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Targeting IL-17B–IL-17RB signaling with an anti–IL-17RB antibody blocks pancreatic cancer metastasis by silencing multiple chemokines

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Pancreatic cancer has an extremely high mortality rate due to its aggressive metastatic nature. Resolving the underlying mechanisms will be crucial for treatment. Here, we found that overexpression of IL-17B receptor (IL-17RB) strongly correlated with postoperative metastasis and inversely correlated with progression-free survival in pancreatic cancer patients. Consistently, results from ex vivo experiments further validated that IL-17RB and its ligand, IL-17B, plays an essential role in pancreatic cancer metastasis and malignancy. Signals from IL-17B–IL-17RB activated CCL20/CXCL1/IL-8/TFF1 chemokine expressions via the ERK1/2 pathway to promote cancer cell invasion, macrophage and endothelial cell recruitment at primary sites, and cancer cell survival at distant organs. Treatment with a newly derived monoclonal antibody against IL-17RB blocked tumor metastasis and promoted survival in a mouse xenograft model. These findings not only illustrate a key mechanism underlying the highly aggressive characteristics of pancreatic cancer but also provide a practical approach to tackle this disease.

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Abbreviations used: AML1, acute myeloid leukemia 1 protein; AP-1, activator protein-1; ATF2, activating transcription factor 2; CCL20, chemokine (C-C motif) ligand 20; ChIP, chromatin immunoprecipitation; CXCL1, chemokine (C-X-C motif) ligand 1; CXCR4, C-X-C chemokine receptor type 4; EGFR, epidermal growth factor receptor; ERK1/2, extracellular-signal-regulated-kinase1/2; IHC, immunohistochemistry; SDF1, stromal cell–derived factor 1; TFF1, trefoil factor 1; TRAF, TNF receptor associated factor.

Pancreatic cancer is considered largely incurable, even when diagnosed at an early stage. Due to a lack of early symptoms and the aggressive nature of pancreatic tumors, pancreatic cancer patients are often diagnosed at a late stage, when metastasis has already occurred. The poor prognosis of pancreatic cancer has been mainly attributed to its aggressive local invasion and early metastasis (Niedergethmann et al., 2007; Rhim et al., 2012). Factors derived from both genetic and surrounding microenvironment may contribute to this aggressive nature. For example, genetic mutations in oncogene *KRAS* (Almoguera et al., 1988); tumor suppressor genes *TP53*, *SMAD4*, and *BRCA2* (Hong et al., 2011); chromatin modification genes *EPC1* and *ARID2*; and DNA damage repair gene *ATM* (Biankin et al., 2012) have been associated with pancreatic cancer progression. However, surrounding stromal cells also contribute to pancreatic cancer malignancy. It was reported that pancreatic stellate cells secrete growth factors and cytokines to promote cancer cell proliferation and migration (Erkan et al., 2012), facilitate tumor growth and metastasis (Hwang et al., 2008; Vonlaufen et al., 2008), and enhance pancreatic cancer stem cell phenotypes (Hamada et al., 2012; Lonardo et al., 2012). Chronic inflammation is also known to serve as a crucial driving force for pancreatic cancer progression (Farrow and Evers, 2002; Clark et al., 2007; Guerra et al., 2007; Rhim et al., 2012). Upon stimulation by inflammatory cytokines, cancer cells express chemokines to promote tumor growth, invasion,

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metastasis, and angiogenesis via autocrine or paracrine loop (Coussens and Werb, 2002; Balkwill, 2004). Factors such as IL-1, IL-6, IL-8, and stromal cell-derived factor 1 (SDF1), and receptors such as C-X-C chemokine receptor type 4 (CXCR4) and epidermal growth factor receptor (EGFR), have all been shown to play crucial roles in tumorigenesis and chemoresistance in pancreatic or other cancers (Sawai et al., 2003; Mori et al., 2004; Li et al., 2005; Grandal and Madshus, 2008; Matsuo et al., 2009; Lesina et al., 2011). However, unlike other well-studied cytokines (McAllister et al., 2014), the importance of IL-17B–IL-17RB signaling in pancreatic cancer is unknown.

The IL-17 family consists of six cytokines, IL-17A to IL-17F, with 20–50% sequence homology. IL-17A and IL-17F are proinflammatory cytokines exclusively secreted by activated T cells (Fossiez et al., 1996). IL-17B, IL-17C, IL-17D, and IL-17E are expressed in various tissues in a low amount. The cognate receptors for the IL-17 family, IL-17RA to IL-17RE, have been identified, but the physiological roles of these receptors have yet to be fully characterized (Song and Qian, 2013).

IL-17RB has been detected in kidney, pancreas, liver, brain, and intestine (Kolls and Lindén, 2004), and up-regulation of IL-17RB expression was found in intestinal inflammation (Shi et al., 2000). We have previously shown that IL-17RB overexpression was associated with poor breast cancer prognosis (Furuta et al., 2011; Huang et al., 2013). Depletion of IL-17RB resulted in reduction of tumorigenic ability of breast cancer cells (Huang et al., 2013). It is likely that IL-17B–IL-17RB autocrine signaling may contribute to the malignant nature of pancreatic cancer.

In this study, we found that IL-17B/RB signaling is essential for pancreatic cancer malignancy. IL-17B–IL-17RB signal pathway enhanced tumor malignancy through two distinct pathways. One was to activate IL-8 expression via transcription factors nuclear factor κB (NF-κB) and activator protein-1 (AP-1) to promote invasion and vasculogenic endothelial cell recruitment. The other was to up-regulate chemokine (C-C motif) ligand 20 (CCL20), chemokine (C-X-C motif) ligand 1 (CXCL1) and trefoil factor 1 (TFF1) expression via transcription factors NF-κB, activating transcription factor 2 (ATF2) and acute myeloid leukemia 1 protein (AML1) to facilitate pancreatic cancer cell recruitment of macrophages (MQ) and enhance cancer cell survival in distant organs. Clinical evidence also indicated that IL-17RB overexpression strongly correlated with postoperative metastasis and poor prognosis. Importantly, treatment with a newly developed monoclonal antibody against IL-17RB blocked tumor growth and metastasis, and also promoted survival in a mouse xenograft model. These findings shed light on the underlying mechanism of pancreatic cancer malignancy and provide a promising therapeutic target to inhibit pancreatic cancer progression.

RESULTS

Overexpression of IL-17RB associates with metastasis and poor clinical outcome in pancreatic cancer patients

To investigate whether IL-17RB is clinically important for pancreatic cancer, we performed immunohistochemistry (IHC) to analyze IL-17RB expression in 111 pancreatic cancer specimens (Fig. 1 A). The characteristics of these cases are given in Table 1. The specimens can be grouped into three categories based on the percentage of cancer cells expressing IL-17RB: negative (0%), low positive (<10%), and high positive (≥10%). High expression of IL-17RB was strongly correlated with poor differentiation ($P = 0.046$), metastasis ($P = 0.009$), and tumor stage using the TNM (tumor, node, metastasis) staging system ($P < 0.001$; Table 1). Moreover, high IL-17RB expression was associated with postoperative metastasis ($P = 0.029$, Fig. 1 B), marginally associated with recurrence ($P = 0.057$; Fig. 1 B), and correlated with poor prognosis (IL-17RB positive vs. negative: $P = 0.035$; IL-17RB high positive vs. negative: $P = 0.007$; Fig. 1 C). The hazard ratio of progression-free survival in patients with high IL-17RB expression was 1.53-fold (95% C.I., 1.03–2.27; $P = 0.034$) of those without detectable IL-17RB expression after being adjusted for age, gender, tumor location, differentiation status, pathological type, stage, and adjuvant therapy (Fig. 1 D). Overall, patients with elevated IL-17RB expression had worse prognosis and enhanced tumor malignancy, indicating the importance of IL-17RB in pancreatic cancer progression.

IL-17RB has an essential role in pancreatic cancer growth, invasion, and metastasis

To evaluate the role of IL-17RB in pancreatic cancer, we first examined the expression of IL-17RB in a panel of human pancreatic cancer cell lines. High IL-17RB expression was detected in HPAF-II, BxPC3, Capan2, and CFPAC-1 cells (Fig. 2 A). In contrast, low expression was observed in HPAC, SU.86.86, and MIA PaCa-2 cells. We then performed a series of experiments using cells with perturbed IL-17RB expression (Fig. 2, B–G). Depletion of IL-17RB in CFPAC-1 and BxPC3 cells reduced soft agar colony formation and invasion abilities (Fig. 2, B–D). Conversely, colony formation and invasion ability were enhanced in SU.86.86 and HPAC cells when full-length IL-17RB was ectopically expressed (Fig. 2, E–G). In contrast, expression of IL-17RB lacking a ligand binding domain (ΔLBD) had no effect (Fig. 2, E–G). These results indicated that the IL-17B–IL-17RB signal is important for enhancing pancreatic malignancy.

To further substantiate its role in pancreatic cancer, we performed subcutaneous xenograft assays using IL-17RB–depleted CFPAC-1 cells (Fig. 2 H) and demonstrated that depletion of IL-17RB resulted in inhibition of tumor growth when compared with the control (shLacZ). Consistent with this observation, when we performed orthotopic xenografts with IL-17RB–depleted CFPAC-1 cells (Fig. 2 I), they formed smaller tumors. Expression of IL-17RB in these orthotopic tumors was confirmed by IHC (Fig. 2 J). Notably, none of the IL-17RB–depleted xenograft mice developed metastasis. In contrast, four out of six mice implanted with the control CFPAC-1 cells (Fig. 2 K) developed lung metastasis and two of these also developed liver metastasis. Also, injection with IL-17RB–depleted cells through tail vein caused none or little lung metastasis, whereas injection with control cells led to severe lung tumor burden (Fig. 2 L).

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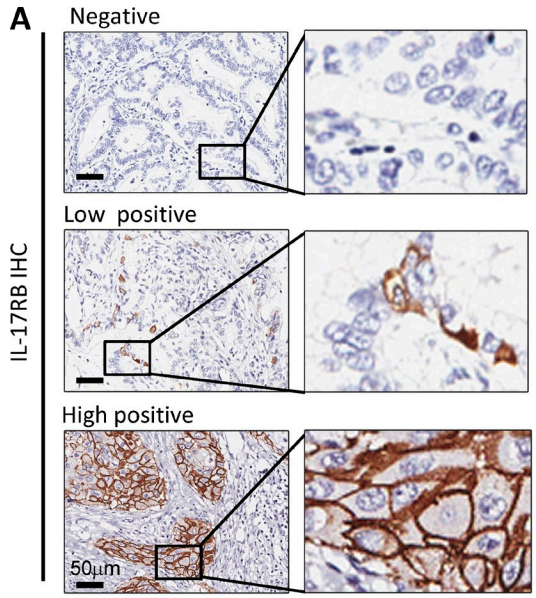
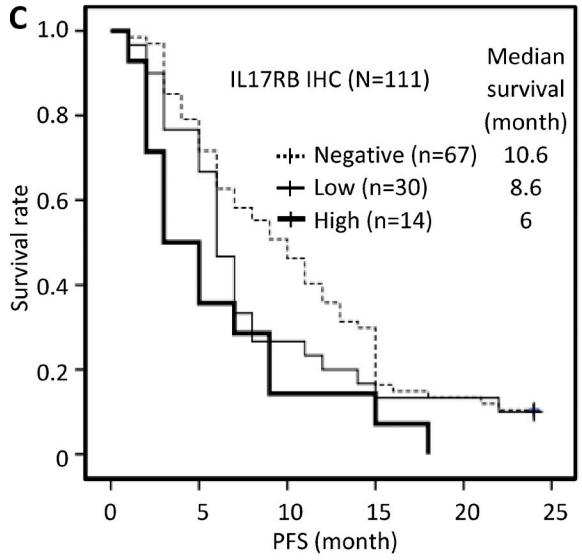


B Correlation between IL-17RB expression with the post-operative recurrence and metastasis in 111 pancreatic cancer cases

	Total N	IL-17RB IHC Negative n (%)	Low n (%)	High n (%)	*P* value[a]
	111	67 (60)	30 (27)	14 (13)	
Recurrence					
No	38	24 (63)	13 (34)	1 (3)	0.057
Yes	73	43 (59)	17 (23)	13 (18)	
Post-operative metastasis					
No	50	37 (74)	9 (18)	4 (8)	0.029
Yes	61	30 (49)	21 (34)	10 (16)	

[a] Chi-square was used for statistic analysis



D Univariate and multivariate Cox regression analysis of the influence of IL-17RB on the clinical outcome (progression free survival) of pancreatic cancer patients after surgical therapy

Variants	Patient no. (%)	Hazard Ratio (95% CI)	*P* value
Univariate:			
IL17RB : Positive (vs. Negative)	44 (40)	1.47 (1.00-2.17)	0.048
Multivariate:			
IL17RB : Positive (vs. Negative)	44 (40)	1.53 (1.03-2.27)	0.034
Age (per 10-yr increment)	56 (50)	1.06 (0.88-1.28)	0.524
Gender : Male (vs. Female)	71 (74)	0.98 (0.79-1.21)	0.979
Tumor location : Tail (vs Head)	14 (13)	0.55 (0.40-0.77)	<0.001
Differentiation			0.037
Moderately (vs. Well)	75 (68)	1.26 (0.87-1.83)	
Poor (vs. Well)	13 (12)	1.36 (0.99-1.79)	
Pathological type: PDAC (vs. Others)	96 (86)	1.38 (1.00-1.89)	0.048
Stage (per stage increment)	16 (14)	2.02 (1.16-3.54)	0.013
Adjuvant therapy (vs. no adjuvant therapy)	44 (40)	0.74 (0.56-0.95)	0.016

Figure 1. Overexpression of IL-17RB is associated with metastasis and poor clinical outcome in pancreatic cancer patients. (A) Representative IHC results of patients with IL-17RB–negative (0%), low positive (< 10%), and high positive (>10%) expression in cancer cells. Bar, 50 µm. Boxes show the enlarged area. (B) Correlation of IL-17RB expression in cancer cells and clinical parameters (2 yr after operation) in 111 pancreatic cancer cases. χ^2 test was used. (C) Comparison of the progression free survival (PFS) of patients with different levels of IL-17RB expression using the Kaplan-Meier method. P = 0.035. (D) Univariate and multivariate Cox regression analysis of the influence of IL-17RB expression on the PFS of 111 pancreatic cancer patients after surgical therapy.

Similar results were observed using BxPC3 cells (unpublished data). Together, these data indicate that elevated IL-17RB expression is critical for pancreatic cancer malignancy.

IL-17B–IL-17RB signaling promotes pancreatic cancer malignancy

Interestingly, pancreatic tumor cells with high level of IL-17RB also expressed IL-17B, an IL-17RB ligand (Fig. 3 A). Depletion of IL-17B by shRNA (Fig. 3, B–D) or treatment with the IL-17B neutralizing antibody (Fig. 3, E–F) in these cells reduced colony formation (Fig. 3, C and E), invasion (Fig. 3, D and F), tumor growth (Fig. 3, G–H), and metastasis assayed by mouse xenograft models (Fig. 3 I), or by tail vein injection experiment (Fig. 3 J). Thus, similar to IL-17RB, expression of the ligand, IL-17B, also has a critical role in pancreatic cancer malignancy.

Consistent with this notion, treating CFPAC-1 and BxPC3 cells with additional recombinant IL-17B (rIL17B) enhanced colony formation and invasion abilities (Fig. 3,

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Table 1. The correlation between IL-17RB expression and the clinical parameters in 111 pancreatic cancer cases

Parameter	Total *n*	IL-17RB IHC			P-value[a]
		Negative *n* (%)	Low *n* (%)	High *n* (%)	
	111	67 (60)	30 (27)	14 (13)	
Age[b]					
<67 yr	55	34 (62)	14 (25)	7 (13)	0.933
≥67 yr	56	33 (59)	16 (29)	7 (12)	
Gender					
Male	40	23 (58)	13 (33)	4 (10)	0.572
Female	71	44 (62)	17 (24)	10 (14)	
Tumor location[c]					
Head	97	59 (61)	26 (27)	12 (12)	0.963
Tail	14	8 (57)	4 (29)	2 (14)	
Differentiation					
Well	23	15 (65)	7 (30)	1 (4)	0.046
Moderately	75	46 (61)	21 (28)	8 (11)	
Poorly	13	6 (46)	2 (15)	5 (39)	
Pathological type					
PDAC	96	60 (63)	27 (28)	9 (9)	0.071
Others[d]	15	7 (47)	3 (20)	5 (33)	
T stage					
2	4	1 (25)	1 (25)	2 (50)	0.063
3	95	61 (64)	26 (27)	8 (8)	
4	12	5 (42)	3 (25)	4 (33)	
Nodal involvement					
Negative	42	23 (55)	13 (31)	6 (14)	0.642
Positive	69	44 (64)	17 (25)	8 (12)	
Metastasis					
No	107	66 (62)	30 (28)	11 (10)	0.009
Yes	4	1 (25)	0 (0)	3 (75)	
Stage[e]					
I and II	95	61 (64)	27 (28)	7 (7)	<0.001
III and IV	16	6 (37)	3 (19)	7 (44)	

[a]χ^2 was used for statistical analysis.
[b]Median of Age = 67 yr old.
[c]Head including head, neck, and body.
[d]Including 3 mucinous adenocarcinoma, 4 mucinous cystadenocarcinoma, 3 adenosquamous cell carcinoma, 2 acinar cell adenocarcinoma, 1 pleomorphic carcinoma, and 1 undifferentiated carcinoma.
[e]Classified by AJCC staging system.

K–L). Moreover, adding rIL17B increased the colony formation and invasion of SU.86.86 and HPAC cells ectopically expressing IL-17RB, but not cells overexpressing ΔLBD (Fig. 3, M–N). These results suggest a critical role of IL-17B–IL-17RB signal in promoting pancreatic cancer malignancy.

IL-17B–IL-17RB signaling activates chemokines CCL20, CXCL1, IL-8, and TFF1 to enhance pancreatic cancer malignancy

To explore potential downstream targets of IL-17B–IL-17RB signaling, we compared the mRNA expression profiles between shLacZ-transduced and IL-17RB–depleted CFPAC-1 cells, and between lenti-neo and IL-17RB ectopically expressing HPAC cells. As shown in Fig. 4 A, 71 genes were down-regulated in IL-17RB–depleted CFPAC-1 cells but up-regulated in IL-17RB–expressing HPAC cells, indicating that these genes are potential targets of IL-17RB signaling. Among these, the four highest encoded chemokines *CCL20*, *CXCL1*, *IL-8*, and *TFF1* were identified (Fig. 4 B). To further confirm this result, we performed qRT-PCR to measure each mRNA expression in those cells. As shown in Fig. 4 C, IL-17RB–depleted CFPAC-1 showed down-regulation of *CCL20*, *CXCL1*, *IL-8*, and *TFF1* expression, whereas ectopic expression of IL-17RB, but not ΔLBD mutant, in HPAC1 cells resulted in up-regulation of these genes (Fig. 4 C). A similar expression pattern at the protein level was also observed (Fig. 4 D). Using TFF1 as an example of

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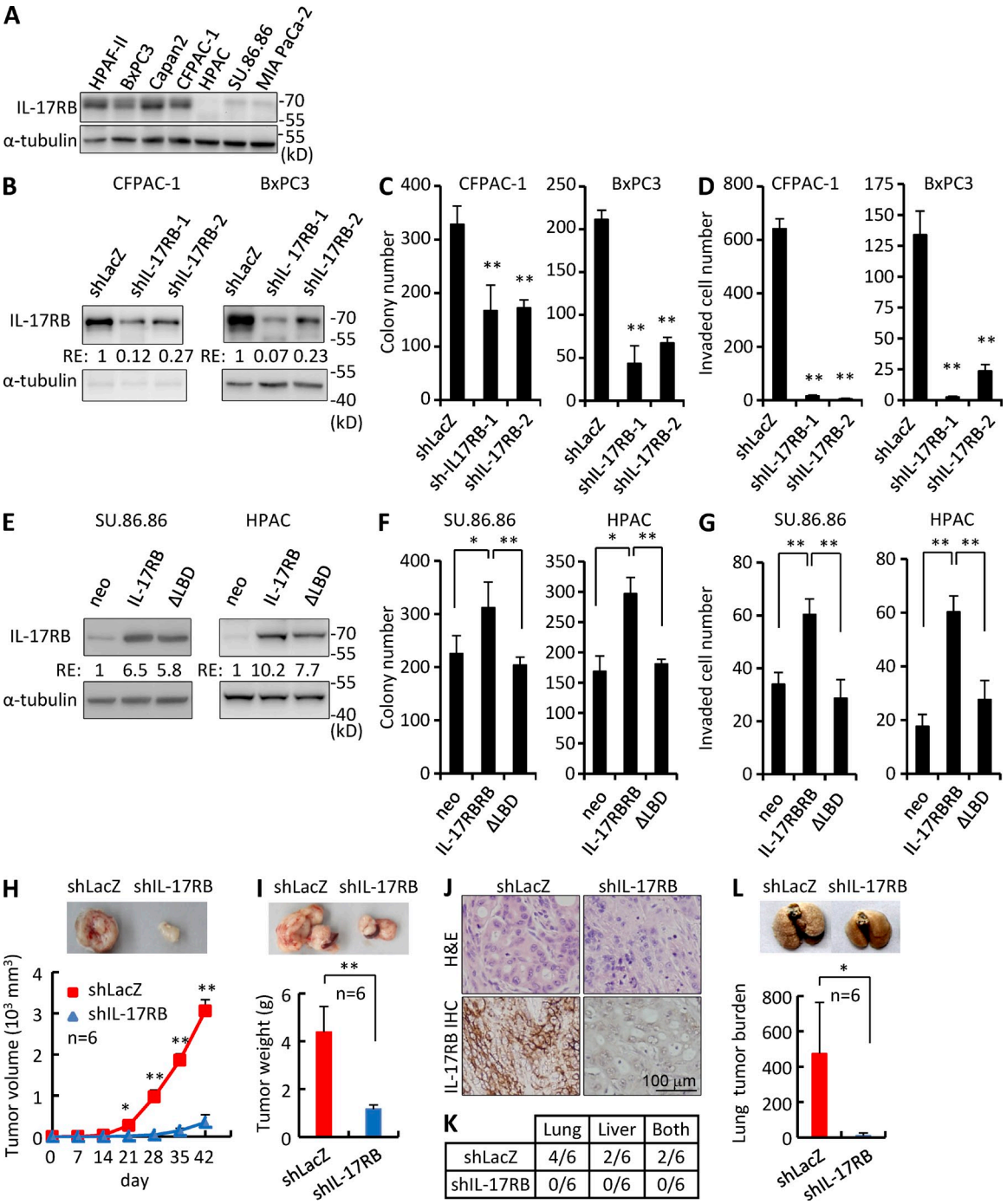

Figure 2. IL-17RB expression has an essential role in tumorigenesis and metastasis of pancreatic tumor cells. (A) Immunoblot analysis of IL-17RB in pancreatic cancer cell lines. α-Tubulin was used as a loading control. (B) IB analysis of IL-17RB in CFPAC-1 and BxPC3 cell lines transduced with lentiviral-control shRNA (shLacZ) or shIL-17RB. Tubulin was used as a loading control. RE, relative expression. (C and D) Soft agar colony formation (SACF) assay (C) and invasion assay (D) using CFPAC-1 and BxPC3 cells infected with shLacZ or shIL-17RB. (E) IB analysis of IL-17RB in control (neo), IL-17RB full-length or ΔLBD overexpressing SU86.86 and HPAC cell lines. Tubulin was used as a loading control. RE, relative expression. (F and G) Assays for soft agar colony formation (SACF; F) and invasion (G) were performed using SU.86.86 and HPAC cells overexpressing full-length or ΔLBD IL-17RB. (H) Tumorigenesis assay of NOD/SCIDγ mice subcutaneously injected with shLacZ-transduced or IL-17RB–depleted CFPAC-1 cells. Cell dose: 1×10^6 cells per mouse. Six mice were used for each group. (I) Tumor weight of NOD/SCIDγ mice orthotopically implanted with shLacZ-transduced or IL-17RB–depleted CFPAC-1 cells. Cell dose: 2.5×10^5 cells per mouse. Six mice were used for each group. (J) IHC of IL-17RB in tumors derived from mice orthotopically implanted with shLacZ-transduced or IL-17RB–depleted CFPAC-1 cells. (K) Summary table of lung and liver metastasis derived from orthotopic xenograft. (L) Lung metastasis of NOD/SCIDγ mice intravenously injected with shLacZ-transduced or IL-17RB–depleted CFPAC-1 cells. Cell dose: 5×10^5 cells per mouse. Six mice were used for each group. Data shown are means ± SD. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test). All experimental data verified in at least two independent experiments.

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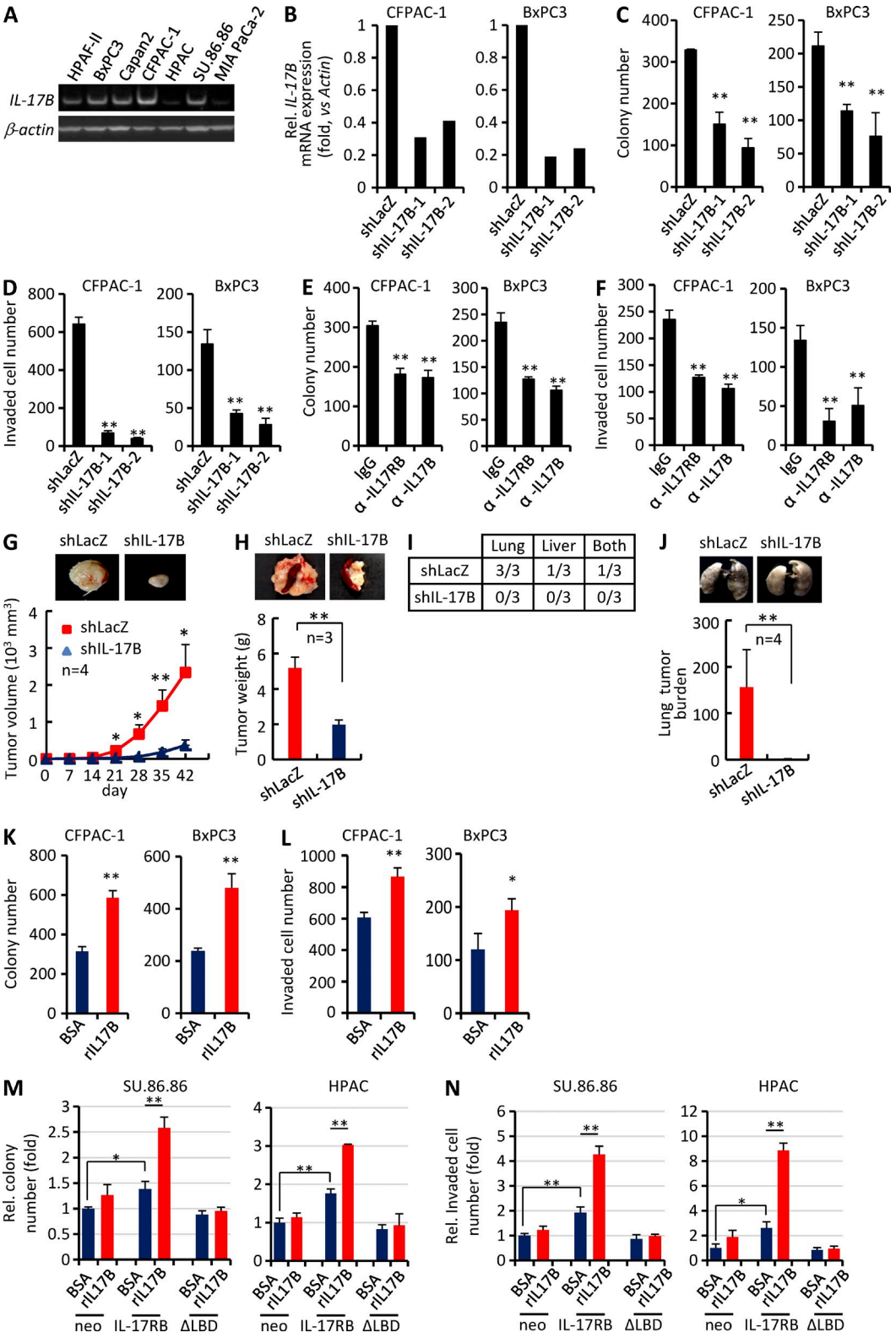

Figure 3. The IL-17B–IL-17RB signaling pathway is essential for tumorigenic and metastatic abilities of pancreatic cancer cell lines. (A) RT-PCR analysis of IL-17B in pancreatic cancer cell lines. β-actin was used as a loading control. (B) mRNA expression of IL-17B determined by RT-qPCR in CFPAC1 and BxPC3 cells infected with shLacZ or shIL-17RB. (C and D) SACF assay (C) and invasion assay (D) using CFPAC-1 and BxPC3 cells infected with shLacZ or shIL-17B. (E and F) SACF assay (E) and invasion assay (F) using CFPAC-1 and BxPC3 cells supplemented with IgG control, anti–IL-17RB, or anti–IL-17B (1 µg/ml). (G) Tumorigenesis assay of NOD/SCIDγ mice subcutaneously injected with shLacZ-transduced or IL-17B–depleted CFPAC-1 cells. Cell

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IL-17RB–regulated chemokines, a positive correlation was observed between IL-17RB and TFF1 expressions in the pancreatic cancer specimens by IHC (Fig. 4, E–F; $P = 0.007$). Consistent with these results, treating CFPAC-1 cells with rIL17B increased mRNA levels of these four chemokines within 3 h (Fig. 4 G). Similarly, increases of these four chemokines were also observed in those cells upon treatment with rIL17B in a dose-dependent manner after serum-starvation (Fig. 4 H). These results suggest that IL-17B–IL-17RB signal activates at least the expression of these four chemokines.

Because chemokines secreted from cancer cells frequently mediate recruitment of the myeloid cells to tumors to support cancer cell survival and to facilitate metastasis (Acharyya et al., 2012), we then examined whether CCL20, CXCL1, IL-8, and TFF1 are downstream factors mediating biological function of IL-17B–IL-17RB signal. First, we observed that depletion of the chemokine expression suppressed the invasion activity, but not the colony formation activity (Fig. 5, A and B). Second, MQ recruitment in mouse lungs injected with the IL-17RB–depleted CFPAC-1 cells was significantly reduced (Fig. 5 C). Importantly, depletion of CCL20, CXCL1, or TFF1 also significantly decreased the percentage of CFPAC-1 cells interacting with MQ (Fig. 5 C); however, the effect was less dramatic than in IL-17RB–depleted cells. Consistent with these results, IL-17RB, CCL20, CXCL1, and TFF1 were all required for pancreatic cancer cell survival in lung metastasis, as depletion of any of these genes significantly increased the proportion of apoptotic CFPAC-1 cells by TUNEL assay (Fig. 5 D). In contrast, IL-8 depletion did not affect cancer cell survival in lung (Fig. 5 D). Instead, depletion of IL-8 significantly decreased the recruitment of $CD31^+$ vasculogenic endothelial cells (Fig. 5 E), presuming that the recruitment is important for angiogenesis and a crucial step in metastasis (DeLisser et al., 1997). Similarly, depletion of IL-17RB, CCL20, CXCL1, or TFF1 also inhibited $CD31^+$ cell recruitment (Fig. 5 E), whereas high IL-17RB expression was significantly correlated with $CD31^+$ microvessel formation by IHC analysis of cancer specimens (Fig. 5, F and G; $P < 0.001$). Together, these results suggest that IL-17RB and its activated downstream chemokines have an important role in pancreatic cancer malignancy (Fig. 5 H).

IL-17B–IL-17RB up-regulates CCL20, CXCL1, TFF1, and IL-8 via activation of ERK signal transducing pathway

To elucidate mechanisms of IL-17B–IL-17RB–mediated chemokine up-regulation, we first compared kinase activation profiles of IL-17RB–depleted CFPAC-1 and IL-17RB–overexpressing HPAC cells using human phosphokinase arrays (Fig. 6, A and B). The analysis showed about a fourfold decrease in extracellular-signal-regulated-kinase (ERK)1/2 activation in IL-17RB–depleted CFPAC-1 cells, whereas there was a fivefold increase in IL-17RB–overexpressing HPAC cells (Fig. 6, C and D), indicating that ERK1/2 activity is positively regulated by IL-17RB. To further confirm this result, we performed direct Western blot analysis using two IL-17RB–depleted cell lines, CFPAC-1 and BxPC3, and two ectopically IL-17RB–overexpressing cell lines, HPAC and SU.86.86, and obtained a similar result (Fig. 6 E). To further test whether ERK1/2 mediates the activation of these chemokines, we added rIL17B to CFPAC-1 and HPAF-II cells and examined the potential signal cascade, including activations of ERK1/2 by phosphorylation, activation of IKK by phosphorylation, activation of NF-κB, and the expression of these chemokines (Fig. 6, F and G). As shown in Fig. 6 (F and G; #1 vs. #4), activation of IL-17B–IL-17RB signaling resulted in ERK1/2 and IKK phosphorylation (Fig. 6 F, #4), subsequent translocation of NF-κB into the nucleus (Fig. 6 H), and increased expressions of the chemokines identified above (Fig. 6 G). Consistent with this, phosphorylation of ERK1/2 and IKK and chemokine expression induced by rIL17B were abolished by the MEK/ERK inhibitor, U0126 (Fig. 6, F and G; #5). When cells were treated with the IKK/NF-κB inhibitor, BAY117082, the phosphorylation of IKK, but not ERK1/2, was diminished (Fig. 6 F, #6). However, chemokine gene expression was only moderately reduced (Fig. 6 G, #6), suggesting that additional factors downstream of IL-17B–IL-17RB–ERK signaling may also contribute to the up-regulation of *CCL2*, *CXCL1*, *TFF1*, and *IL-8* in CFPAC-1 cells. Similar results were observed using HPAF-II cells (unpublished data).

To explore additional factors mediating IL-17B–IL-17RB/ERK signaling regulation of CCL20, CXCL1, TFF1 and IL-8 expression, we examined the promoter regulation of each of these chemokine genes by IL-17B–IL-17RB signaling. Within three hours of rIL17B treatment, the proximal promoter (–1,000 to +1 bp) activities of these chemokines were up-regulated in transient reporter assays (Fig. 6 I), suggesting that these proximal promoters may harbor IL-17B–IL-17RB–responsive elements. We next analyzed these promoters in silico (Heinemeyer et al., 1998; Messeguer et al., 2002; Farré et al., 2003; Karolchik et al., 2004; Fig. 6 J). In addition to the binding sites of NF-κB on the *CCL20*, *CXCL1*, *TFF1*, and *IL-8* promoters, binding sites for two well-known ERK downstream effectors, ATF2 (Lopez-Bergami et al., 2010)

dose: 1×10^6 cells per mouse. Four mice were used for each group. (H) Tumor weight of NOD/SCIDγ mice orthotopically implanted with shLacZ-transduced or IL-17B–depleted CFPAC-1 cells. Cell dose: 2.5×10^5 cells per mouse. Three mice were used for each group. (I) Summary table of lung and liver metastasis derived from orthotopic xenograft. (J) Lung metastasis of NOD/SCIDγ mice intravenously injected with shLacZ-transduced or IL-17B depleted CFPAC-1 cells. Cell dose: 5×10^5 cells per mouse. Four mice were used for each group. (K and L) SACF assay (K) and invasion assay (L) using CFPAC-1 and BxPC3 cells treated with BSA or rIL17B. (M and N) SACF assay (M) and invasion assay (N) using IL-17RB full-length or ΔLBD overexpressing SU.86.86 and HPAC cells treated with BSA or rIL17B. Data shown are means ± SD. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test). All experimental data verified in at least two independent experiments.

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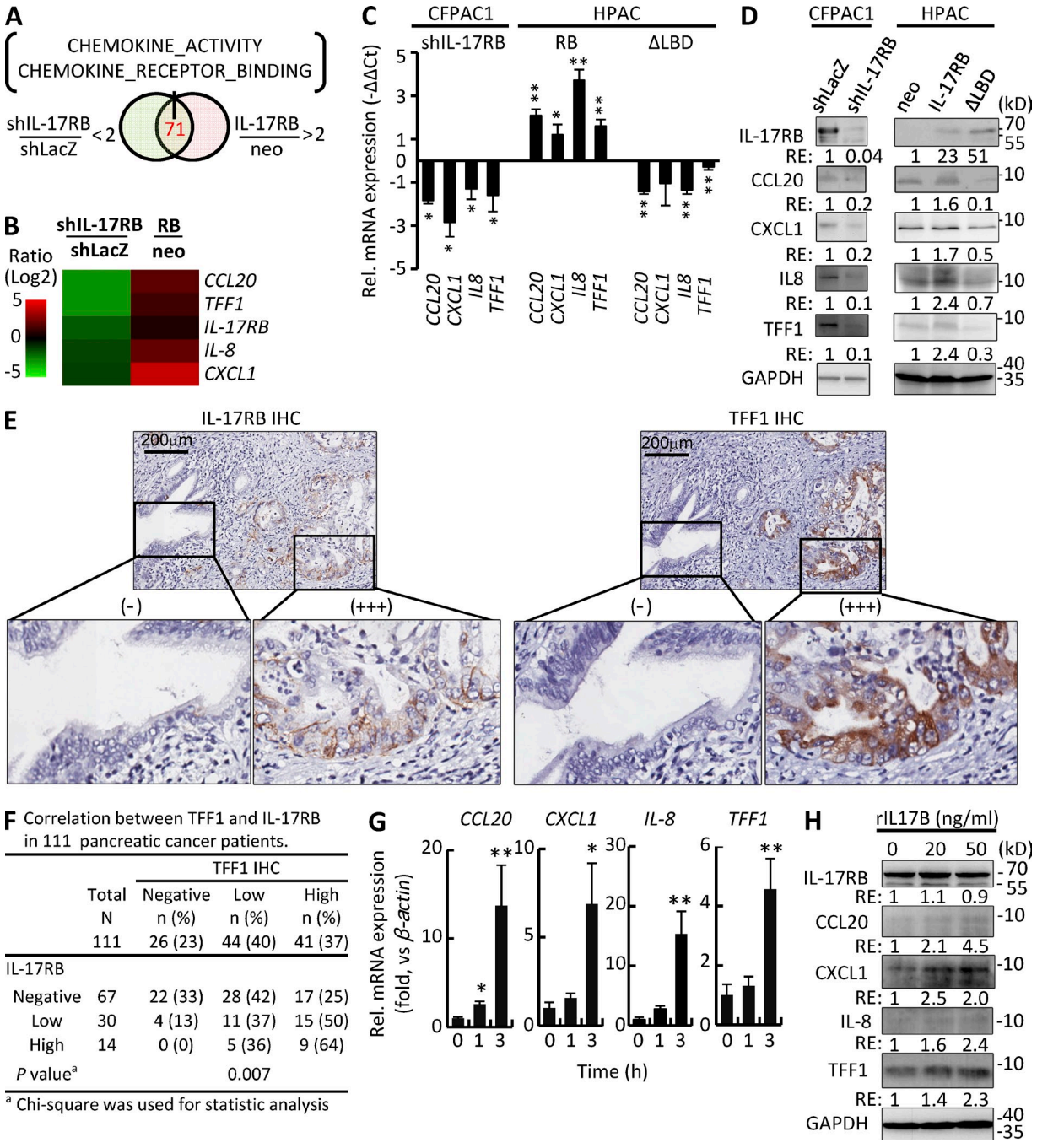

Figure 4. Chemokines CCL20, CXCL1, IL-8, and TFF1 are the downstream targets of the IL-17B–IL-17RB signaling. (A) Summary of cDNA microarray analyses. Total of 71 genes expressed at least 2-fold higher in IL-17RB–overexpressing HPAC cells and 2-fold lower in IL-17RB–depleted CFPAC-1 cells compared with the proper control were identified by Affymetrix microarray analyses. (B) Four chemokines, CCL20, CXCL1, IL-8, and TFF1, were identified among the 71 genes. (C) qRT-PCR analysis to reconfirm the expression profile of the four chemokines in IL-17RB–depleted CFPAC-1, IL-17RB–, and ΔLBD-overexpressing HPAC cells. β-actin was used as an internal control. (D) Immunoblot analysis of IL-17RB and the four chemokines in shLacZ-transduced or IL-17RB–depleted CFPAC-1 cells, and lenti-neo, IL-17RB, or ΔLBD-overexpressing HPAC cells. GAPDH was used as a loading control. RE, relative expression. (E) Representative pictures of the IHC analyses of IL-17RB and TFF1 in serial sections of a PDAC case. Boxes show the enlarged area of IL-17RB high (+++) and negative (−) regions. (F) Correlation between TFF1 and IL-17RB in 111 pancreatic cancer cases from IHC assays. χ^2 test was used. (G) mRNA expression of the four chemokines were measured by RT-qPCR in CFPAC-1 cells treated with 50 ng/ml rIL17B for 0, 1, and 3 h after serum-starvation. β-actin was used as internal control. Data shown are means ± SD. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test). (H) Protein expression of IL-17RB and the four chemokines were measured by immunoblotting in CFPAC-1 cells treated with 0, 20, or 50 ng/ml rIL17B for 6 h after serum starvation, respectively. GAPDH were used as internal controls. RE, relative expression. All experimental data was verified in at least two independent experiments.

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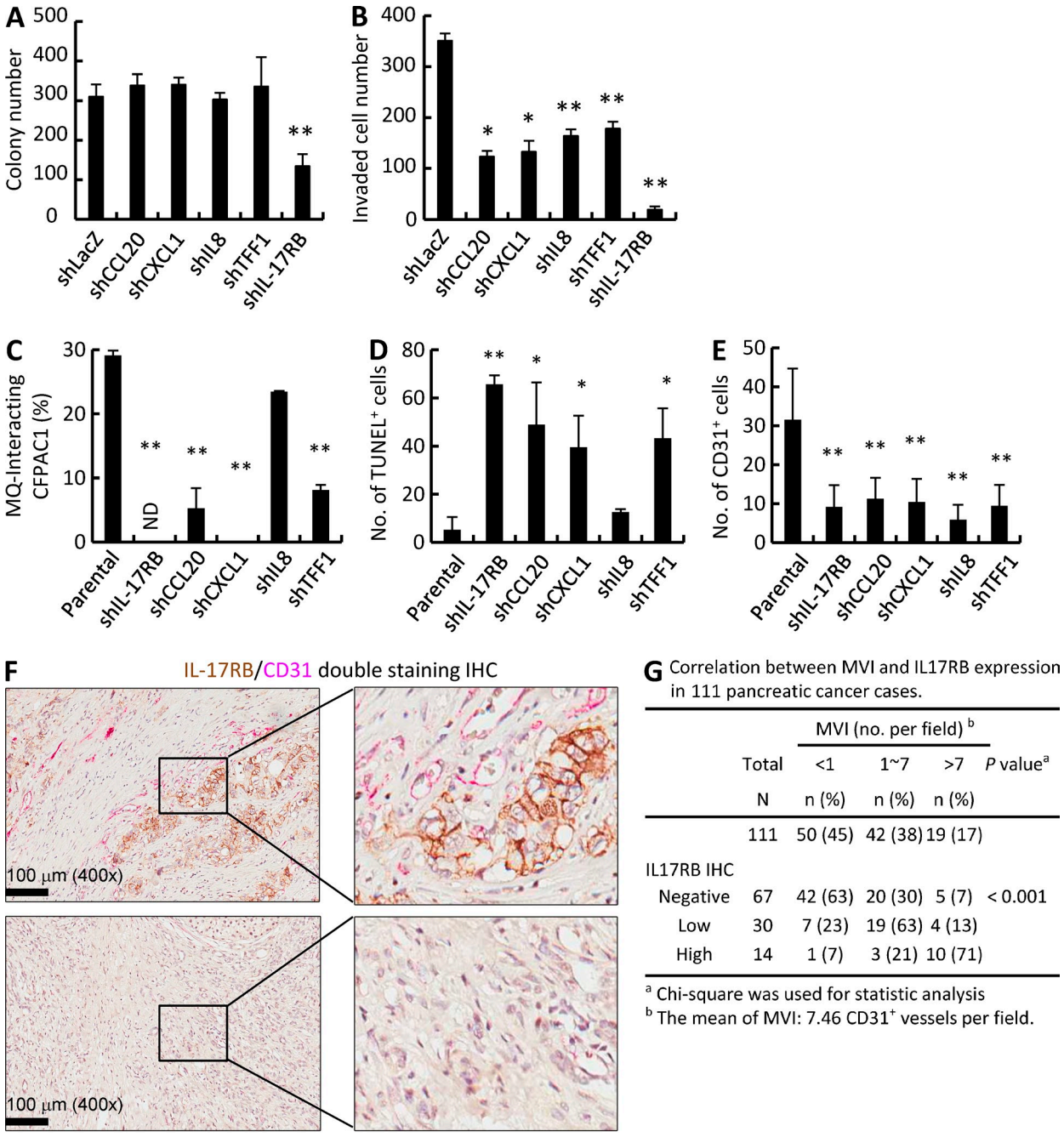

G Correlation between MVI and IL17RB expression in 111 pancreatic cancer cases.

		MVI (no. per field) [b]			
	Total	<1	1~7	>7	*P* value[a]
	N	n (%)	n (%)	n (%)	
	111	50 (45)	42 (38)	19 (17)	
IL17RB IHC					
Negative	67	42 (63)	20 (30)	5 (7)	< 0.001
Low	30	7 (23)	19 (63)	4 (13)	
High	14	1 (7)	3 (21)	10 (71)	

[a] Chi-square was used for statistic analysis
[b] The mean of MVI: 7.46 $CD31^+$ vessels per field.

Figure 5. The downstream chemokines of IL-17B–IL-17RB signaling promote MQ and endothelial cell recruitment for enhancement of cancer cell survival and metastasis. (A and B) Soft agar colony formation assay (A) and invasion assay (B) using CFPAC-1 cells infected with shLacZ, shCCL20, shCXCL1, shIL8, shTFF1, and shIL-17RB. (C and D) Quantification of cancer cells, F4/80-expressing MQs (C) and TUNEL (D) staining in lung of mice 8 and 24 h, respectively, after intravenously injected with parental, or IL-17RB–depleted, GFP/LUC-tagged CFPAC-1 cells from immunofluorescence assays. (E) Quantification of $CD31^+$ cells in tumors derived from mice 2 wk after being orthotopically injected with parental, IL-17RB, CCL20, CXCL1, IL-8, or TFF1-depleted GFP-LUC-tagged CFPAC-1 cells from IHC assays. (F) IHC of IL-17RB (brown) and CD31 (red; used as a marker for microvessel invasion [MVI]) in human pancreatic cancer. Boxes show the enlarged areas. (G) Correlation between IL-17RB expression and MVI in 111 pancreatic cancer cases. Data were derived from IHC analysis (F) and the means ± SD are shown. χ^2 test was used.

and AML1 (Tanaka et al., 1996), were found on the *CCL20*, *CXCL1*, and *TFF1* promoters (Fig. 6 J). Binding sites for another ERK downstream transcription factor, AP1 (Whitmarsh and Davis, 1996), were found on *TFF1* and *IL-8* promoters (Fig. 6 J). Subsequently, we performed reporter activity analysis in response to rIL-17B treatment and found that the full activity responding to rIL17B of *TFF1* was located on the −250 to +1 bp promoter region, which contains the binding

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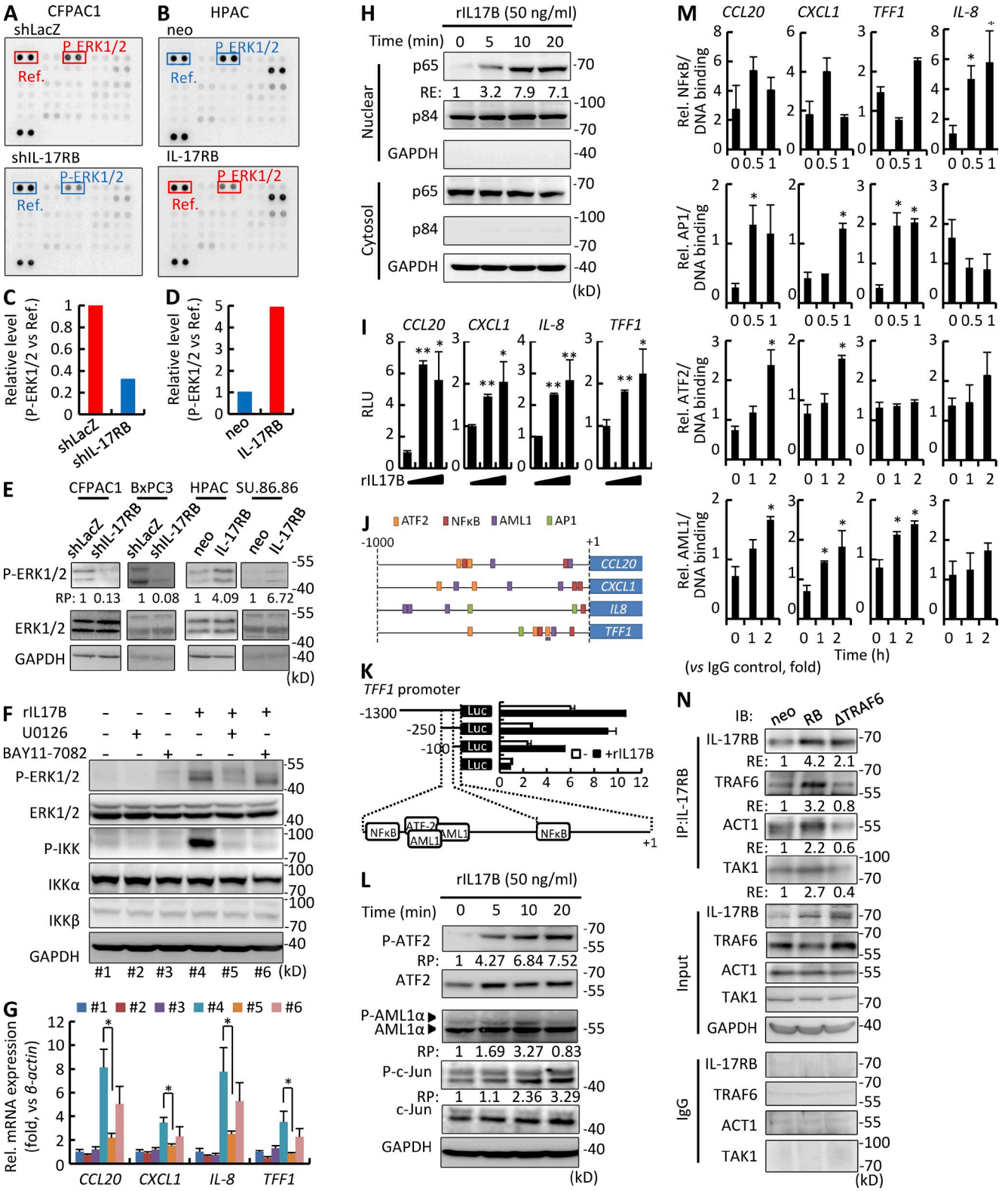

Figure 6. IL-17B–IL-17RB signaling modulates CCL20, CXCL1, TFF1, and IL-8 expression through transcription factors NF-κB, ATF2, AML1, and AP1 via the TRAF6–ACT1–TAK1–ERK1/2 pathway. (A–D) Phospho-kinase array detected ERK1/2 phosphorylation in shLacZ-transduced or IL-17RB–depleted CFPAC-1 cells (A), and lenti-neo or IL-17RB overexpressing HPAC cells (B). Relative phosphorylation level of ERK1/2 protein to reference (Ref) is indicated (C and D). (E) Immunoblot analysis of ERK1/2 phosphorylation in shLacZ-transduced or IL-17RB–depleted CFPAC-1 and BxPC3 cells, and lenti-neo or IL-17RB overexpressing HPAC and SU.86.86 cells. GAPDH was used as a loading control. Relative phosphorylation (RP) level of ERK1/2

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sites of ATF2 and AML1, in addition to NF-κB (Fig. 6 K). Also, upon rIL17B treatment, phosphorylation of ATF2, AML1, and c-Jun (the subunit of AP1) was detected (Fig. 6 L), reflecting a transcriptional activation of *TFF1* gene (Tanaka et al., 1996; Whitmarsh and Davis, 1996; Lopez-Bergami et al., 2010). Similarly, the occupancy of NF-κB quantified by chromatin immunoprecipitation (ChIP) assay increased 4–6-fold in the *IL-8* proximal promoter within 1 h after rIL17B treatment, although it was only slightly increased in *CCL20*, *CXCL1*, and *TFF1* proximal promoters (Fig. 6 M). Interestingly, binding of AP1 and AML1 was detected in *CCL20*, *CXCL1*, and *TFF1* but not *IL-8* proximal promoters, and binding of ATF2 was detected in *CCL20* and *CXCL1* but not *TFF1* or *IL-8* proximal promoters (Fig. 6 M), suggesting that IL-8 may be modulated differently.

Because IL-17RB is known to interact with TNF receptor associated factor 6 (TRAF6) and ACT1 (also known as TRAF3IP2, TRAF3-interacting protein 2; Maezawa et al., 2006), and TRAF6-TAK1 is known to activate the ERK–MAPK signaling for cell survival (Nishimura et al., 2009), it is likely that IL-17B–IL-17RB signal may transduce through this path. To test this possibility, we performed the co-immunoprecipitation assay and found that the interactions of TRAF6, ACT1, and TAK1 (also known as NR2C2, nuclear receptor subfamily 2, group C, member 2) with IL-17RB were enhanced upon ectopic expression of IL-17RB in HPAC cell (Fig. 6 N). Treatment of rIL17B in both CFPAC-1 and HPAF-II cells also increased their interaction (not depicted), whereas the interactions were all diminished when the TRAF6 interaction domain of IL-17RB was deleted (Fig. 6 N). This suggested that the interaction between TRAF6 and IL-17RB was critical for the signal transduction. Collectively, these results suggest that IL-17B–IL-17RB signaling modulates transcriptional activation of CCL20, CXCL1, TFF1, and IL-8 chemokines via coordinated activation of ERK1/2 and its downstream transcription factors.

Treatment with a newly developed monoclonal IL-17RB antibody blocks tumor growth and metastasis, and extends survival in a mouse orthotopic xenografted model

To develop a useful and specific anti–IL-17RB antibody for blocking elevated IL-17RB signal in pancreatic cancer, we used recombinant IL-17RB extracellular domain (amino acids 18–289) that carries only a single N-linked GlcNAc at each of the six glycosylation sites as an immunogen. Two different monoclonal IL-17RB antibodies were obtained and used in this study. The A68 monoclonal antibody was used for Western blotting and IHC, and the D9 monoclonal antibody was used for immunoprecipitation and immunofluorescent staining (unpublished data). Notably, treatment with D9 inhibited both colony formation and invasion abilities of pancreatic cancer cells overexpressing IL-17RB (Fig. 7, A-C). To test the efficacy of D9 in vivo, nonobese diabetic/severe combined immunodeficient *IL2Rγ*^{null} (NOD/SCIDγ) mice were orthotopically implanted with luciferase-labeled CFPAC-1 cells and subjected to 8 doses of D9 or control IgG treatment (1 mg/kg per dose) intravenously (Fig. 7 D), followed by evaluation of the tumor progression. Consistent with the in vitro result, treatment of D9 significantly suppressed tumor growth (Fig. 7, E and F) and lung metastasis (Fig. 7 G). In tumors derived from CFPAC-1–xenografted mice, D9 treatment reduced expression levels of IL-17RB protein (Fig. 7, H and I) and IL-17B–IL-17RB–activated chemokines (Fig. 7, J and K). Importantly, D9 but not mock treatment significantly extended the life span of CFPAC-1–xenografted mice (Fig. 7 L; $P < 0.001$). These results suggest that targeting IL-17RB with specific antibodies is an efficient strategy to suppress malignancy of pancreatic cancer with elevated IL-17RB expression.

DISCUSSION

In this work, we demonstrate that IL-17B–IL-17RB signaling has an essential role in pancreatic tumor growth, invasion and metastasis. This signaling activated downstream transcription

protein in IL-17RB–perturbed cells relative to control is indicated. (F) Immunoblot analysis of ERK1/2 and IKK phosphorylation in CFPAC-1 cells treated with 50 ng/ml rIL17B after pretreatment of 10 µM MEK/ERK inhibitor U0126 and/or 10 µM NF-κB inhibitor BAY117082 in a serum-free condition. GAPDH was used as a loading control. (G) mRNA expressions of *CCL20, CXCL1, TFF1* and *IL-8* were measured by qRT-PCR in CFPAC-1 cells treated rIL17B after pretreatment of 10 µM U0126 and/or 10 µM BAY117082 in a serum free condition. β-*actin* was used as an internal control. Relative expression of chemokines in rIL17B and/or kinase inhibitor treated cells relative to nontreated cells is indicated. (H) IB analysis of NF-κB subunit p65 in nuclear and cytosolic fractions of CFPAC1 cells. p84 was used as a loading control for nuclear fraction and GAPDH was used as a loading control for cytosolic fraction. (I) Reporter assay was performed using rIL17B-treated CFPAC-1 cells transfected with the promoter construct of *CCL20, CXCL1, IL-8*, or *TFF1*. Relative fold-change in luciferase activity (RLU) was shown. Data shown are means ± SD. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test). (J) Diagram shows the predicted binding sites of NF-κB, ATF2, AML1 and/or AP1 on the −1-kb promoter regions of *CCL20, CXCL1, TFF1*, and *IL-8*. (K) Reporter assay using rIL17B-treated CFPAC-1 cells transfected with the segmented promoter construct of *TFF1*. Diagram showed the predicted binding sites of NF-κB, ATF2, and AML1 on the +1–250-bp promoter region of *TFF1*. (L) Time course assay using IB analysis to detect ATF2, AML1a, and c-Jun phosphorylation in CFPAC-1 cells treated with 50 ng/ml rIL17B. GAPDH was used as a loading control. Relative phosphorylation (RP) level of each protein at different time point relative to 0 time point is indicated. (M) Time-course ChIP of NF-κB, AP1, ATF2, or AML1 on the chemokine promoters in CFPAC-1 cells treated with 50 ng/ml rIL17B. Normal immunoglobulin G (IgG) was used as controls for promoter association. (N) Co-IP of IL-17RB, TRAF6, ACT1, and TAK1 in lenti-neo control, IL-17RB, or TRAF6-overexpressing HPAC cells. Normal IgG was used as a control. Relative enrichment (RE) level of IL-17RB-interacting proteins in rIL17B-treated cells at different time points relative to 0 min is indicated. Data shown are means ± SD. All experimental data were verified in at least two independent experiments.

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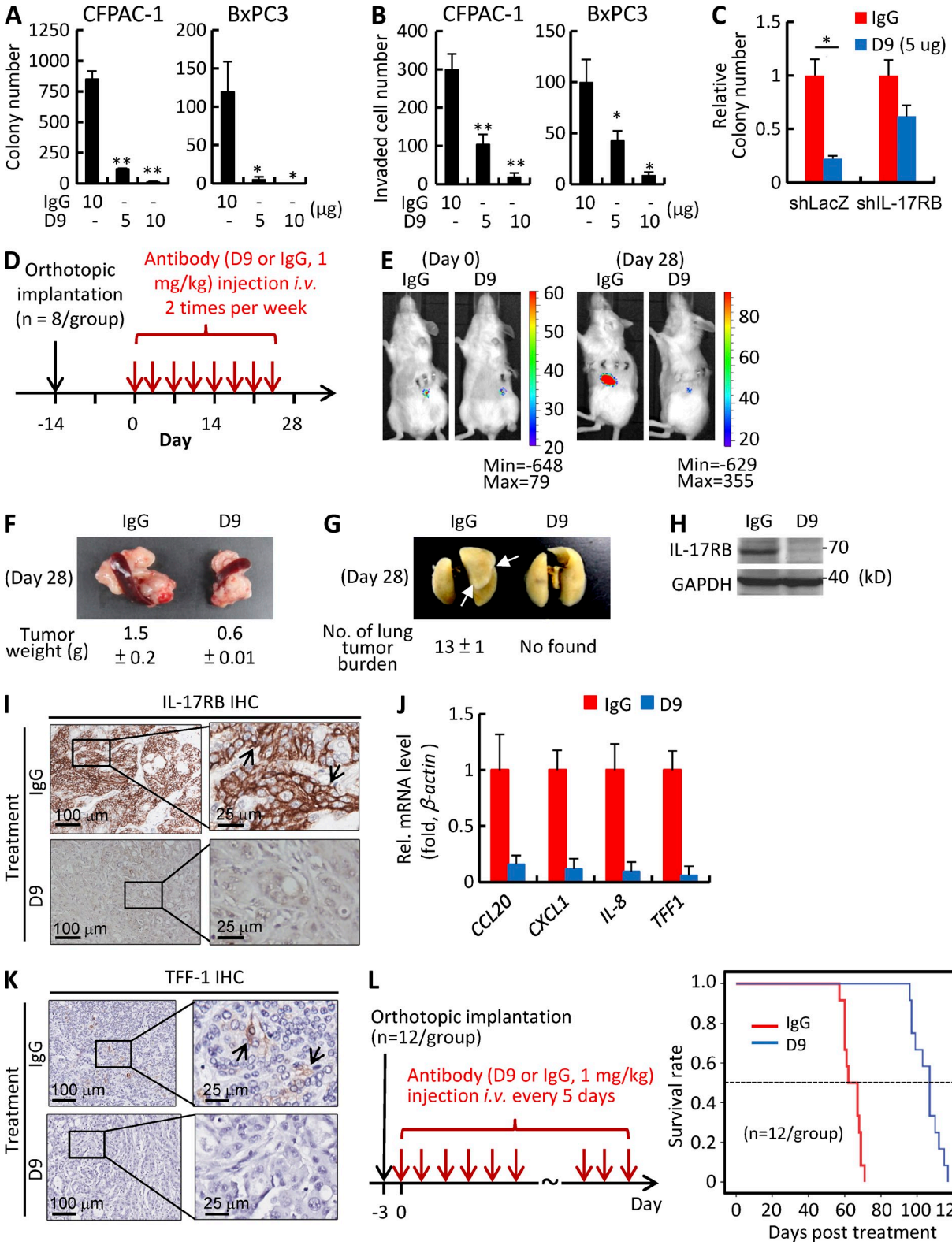

Figure 7. Treatment with a neutralizing monoclonal antibody against IL-17RB blocks tumor growth and metastasis, and extends survivals in a mouse orthotopic xenografted model. (A and B) Soft agar colony formation (A) and invasion (B) assays using CFPAC-1 and BxPC3 cells treated with control IgG or D9 antibody. All experimental data were verified in at least two independent experiments. (C) Relative sensitivity of both control and IL-17RB–KD clones to IgG and anti–IL-17RB in colony formation activities. (D) Schematic diagram of antibody treatment in orthotopically xenografted mice. (E and F) IVIS image (E) and tumor weight (F) of antibody-treated NOD/SCIDγ mice orthotopically implanted with CFPAC-1 cells on day 28. Cell dose:

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factors NF-κB, ATF2, AML1, and AP1 via the TRAF6–ACT1–TAK1–ERK1/2 pathway to induce expression of CCL20, CXCL1, TFF1, and IL-8. Even though there was only an association between IL-17RB expression and MQ recruitment observed in our animal model, these chemokines may contribute to the recruitment of MQ to promote cancer cell local invasion and survival in lung, as well as vasculogenic endothelial cells to facilitate angiogenesis. Clinical evidence suggested that high expression of IL-17RB strongly correlated with advanced pancreatic cancer, postoperative recurrence, and metastasis. Importantly, treatment of a monoclonal antibody recognizing the native form of IL-17RB successfully delayed the malignancy of pancreatic cancer cells expressing IL-17RB and significantly extended animal survival.

Autocrine stimulation of cancer cells and reciprocal paracrine stimulation between cancer cells and stromal cells have been suggested to play crucial roles in tumorigenesis and disease progression (Ben-Baruch, 2006; Raman et al., 2007). The amount of secreted chemokines in the microenvironment and the expression level of the chemokine receptors on cancer cells contribute to the extent of cancer malignancy. In pancreatic cancer, severe desmoplastic response is usually observed around the primary tumor (Olive et al., 2009; Rhim et al., 2014). These stromal cells secret cytokines, growth factors, and angiogenic factors to promote tumor growth (McCawley and Matrisian, 2001) and metastasis (Farrow and Evers, 2002; Ben-Baruch, 2006). Likewise, chemokines induced by IL-17B–IL-17RB as described in this study may also be secreted from stromal cells and participate in MQ and endothelial cell recruitment to promote pancreatic cancer progression. Indeed, we observed that, except for TFF1, which was predominantly expressed in cancer cells, CCL20, CXCL1, and IL-8 were detected in both pancreatic cancer cells and the surrounding stroma, especially in inflammatory cells (unpublished data). TFF1 overexpression in pancreatic cancer cells has been observed to greatly enhance metastasis but not affect primary tumor growth (Arumugam et al., 2011). Increased levels of three other chemokines in the tumor microenvironment have been shown to stimulate tumor growth, migration, invasion, angiogenesis, metastasis, and chemoresistance (Li et al., 2003; Campbell et al., 2005; Fernando et al., 2011; Acharyya et al., 2012). The dynamic interaction between cancer cells and the microenvironment is complicated. Based on our observations, we hypothesize that IL-17B–IL-17RB signaling increases the tumorigenic and metastatic potential in the cancer cell itself first, and then facilitates the reconstitution of its microenvironment (i.e., MQ and vasculogenic endothelial cell recruitment) to be prone to metastasis, in part, by secreting these chemokines. Thus, the IL-17B–IL-17RB signaling emerges as an important regulator of pancreatic cancer growth and metastasis and may serve as an effective immunotherapeutic target (Laheru and Jaffee, 2005).

Up-regulation of chemokines in cancer cells has been attributed to constitutively activated NF-κB in many cancers, including pancreatic cancer (Rayet and Gélinas, 1999; Farrow and Evers, 2002). Besides chemokines, numerous target genes of NF-κB, which promote cell cycle activity, angiogenesis, anti-apoptosis, metastasis, and tumor progression, have been identified (Baldwin, 2001; Tak and Firestein, 2001; Yamamoto and Gaynor, 2001; Karin, 2006). Our study indicated that IL-17B–IL-17RB signaling not only activated transcriptional activity of NF-κB but also induced the expression of three oncogenic transcription factors: ATF2, AML1, and AP1 via ERK1/2 to modulate chemokine expression (Fig. 6). This coordinated regulation of the expression of multiple chemokines places the IL-17B–IL-17RB signaling in the key position of a multifaceted regulatory network for pancreatic cancer progression.

There has been a long-running debate about whether chronic pancreatitis leads to pancreatic cancer because most pancreatic cancer develops spontaneously in the absence of apparent inflammation (Algül et al., 2007). Recently, McAllister et al. (2014) used a murine model of pancreatic intraepithelial neoplasia (PanIN) to demonstrate such an association. They found that recruitment of IL-17A–secreting $CD4^+$ and γδ T cells to the pancreatic microenvironment is required for pancreatic cancer initiation and progression in mice with activated Kras and chronic pancreatitis. In parallel, our study found that IL-17RB expression and IL-17B–IL-17RB signaling is critical for 40–50% of pancreatic tumor growth, invasion, and metastasis. Our data also showed that autocrine/paracrine IL-17B–IL-17RB signaling stimulates at least three chemokines, including IL-8, CXCL1, and CCL20, known to enhance inflammation by recruiting neutrophils, MQ, and lymphocytes (Onishi and Gaffen, 2010), suggesting a similar proinflammatory effect as IL-17A–IL-17RA

2.5×10^5 cells per mouse. Eight mice were used for each group. Two mice from each group were sacrificed on day 28 for measurement of tumor weight and lung metastasis. (G) Lung metastasis of antibody-treated NOD/SCIDγ mice orthotopically injected with CFPAC-1 cells. Cell dose: 2.5×10^5 cells per mouse. Six mice were used for each group. (H) Immunoblot analysis of IL-17RB using tumors derived from antibody-treated mice xenografted with CFPAC-1 cells. GAPDH was used as a loading control. All experimental data verified in at least two independent experiments. (I) Representative pictures of the IHC analyses of IL-17RB in tumors from control IgG or mAb-treated xenografted mice. Bar, 100 µm. Boxes show the enlarged area. (J) mRNA expressions of *CCL20*, *CXCL1*, *IL-8*, and *TFF1* were measured by qRT-PCR in tumors derived from antibody-treated mice xenografted with CFPAC-1 cells. β-actin was used as an internal control. Relative expression of chemokines in D9-treated cells relative to control IgG-treated cells is shown. (K) Representative pictures of the IHC analyses of TFF-1 in tumors from control IgG or mAb-treated xenografted mice. Bar, 100 µm. Boxes show the enlarged area. All experimental data verified in at least two independent experiments. (L) Comparison of the survival periods of the antibody-treated NOD/SCIDγ mice orthotopically injected with CFPAC-1 cells using the Kaplan-Meier method. Data shown are means ± SD. $P < 0.001$. All xenograft experiments were performed once with CFPAC-1 cells and similar results were observed using BxPC3 cells (not depicted).

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signaling in pancreas. Interestingly, whether oncogenic KRAS modulates the expression of IL-17RB in pancreatic cancer cells in a manner similar to IL-17RA (McAllister et al., 2014) is unclear. Because KRAS is mutated and activated in 80–95% of pancreatic cancer, whereas only 40% of the patients are IL-17RB$^+$ (Fig. 1), KRAS may not be the sole pathway regulating IL-17RB expression. This is consistent with the observation that KRAS in most of the pancreatic cancer cell lines is activated, but not every cell line expresses high levels of IL-17RB. It is also noted that BxPC3, one cell line used in this study, expresses wild-type KRAS and still has relatively high expression of IL-17RB. Genetic amplification of IL-17RB was not detected in cancer cells with IL-17RB overexpression. It is likely that other epigenetic factors and posttranslational mechanisms may contribute to IL-17RB up-regulation, which is currently under investigation.

Overall, the data presented here define a novel regulatory pathway mediated by IL-17B–IL-17RB in pancreatic cancer cells. Our findings show that IL-17RB plays a critical role in pancreatic cancer malignancy, especially metastasis, and that the treatment with D9 monoclonal antibody effectively prolongs diseased animal lifespan. This suggests that targeting IL-17RB may be an effective adjuvant treatment. Determining how IL-17RB is up-regulated in pancreatic cancer cells, identifying additional downstream targets involved in metastasis, and pinpointing how IL-17B–IL-17RB participates in pancreatic cancer microenvironment remodeling will help provide a complete picture of this IL-17B–IL-17RB–centered regulatory network. Such information will be essential to further develop therapeutic strategies for treating pancreatic cancers.

MATERIALS AND METHODS

Ethics statement. All pancreatic cancer tissue specimens are from the National Taiwan University Hospital, Taipei, Taiwan. All patients were given informed consent, which was approved by the institutional review board of the NTUH (201303029RINC). Animal care and experiments were approved by the Institutional Animal Care and Utilization Committee of Academia Sinica (IACUC#10-04-065). All data points of cell line experiments were performed in at least triplicate, and all experiments were performed at least two times with similar results. One representative result is shown.

Cell culture. Human pancreatic cancer cell lines HPAF-II, BxPC3, Capan2, CFPAC-1, HPAC, SU.86.86, and MIA PaCa-2 cells were obtained from American Type Culture Collection (ATCC) and cultured in ATCC-suggested complete growth medium in a humidified 37°C incubator supplemented with 5% CO_2. To assess the effect of IL-17B on IL-17RB signaling, cells were serum starved before rIL17B treatment to remove the endogenous IL-17B.

Plasmids and reagents. Lenti-neo control, IL-17RB, ΔLBD, and ΔTRAF6 were cloned as previously described (Huang et al., 2013). The lentiviral shRNA expression vectors of pLKO.1-shLacZ, shIL-17RB (TRCN58814, 58815), shIL17B (TRCN8595, 8596), shCCL20 (mixture of TRCN57963-7), shCXCL1 (mixture of TRCN57939-57941, 371953–4), shTFF1 (mixture of TRCN33614-5, 373750–1, 373674), and shIL8 (mixture of TRCN58028, 58030–1, 232050–1) were purchased from the National RNAi Core Facility (Taipei, Taiwan). *CCL20*, *CXCL1*, *TFF1*, and *IL-8* promoters were amplified from BxPC3 genomic DNA and cloned into a pGL3.basic reporter vector (Promega) using restriction enzymes and primers listed in Table S1. The human Phospho-kinase array was purchased from R&D Systems. MEK kinase inhibitor U0126 was purchased from LC Laboratories and NF-κB inhibitor BAY11-7082 was purchased from Sigma-Aldrich.

RNA isolation, reverse-transcription, (real-time) PCR assay, and gene expression using microarray analysis. Total RNA from cell culture and tumor tissue was isolated using TRIzol reagent (Invitrogen) and reverse-transcribed with Transcriptor First Strand cDNA Synthesis kit (Roche) for gene expression analysis according to instructions from the manufacturers. Quantitative real-time RT–PCR was performed using KAPA SYBR FAST qPCR kit (KAPA Biosystems) for gene expression according to the manufacturer's instruction and analyzed on a StepOnePlus Real-Time PCR system (Applied Biosystems). β-actin mRNA was used as an internal control for mRNA expression. Expression levels were calculated according to the relative ΔC_t method. All primers are listed in Table S2. Affymetrix U133 Plus 2.0 human oligonucleotide microarrays (Phalanx Biotech Group) were used to detect gene expression in IL-17RB–depleted CFPAC-1– and IL-17RB–overexpressing HPAC cells.

Soft agar colony formation assay. Soft agar colony formation assay was performed as previously described (Hwang-Verslues et al., 2009). In brief, 2,500 cells were seeded in a layer of 0.35% agar/complete growth medium over a layer of 0.5% agar/complete growth medium in a well of a 12-well plate. Additional 50 µl of serum-free media containing 50 ng rIL17B or 1 µg anti–IL-17RB antibody was added weekly. On day 14 or 28 after seeding, crystal violet–stained colonies were counted.

Invasion assay. Cells (10^4) were seeded in the top chamber with Matrigel-coated membrane (24-well Falcon HTS Fluoro Block insert; pore size, 8 µm; BD) in serum-free media containing 50 ng/ml rIL17B or 1 µg/ml anti–IL-17RB antibody. Medium supplemented with serum was used as a chemoattractant in the lower chamber. After 48h incubation, the invaded cells were fixed with methanol, stained with 4′,6-diamidino-2-phenylindole (DAPI) and counted with fluorescence microscopy.

IL-17RB antibody production. Recombinant IL-17RB extracellular domain that carried only a single N-linked GlcNAc at each glycosylation sites was generated by ectopic overexpression in a suspension cell culture of *N*-acetylglucosaminyltransferase I-deficient (GnTI$^-$) strain HEK293 cells (Reeves et al., 2002). The resulting N-glycan, GlcNAc2Man5, was then treated with endoglycosidase Endo H to remove residual glycans. Polyclonal antibody (FGRB for Co-IP) and monoclonal antibody (A68 for IB and IHC, D9 for IF and functional analysis) generated through this immunogen displayed a high specificity against endogenous IL-17RB.

Immunoblotting. Immunoblot analysis was performed after 8 or 12% SDS-PAGE, with overnight incubation with a 1:1,000 dilution of primary antibody and followed by a 1:5,000 dilution of horseradish peroxidase–conjugated anti–rabbit, anti–mouse, or anti–goat antibody (GeneTex). Signals were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore). The homemade antibody against IL-17RB (A68) was used. Antibodies against CCL20 (67310), CXCL1 (20326), and IL-8 (6217) were purchased from R&D Systems. Antibodies against TFF1 (EPR3972), TAK1 (GTX107452), p84 (GTX118740), ATF2-phosphoThr71 (E268), ATF2 (E242), IKKα (GTX27609), IKKβ (GTX105690) and p65 (GTX102090) were purchased from Genetex. Antibodies against p-ERK1/2 (4370), ERK1/2 (4695), p-IKK (2687), c-JUN-phosphoSer63 (9261), Akt (9272), and p-Akt (4058) were purchased from Cell Signaling Technology. Antibodies against TRAF6 (Sc-8409), ACT1 (H300), AML1α (N20), and c-JUN (H79) were purchased from Santa Cruz Biotechnology. Protein concentration was determined by the Bradford assay (Bio-Rad Laboratories) before loading and verified by α-tubulin or GAPDH level using a 1:100,000 dilution of anti-tubulin antibody (GeneTex).The optical density was determined using the National Institutes of Health ImageJ program.

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IHC. Formalin-fixed paraffin embedded primary tumor tissue sections were used for IHC. Heat-induced antigen retrieval was performed using 0.1 M citrate buffer, pH 6.0, and autoclaved for 20 min. Endogenous peroxidase was eliminated with 3% H_2O_2. Slides were blocked with a homemade anti–IL-17RB antibody (A68; 1:100) in PBS/10% FBS and anti-TFF1 antibody (1:100, EPR3972; Genetex) or anti-CD31 rabbit polyclonal antibody (1:50, GTX81432) in PBS/5% FBS overnight at 4°C. After washing, slides were incubated with HRP rabbit/mouse polymer before visualization with liquid diaminobenzidine tetrahydrochloride plus substrate DAB chromogen from Dako REAL EnVision. All slides were counterstained with hematoxylin. The images were captured by an Aperio Digital Pathology system. For $CD31^+$ endothelial cell counting in mice, 1 tumor section per mouse ($n = 6$) was used and 4 fields (400×) of each section were examined. 111 pancreatic cancer specimens were used for CD31 and IL-17RB double staining. Four fields (400×) of each case, with IL-17RB negative or high expression were evaluated.

Transient reporter assay. CFPAC-1 cells of 80% confluence were transfected using Lipofectamine 2000 (Invitrogen). Luciferase reporter gene construct (1.6 µg) and 10 ng pGL4-74 *Renilla* luciferase construct (for normalization) were co-transfected per well of a 12-well plate. Six hours after transfection, cells were treated with rIL17B (range from 50 to 200 ng/ml) for 16h. Cell extracts were prepared at 22h after transfection, and the luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega).

ChIP assay. Chromatin immunoprecipitation assay was performed as previously reported (Hwang-Verslues et al., 2013). Immunoprecipitations were performed with anti–NF-κB (GTX102090; GeneTex), anti-ATF2 (E242; GeneTex), anti-AML1 (N20; Santa Cruz Biotechnology), anti-cJUN (H79; Santa Cruz Biotechnology), and corresponding control (immunoglobulin G) antibodies and protein A/G plus-agarose (Santa Cruz Biotechnology). qPCR was performed to detect protein associated promoter regions using primers listed in Table S3.

Co-immunoprecipitation (Co-IP) assays. Whole-cell lysates were prepared using a lysis buffer containing 10 mM Tris-Cl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 2 mM $MgCl_2$, protease and phosphatase inhibitors cocktail (Roche) followed by 30 min end-to-end rotation and centrifugation at 12,000 *g* at 4°C. For Co-IP, 500 µg of the crude whole-cell extract was incubated with 5 µg anti–IL-17RB (FGRB, homemade, polyclonal) or control IgG antibodies at 4°C overnight. Then, 50 µl prewashed protein A/G agarose was added to the mixture and incubated at 4°C for 2 h with gentle agitation. After extensive washing with a diluted lysis buffer (0.01% Triton X-100), IL-17RB–interacting proteins were eluted with SDS buffer and analyzed by immunoblot. In rIL17B treatment experiment, the cells were serum starved before treated with rIL17B.

Tumorigenicity and metastasis assays in mice. For tumorigenicity assay, NOD/SCIDγ mice were injected with 2.5×10^5 GFP-LUC-tagged CFPAC-1 cells orthotopically. IVIS kinetics imaging system (Caliper LifeSciences) was used to monitor tumor growth and metastasis. For subcutaneous implantation, 10^6 GFP-LUC-tagged CFPAC-1 cells were injected. Tumor volumes were evaluated every 7 d. Mice were sacrificed 56 d after orthotopic implantation or 42 d after subcutaneous injection. The tumors were weighed for tumorigenesis evaluation and liver and lung were examined for metastatic cancer cells. For in vivo metastasis assay, 5×10^5 GFP-LUC-tagged CFPAC-1 cells were injected intravenously. The lungs were inspected for tumor burden 70 d after injection.

Immunofluorescence microscopy for paraffin sections. 5×10^5 GFP-LUC-tagged CFPAC-1 cells were injected intravenously per mouse. Six mice for each time point were used. Mice were sacrificed and the lungs were obtained for formalin fixation and paraffin embeds for immunofluorescence 8 or 24 h after injection for MQ counting or TUNEL assay, respectively. Heat-induced antigen retrieval was performed using Trilogy, pH 6.0, and heated at 95°C for 10 min. Slides were blocked with rat anti–mouse F4/80 monoclonal antibody (1:100, CI:A3-11, Genetex), anti-MUC1 mouse monocloncal antibody (1:200; MA1-38211; Thermo Fisher Scientific), or rabbit anti-GFP (1:100; GTX113617) monoclonal antibody in antibody diluent (Dako) overnight at 4°C. After washing, slides were incubated with an appropriate fluorochrome-conjugated secondary antibody (1:100; Alexa Fluor 488 goat-α–rat or Alexa Fluor 647 goat-α–rabbit; Invitrogen). For TUNEL staining, DeadEnd Fluorometric TUNEL System (Promega) was used. Coverslips with stained tissues were then washed with PBS twice, stained with DAPI (1:2,000), and mounted onto glass slides with Dako fluorescent mounting medium and examined with a Leica TCS-SP5-MP-SMD confocal microscope (100× objective) fitted with appropriate fluorescence filters. To determine the numbers of cancer cells directly contacting MQ, 5–8 fields of each slide (×40 objective) and a total of two slides of each mouse were evaluated. For TUNEL assay, two slides of each mouse were used for staining, and 100 cancer cells of each slide were examined. All localization experiments were performed at least twice independently.

Statistical analysis. Except for the clinical correlation and quantification for specific immunoblots, all data are presented as means ± SD, and Student's *t* test was used to compare control and treatment groups. * and ** indicate statistical significance with $P < 0.05$ and < 0.01, respectively. The following analyses were performed using SPSS statistics software: an χ^2 (χ^2) test was used to examine the correlation between IL-17RB expression and the clinical parameters in 111 pancreatic cancer cases. The Kaplan-Meier estimation method was used for overall progression free survival analysis, and a log-rank test was used to compare differences. Univariate and multivariate Cox regression analyses were performed to evaluate the influence of IL-17RB expression on the clinical outcome of pancreatic cancer patients after surgery.

Online supplemental material. Table S1 shows primer sequence and restriction enzyme for cloning. Table S2 shows primer sequence for RT-qPCR. Table S3 shows primer sequence for ChIP. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20141702/DC1.

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Author contributions: H.-H. Wu, W.W. Hwang-Verslues, and W.-Hwa Lee conceived and designed the experiments. H.-H. Wu performed most of the experiments; W.W. Hwang-Verslues, C.-K. Huang, and P.-C. Wei performed some experiments. Y.-M. Jeng and Y.-W. Tien provided the primary pancreatic cancer specimens, and Y.-M. Jeng evaluated the results of IHC analyses. W.-Hwa Lee, J.-Y. Shew, W.-Hsin Lee, C.-L. Chen, and C. Ma produced IL-17RB/B antibodies. E.Y.-H.P. Lee, W.-Hwa Lee, H.-H. Wu, W.W. Hwang-Verslues, and P.-C. Wei analyzed the data. W.W. Hwang-Verslues and W.-Hwa Lee wrote and completed the paper. W.-Hwa Lee supervised the entire project.

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