Whitney *U* test, and *p* < 0.05 was considered significant. Levels of significance are indicated as *, *p* < 0.05; **, *p* < 0.01; and ***, *p* < 0.001.

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