Journal of Pathology
J Pathol 2009; **219**: 205–213
Published online 1 June 2009 in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/path.2585

Original Paper

The epigenetic regulators Bmi1 and Ring1B are differentially regulated in pancreatitis and pancreatic ductal adenocarcinoma

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No conflicts of interest were declared.

Received: 18 February 2009
Revised: 20 May 2009
Accepted: 25 May 2009

Abstract

Chronic pancreatitis and pancreatic ductal adenocarcinoma (PDAC) are associated with major changes in cell differentiation. These changes may be at the basis of the increased risk for PDAC among patients with chronic pancreatitis. Polycomb proteins are epigenetic silencers expressed in adult stem cells; up-regulation of Polycomb proteins has been reported to occur in a variety of solid tumours such as colon and breast cancer. We hypothesized that Polycomb might play a role in preneoplastic states in the pancreas and in tumour development/progression. To test these ideas, we determined the expression of PRC1 complex proteins (Bmi1 and Ring1b) during pancreatic development and in pancreatic tissue from mouse models of disease: acute and chronic pancreatic injury, duct ligation, and in K-*Ras*^{G12V} conditional knock-in and caerulein-treated K-*Ras*^{G12V} mice. The study was extended to human pancreatic tissue samples. To obtain mechanistic insights, Bmi1 expression in cells undergoing *in vitro* exocrine cell metaplasia and the effects of Bmi1 depletion in an acinar cancer cell line were studied. We found that Bmi1 and Ring1B are expressed in pancreatic exocrine precursor cells during early development and in ductal and islet cells — but not acinar cells — in the adult pancreas. Bmi1 expression was induced in acinar cells during acute injury, in acinar–ductal metaplastic lesions, as well as in pancreatic intraepithelial neoplasia (PanIN) and PDAC. In contrast, Ring1B expression was only significantly and persistently up-regulated in high-grade PanINs and in PDAC. Bmi1 knockdown in cultured acinar tumour cells led to changes in the expression of various digestive enzymes. Our results suggest that Bmi1 and Ring1B are modulated in pancreatic diseases and could contribute differently to tumour development.

Keywords: Polycomb; pancreas; pancreatitis; PDAC; acinoductal metaplasia; immunohistochemistry

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal human cancers. This disease only becomes clinically apparent during the late stages, at a time when it is refractory to conventional chemotherapy and radiotherapy [1]. Therefore, understanding the pathogenesis of the pre-invasive stages of PDAC is critical for developing diagnostic tests that may identify the disease at a time when therapy might be more effective [2].

The pathogenesis of pancreatic cancer includes both genetic and environmental factors. At the genetic level, PDAC usually harbours activating mutations in K-*RAS* and inactivating mutations in *p16*^{INK4A}, *TP53*, and *SMAD4* [3]. Among the environmental factors, cigarette smoking is the best established risk factor [4]. Other risk factors include age, a family history of PDAC [5], and chronic pancreatitis, especially

among individuals with hereditary forms of the disease [6].

A model for pancreatic carcinogenesis has been proposed which describes the progression from precursor lesions termed pancreatic intraepithelial neoplasia (PanIN) to invasive adenocarcinoma [7]. Several mouse models have been developed trying to recapitulate human PDAC progression at the molecular and histological level. Most of them use promoters of genes involved in pancreatic development or in acinar differentiation to drive the expression of oncogenes such as K-*Ras* or *myc*. Recently, knock-in mice in which the expression of an oncogenic K-*Ras* allele can be activated at will have been generated. When K-Ras^{G12D} is expressed in all pancreatic lineages during mouse embryonic development at embryonic day 8.5 (E8.5), the full spectrum of PanIN and PDAC lesions can be reproduced [8]. However, this mouse model does not provide information about the target cell in which K-*Ras* mutation must take place for PDAC to develop. A more refined model that allows this question to be addressed was reported in 2007: the K-Ras^{G12V} oncogenic allele was expressed in acinar and centroacinar cells during embryonic development driven under the control of the Elastase promoter. In this model, the full spectrum of PanINs and invasive PDAC is reproduced, indicating that these cell types can lead to ductal-type tumours [9]. Strikingly, K-*Ras* activation in acinar and centroacinar cells in adult mouse pancreas does not result in tumours, except in those mice chronically treated with caerulein [9]. This important finding is in agreement with the known fact that chronic pancreatitis constitutes a risk factor for PDAC in humans [6].

Chronic pancreatitis is an inflammatory lesion associated with replacement of acinar cells by tubular complexes, a process commonly designated as acinar–ductal metaplasia [10], and an abundant desmoplastic reaction. Epithelial cell changes are, in part, recapitulated *in vitro* when the pancreatic exocrine fraction is placed in culture [11–14]. Additional evidence for pancreatic tissue plasticity comes from the *in vitro* islet-to-duct [15], acinar-to-duct [12,13], and acinar-to-hepatocyte [16] transdifferentiation processes.

Understanding this morphogenetic plasticity of the adult pancreas is essential to unravel the mechanisms involved in both differentiation and cancer. One key family of proteins involved in cell identity maintenance, plasticity, and proliferation is the Polycomb group (PcG). PcG proteins are highly conserved repressors that form multimeric complexes and exert their functions by modifying the chromatin structure and by regulating both post-translational histone modifications and histone mark recognition [17,18]. PcG proteins function in at least two distinct major complexes, PRC1 and PRC2. Among the PRC2 proteins, Ezh2 is a histone H3-K27-methyltransferase [19]. PRC1 recognizes trimethylated H3-K27 via the chromo-domain of the Polycomb protein. PRC1 also contains the RING finger domain proteins Bmi1 and Ring1B, which mediate the mono-ubiquitination of the histone H2A-K119 [19].

Bmi1 was originally identified as a collaborating oncogene in the induction of lymphoma [20,21] and was subsequently reported to be overexpressed in various human cancers [22]. Its oncogenic function has mainly been ascribed to its repressive effect on the *Ink4a/Arf* tumour suppressor locus [23]. However, recent studies have demonstrated that PcG proteins bind to multiple regions of the genome and Bmi1 could therefore have additional targets [24–28].

We hypothesized that changes in PRC1 expression might contribute to cellular identity changes associated with the development of chronic pancreatitis and PDAC. To investigate this possibility, we analysed Bmi1 and Ring1B expression in embryonic and adult normal mouse pancreas, as well as in samples from mice in which experimental pancreatitis was induced or in which PDAC develops upon activation of a mutant K-*Ras* endogenous allele in Elastase-expressing cells. In addition, we analysed BMI1 and RING1B expression in human normal and neoplastic pancreatic tissues. Finally, we assessed the influence of Bmi1 depletion in acinar tumour cells on the expression of selected pancreatic exocrine markers *in vitro*.

Materials and methods

Cell culture and oligofection

Mouse acinar 266.6 cells were grown in D-MEM (Gibco). Details on oligofection can be found in the Supporting information, Supplementary material. Mock and Bmi1 siRNA sequences have been reported elsewhere [29].

Protein extraction, western blot analysis, and immunohistochemistry

Details about the protocols and antibodies used for the detection and quantification of protein expression by western blot and immunohistochemistry can be found in the Supporting information, Supplementary material.

Acute and chronic treatment with caerulein

Acute pancreatitis was induced in male C57BL/6J mice (22–25 g; Charles River Laboratories) by the administration of seven intraperitoneal injections of caerulein (50 μg/kg; Sigma) given 1 h apart; animals were sacrificed at various time points after caerulein administration [30].

Chronic caerulein treatment was performed as previously described [9] with the following modifications: daily intraperitoneal injections of caerulein (0.1 ml of a 50 μg/ml solution in saline, 5 days per week for 1 month) were administered to 1-month-old mice and animals were sacrificed 12 months later [9].

Duct ligation

Duct ligation was performed in adult Wistar rats as previously described [13]. Briefly, ducts draining the splenic part of the rat pancreas were ligated and 7 days later, the gland was collected for analysis [13].

Exocrine cell culture model

The exocrine fraction from adult mouse pancreas was isolated from collagenase-digested pancreas as previously described [31] and cultured in suspension in RPMI-1640 medium for up to 5 days [13].

PDAC and chronic pancreatic injury-associated PDAC experimental models

K-*Ras*$^{+/+}$; *Elas-tTA*; *TetO-Cre* and K-*Ras*$^{+/LSLG12^{Vgeo}}$; *Elas-tTA*; *TetO-Cre* mouse strains were used as previously described [9]. Mice were bred in the absence of doxycycline and the K-*Ras*^{G12V} oncogene was activated in cells expressing the Elastase gene, starting at E16.5 [9].

All experiments were performed according to the International Guiding Principles for Biomedical Research Involving Animals (Council for International Organizations of Medical Sciences).

Human pancreatic cancer samples

Human pancreatic cancer tissues were obtained from the Pathology Department, Hospital del Mar. We constructed a tissue microarray (TMA) with pancreatic tissues from 35 individuals. An expert pathologist evaluated each core to classify the lesions. These procedures were approved by the Ethical Committee for Clinical Research of our institution. Semi-quantification of the immunohistochemical signal was performed as indicated in the Supporting information, Supplementary material. Statistical significance was assessed using the Mann–Whitney *U*-test.

Results

PRC1 expression in normal mouse pancreas

First, we investigated the mRNA expression of PRC1 members (Bmi1, Ring1B, Mel18, and Phc1) in E14.5 mouse embryos, at the time of the secondary transition [32], by using the public database http://www.genepaint.org. This database is a digital atlas of gene expression patterns in the mouse determined by *in situ* hybridization. Whereas Mel18 and Phc1 were essentially undetectable in the pancreas, Bmi1 and Ring1B yielded a regionalized signal, displaying strong expression at the pancreatic branch tips (Figure 1). As expected, Elastase 3B (Ela3b), Amylase 2 (Amy2), and Carboxypeptidase A1 (Cpa1) were also expressed in this region (Figure 1). In contrast, Neurogenin 3 (Ngn3), a marker of endocrine precursor cells, was confined to the trunk of the branching epithelium. Using immunohistochemistry, Bmi1 was also detected in acinar cell nuclei at E15.5 (Figure 2A), in agreement with the *in situ* hybridization data. In contrast, Ring1B could not be detected at the protein level in pancreatic epithelial cells at E15.5, although it could be detected in mesenchymal cells (Figure 2A).

At E17.5, when endocrine cells increase in number and reorganize to form mature islets, Bmi1 became detectable in islet cell nuclei and was detectable only in a few scattered cells within the exocrine compartment (Figure 2B). We then analysed Bmi1 and Ring1B expression in adult pancreas. While both proteins were strongly expressed in endocrine cell nuclei, they were undetectable or weakly expressed in acinar cells (Figure 2C). In addition, ductal cells as well as some scattered cells in the exocrine compartment exhibited Bmi1 expression. Together, these data suggest that Bmi1 and Ring1B expression is dynamically regulated in exocrine and endocrine cells during pancreatic development and differentiation.

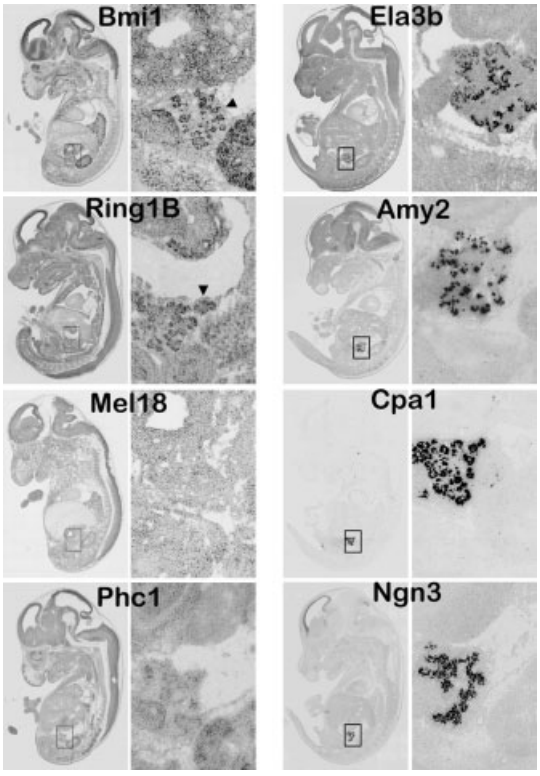

Figure 1. *In situ* hybridization analysis of transcripts for PRC1 components in mouse embryos at E14.5. Left panels: Genepaint *in situ* hybridization detection of Bmi1, Ring1B, Mel18, and Phc1 transcripts in sagittal sections of mouse embryos. Right panels: Genepaint *in situ* hybridization for the exocrine markers Elastase 3b, Amylase 2 and Carboxypeptidase A1, and for the endocrine transcription factor Neurogenin 3. Insets on the right show magnified views of expression in the pancreas. Bmi1 and Ring1B probes label the distal tips (arrowheads), which are also labelled for the exocrine markers Carboxypeptidase A1 and Amylase 2. By contrast, the trunk of the pancreatic branches is largely occupied by Neurogenin 3-positive endocrine cells. Images obtained from the public database http://www.genepaint.org

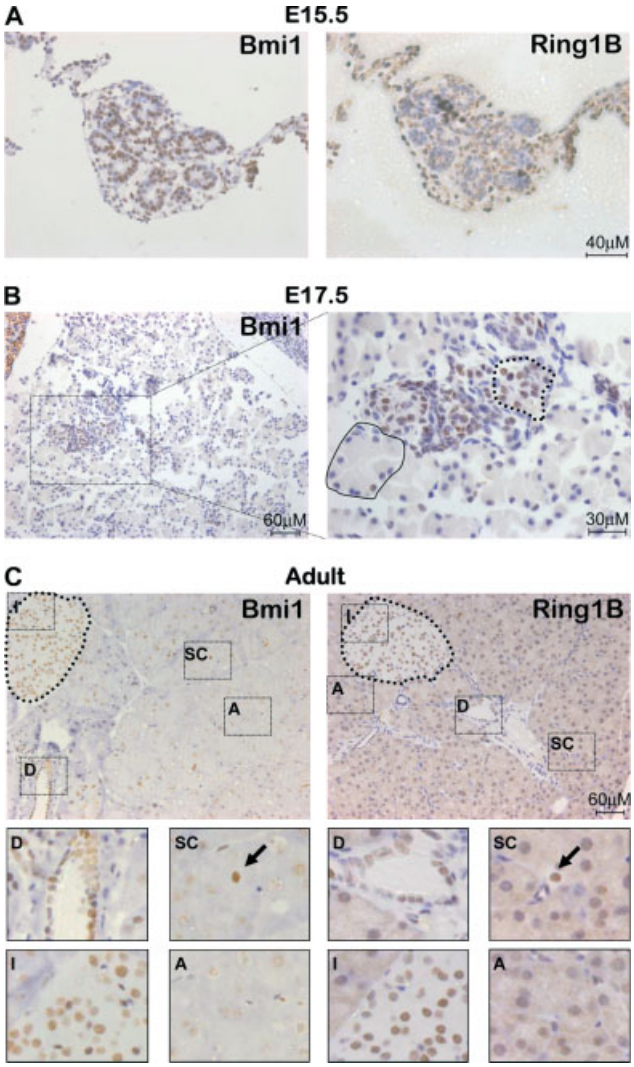

Figure 2. Immunohistochemical analysis of Bmi1 and Ring1B expression in normal mouse pancreas. (A) Sections from formalin-fixed, paraffin-embedded E15.5 mouse embryo pancreas were labelled with Bmi1 or Ring1B antibodies. (B) Immunohistochemistry to detect Bmi1 in whole embryos at E17.5. A higher magnification of the field shown in the left panel is shown. The dashed line indicates endocrine cells and the solid line, acinar/centroacinar cells. (C) Bmi1 and Ring1B expression in adult pancreas. Bmi1 and Ring1B-specific nuclear signal corresponds to the islet compartment, to the ducts, and to some scattered cells within the exocrine compartment. The dashed line indicates islet, I. D = duct; A = acinar cells; SC = Bmi1-positive scattered cells

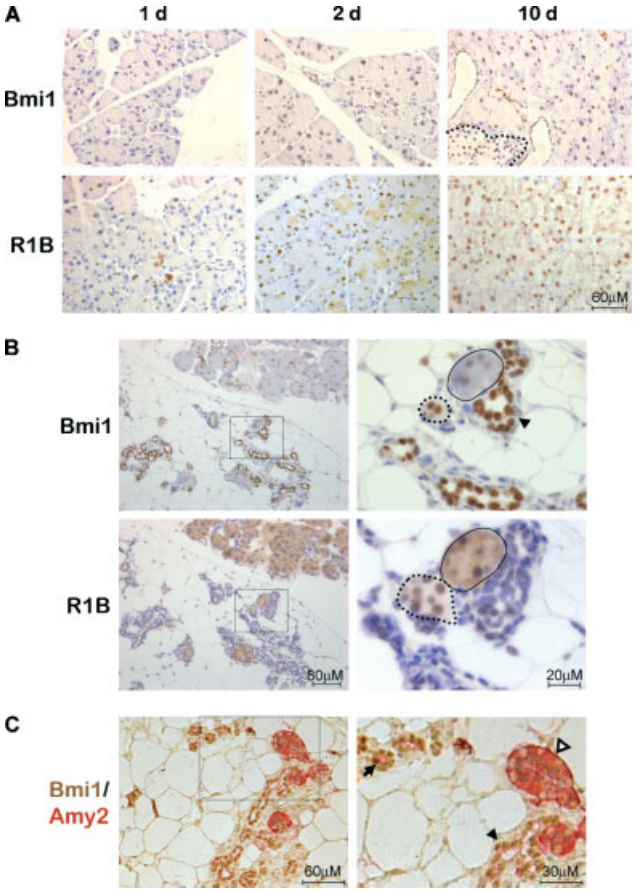

Figure 3. Bmi1 and Ring1B expression in the pancreas from mice acutely or chronically treated with caerulein. (A) Immunohistochemical analysis of Bmi1 and Ring1B expression in the pancreas from mice with caerulein-induced acute pancreatitis euthanized at 1, 2, or 10 days after caerulein administration. The dashed line indicates islet. (B) Bmi1 and Ring1B expression in the pancreas from mice treated with caerulein 5 days per week from the first to the second month of the mouse's life and euthanized at 13 months, at two different magnifications. The solid line indicates acini; the arrowhead, metaplastic duct; and the dashed line, islet cells. (C) Double staining analysis of Bmi1 (brown) and Amylase 2 (red) at two different magnifications of the same field. Empty arrowhead, normal acinus expressing Amylase 2; arrow, atrophic acinar cells expressing Bmi1 and residual Amylase 2; arrowhead, metaplastic duct

PRC1 expression in experimental models of acute and chronic pancreatic injury

We examined Bmi1 and Ring1B expression in mice undergoing experimental caerulein-induced acute pancreatitis. In this model, pancreatic histology and function are completely restored 1 week later [30]. Whereas very subtle changes in the expression of Bmi1 and Ring1B were detected 1 day after caerulein administration, both proteins were induced in more than 50% of acinar cell nuclei at day 2. Importantly, most acinar cell nuclei retained Bmi1 and Ring1B expression 10 days after caerulein administration (Figure 3A).

We used daily caerulein administration as a model of chronic pancreatic injury [9]. Single daily intraperitoneal injections of low-dose caerulein 5 days per week for 1 month cause a mild injury to the pancreas, characterized by panlobular lesions displaying atrophic acini, acinoductal metaplasia, and sparse inflammatory infiltrates. In such conditions, Ring1B was found to be weakly expressed, or undetectable, in the nucleus of the acinar cells and metaplastic ductal cells at 6 months (Figure 3B). By contrast, Bmi1 was readily detected in the nuclei of the atrophic acini and its expression was further enhanced in regions containing acinar-to-ductal metaplasia (Figure 3B). Furthermore, Amy2 and Bmi1 displayed a mutually exclusive expression pattern in atrophic acini and metaplastic ducts (Figure 3C). These data indicate that Bmi1

induction in acinar cells is a shared response to acute and chronic pancreatic injury, and that acinar cells display sustained Bmi1 expression several months after discontinuation of caerulein administration.

Bmi1 expression in acinoductal metaplasia

To determine whether Bmi1 induction is a more general feature of chronic pancreatic injury, we analysed its expression upon pancreatic duct ligation, a well-established model of tissue damage in which acini become metaplastic and convert into duct-like structures [33]. Acinar cells showed very weak Bmi1 expression in the unligated part of the pancreas, whereas Bmi1 was strongly induced in metaplastic areas of the duct ligation-affected regions (Figure 4A).

Next, we investigated Bmi1 expression in an *in vitro* model of exocrine cell metaplasia in which isolated acini are cultured in suspension. In this model, conversion of acinar to duct-like cells occurs spontaneously, similar to the duct ligation model [13]. Whereas in freshly isolated acini only 9% of cells expressed Bmi1, 65% of the metaplastic exocrine cells grown in suspension for 1 day displayed Bmi1 expression, and by day 5 of culture, all of the cells expressed Bmi1 (Figure 4B).

PRC1 expression in pancreatic exocrine cancer models

To test the hypothesis that PRC1 is involved in PDAC development, we took advantage of a conditional knock-in mouse model in which the expression of the endogenous K-Ras^{G12V} oncogene was activated by Cre recombinase at E16.5 in acinar and centroacinar cells [9]. We analysed Bmi1 and Ring1B expression in the pancreas of 12-month-old mice. At this age, 80% of the animals contained multiple PanINs associated with desmoplasia and some of the mice had developed at least one PDAC lesion [9]. As Figure 5A shows, Bmi1 expression ranged from weak in the acini of the transgenic mice to moderate in PanIN and PDAC lesions. These data indicate that Bmi1 expression is up-regulated in preneoplastic PanIN lesions. On the other hand, expression of Ring1B was weak in ductal preneoplastic lesions and strong in PDAC lesions (Figure 5A and Table 1).

Next, we sought to investigate whether Bmi1 and Ring1B expression was linked to PDAC development in the caerulein-associated cancer model [9]. To this end, chronic pancreatic injury was induced in 1-month-old mice with *K-Ras*^{G12V} expression since E16.5 by caerulein administration 5 days per week for 1 month,

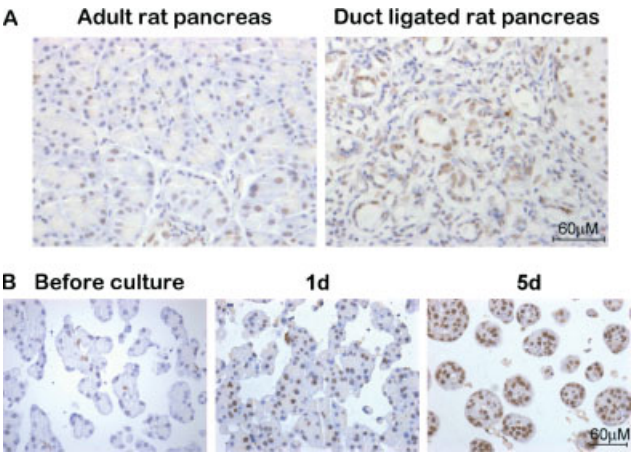

Figure 4. Bmi1 expression in acinoductal metaplasia. (A) Bmi1 expression in sections of normal adult rat pancreas and in duct-ligated pancreas. (B) Bmi1 expression in freshly isolated acini and in acini cultured for 1 or 5 days

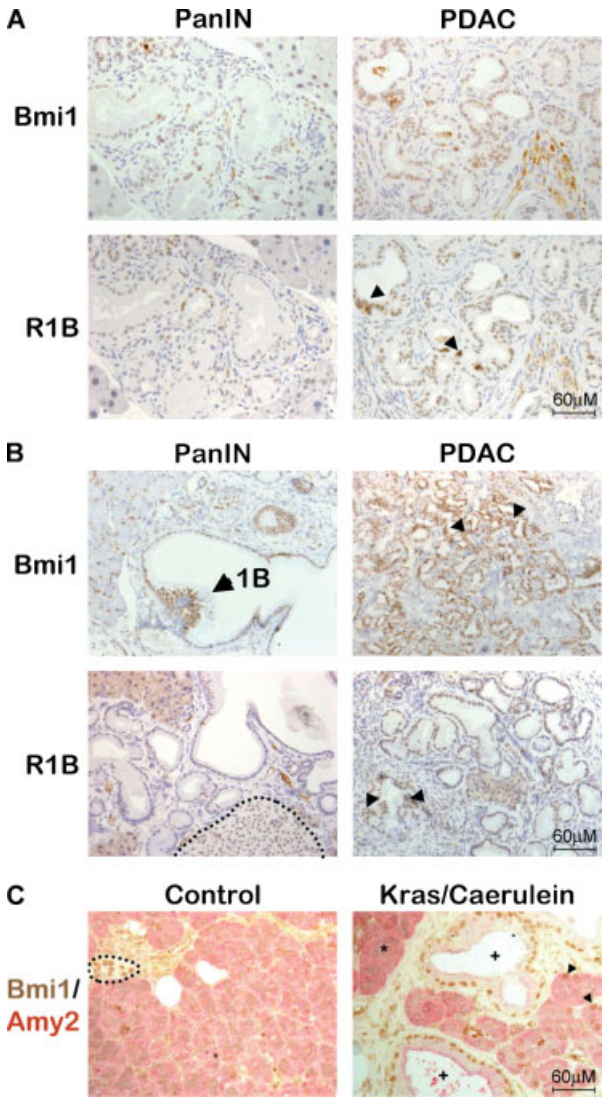

Figure 5. Bmi1 and Ring1B expression in murine experimental models for PDAC and chronic pancreatic injury-associated PDAC. (A, B) Bmi1 (upper panels) and Ring1B (lower panels) expression in pancreatic lesions of K-$Ras^{+/LSLG12Vgeo}$/*Elas-tTA/TetO-Cre* mice (A) and in the pancreas of K-$Ras^{+/LSLG12Vgeo}$/*Elas-tTA/TetO-Cre* mice chronically treated with caerulein (B). In the left panels, low-grade PanIN lesions; in the right panels, PDAC lesions. Arrowheads indicate tumour cells. (C) Double staining analysis of Bmi1 (brown) and Amylase 2 (red) in normal pancreas and in the chronic pancreatic injury-associated PDAC model. Asterisks indicate normal acini expressing Amylase 2; arrowhead, atrophic acinar cells expressing Bmi1 and residual Amylase 2; +, PanIN1 lesions; and dashed lines, islets

Table 1. Semi-quantification of Bmi1 and Ring1B expression in murine experimental models for PDAC and chronic pancreatic injury-associated PDAC. Semi-quantitative analysis of Bmi1 and Ring1B expression detected by immunohistochemistry in pancreatic lesions of K-*Ras*$^{+/LSLG12Vgeo}$ /*Elas-tTA/TetO-Cre* mice and in the pancreas of caerulein chronically-treated K-*Ras*$^{+/LSLG12Vgeo}$/*Elas-tTA/TetO-Cre* mice (see the Materials and methods section for details)

	Bmi1			
	Control	Caerulein	K-*Ras*^{G12V}	K-*Ras*^{G12V}/ caerulein
Acini	0.0 ± 0.0	1.1 ± 0.8	0.9 ± 0.5	0.4 ± 0.4
Atrophic acini		2.0 ± 1.0	1.3 ± 0.7	1.3 ± 0.9
Metaplastic ducts		1.8 ± 0.4	1.7 ± 0.6	1.7 ± 0.7
PanIN 1			1.3 ± 0.6	1.7 ± 0.6
PanIN 2/3			1.8 ± 0.3	2.1 ± 0.4
PDAC			1.8 ± 0.5	2.1 ± 0.7
	Ring1B			
	Control	Caerulein	K-*Ras*^{G12V}	K-*Ras*^{G12V}/ caerulein
Acini	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.3	0.0 ± 0.0
Atrophic acini		0.0 ± 0.0	0.5 ± 0.5	0.0 ± 0.0
Metaplastic ducts		0.2 ± 0.3	1.3 ± 0.6	0.1 ± 0.2
PanIN1			1.2 ± 0.7	0.4 ± 0.4
PanIN2/3			2.4 ± 0.4	1.7 ± 0.5
PDAC			2.7 ± 0.4	2.3 ± 0.5

and mice were sacrificed at 13 months of age. In this experimental condition, a wide spectrum of lesions is observed in the pancreas: acinar hyperplasia, ductal metaplasia, PanINs, and PDAC. Bmi1 was expressed in pancreatic acini of caerulein-treated K-*Ras*^{G12V} mice, in agreement with the findings described above, and its expression was further enhanced in high-grade PanINs and PDAC (Figure 5B, Table 1, and Supporting information, Supplementary Figure 1). As observed in chronically injured pancreas, Bmi1 expression was inversely associated with Amy2 expression, and residual Amy2 staining could be detected in those acini in which Bmi1 was up-regulated (Figure 5C). In contrast, Ring1B expression could be detected only in PanIN2/3 and PDAC (Figure 5B and Table 1).

Bmi1 and Ring1B expression in human pancreas

In an attempt to correlate our findings in the above experimental models with PDAC in humans, we analysed Bmi1 and Ring1B expression in tissues from PDAC patients ($n = 35$) containing a wide range of preneoplastic and neoplastic lesions. As observed in mice, BMI1 expression was significantly up-regulated in areas of chronic pancreatitis when compared with histologically normal exocrine pancreas (1.5 ± 0.4 versus 0.7 ± 0.4; $p < 0.01$) (Figures 6A and 6B). BMI1 expression was up-regulated to a similar extent in preneoplastic ducts and in PDAC, regardless of the level of dysplasia.

In contrast, Ring1B expression was increased in chronic pancreatitis and PanIN1 lesions, but expression was highest in high-grade PanINs and PDAC (2.0 ± 0.8 in PanIN2/3 lesions and 2.1 ± 0.9 in PDAC lesions versus 0.5 ± 0.4 in normal pancreas; $p < 0.001$ for both situations) (Figures 6A and 6B). These data corroborate the findings in the murine PDAC model.

Bmi1 modulates the expression of acinar-specific genes

Next, we analysed whether Bmi1 could modulate the expression of Ptf1a/p48, a pancreatic transcription factor down-regulated during acinar–ductal metaplasia and in experimental pancreatitis. We used the 266.6 acinar cell line [34], which expresses Ptf1a/p48, Elastase, Amylase, and Trypsin [35]. Bmi1 was knocked down using small interference RNAi oligos (siRNA) [29] (Figure 7) and western blotting was performed to detect Ptf1a/p48. Bmi1 depletion (70% knockdown) resulted in a modest trend towards an increase of Ptf1a/p48 expression, in a 2.2-fold increase in Amy2 expression, and in a 1.7-fold decrease in Cpa1 expression (Figure 7). Since Bmi1 is generally associated with gene repression, Cpa1 regulation by Bmi1 must occur by an indirect mechanism. Together these data indicate that Bmi1 depletion impinges on the expression of a limited number of exocrine pancreatic markers, suggesting that Bmi1 can modulate acinar differentiation.

Discussion

In this study, we report the expression of Bmi1 and Ring1B, two members of the PRC1 complex of the Polycomb proteins, in developing and in adult pancreas. At E14.5–15.5, Bmi1 is expressed in exocrine pancreatic progenitors, being down-regulated in differentiated acinar cells in adult pancreas. Bmi1 distribution is reminiscent of the stem cell/progenitor compartment at the tip of the acini [36], a finding that is consistent with the recent demonstration that both mammary and intestinal stem cells are dependent on Bmi1 for self-renewal and maintenance of the differentiated pool of cells [37,38]. In the adult pancreas, Bmi1 expression is down-regulated in acini and becomes restricted to duct, islet cells, and occasional acinar cells, whose nature needs to be further characterized. *Ring1B* mRNA is also expressed at E14.5 in the pancreas but the protein could not be detected at E15.5, suggesting either very low levels of expression or post-transcriptional regulation. Interestingly, it has recently been reported that Ring1B translation depends on a stable IRES present in the 5′ UTR [39].

We focused our studies on Bmi1 and Ring1B expression in exocrine pancreas because most pancreatic tumours display exocrine features. Importantly, we have shown that tissue regeneration associated with

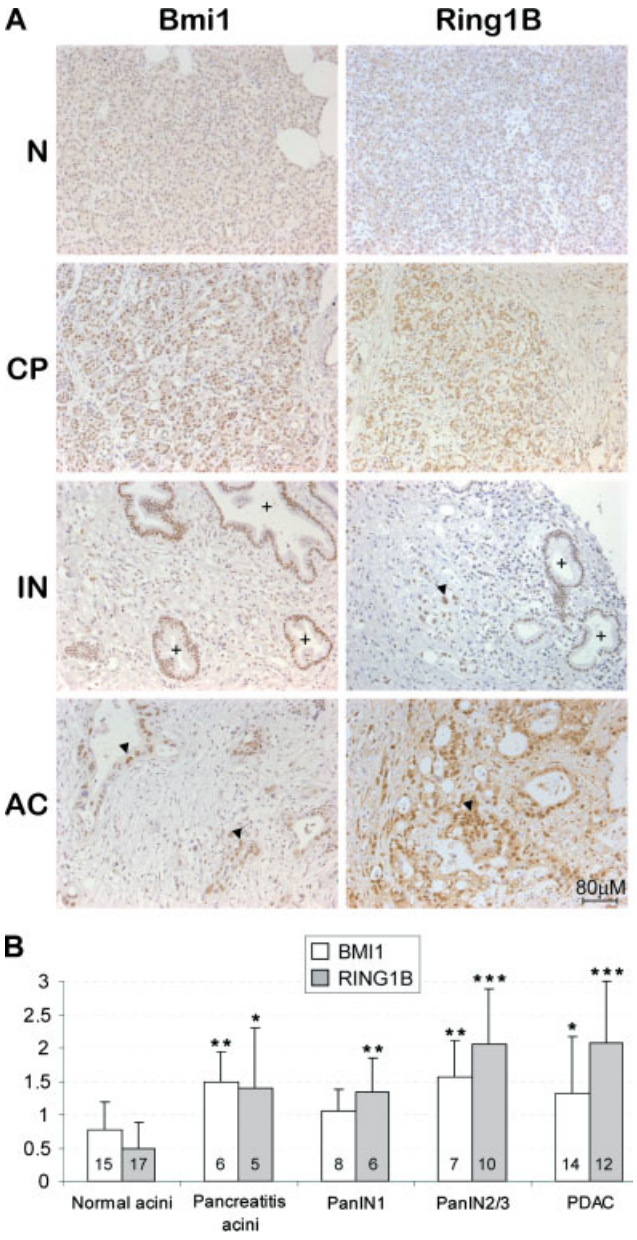

Figure 6. Bmi1 and Ring1B expression in human pancreas. (A) Bmi1 (left) and Ring1B (right) expression in normal (N), chronic pancreatitis (CP), low-grade PanIN lesions (IN), and in PDAC (AC) in human pancreatic samples. += low-grade lesions; arrowheads indicate tumour cells. (B) Quantification of Bmi1 and Ring1B expression detected by immunohistochemistry in human pancreatic tissues (see the Supporting information, Supplementary material for details). The number of samples for each condition is indicated inside the bars. For statistical significance, Bmi1 or Ring1B expression in the different lesions was compared with the expression in normal tissue by the Mann-Whitney *U*-test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

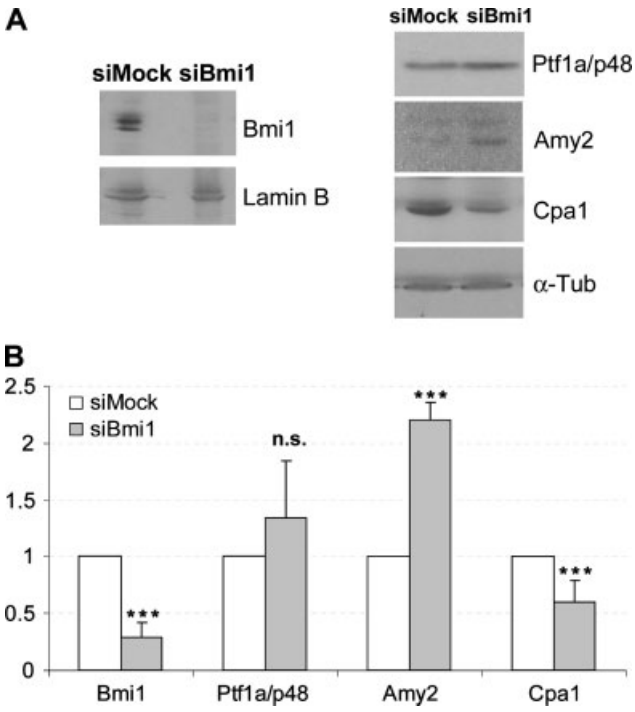

Figure 7. Bmi1 modulates the acinar differentiation programme. (A) Effects of Bmi1 knockdown (left) on the expression of exocrine markers by 266.6 acinar tumour cells (right) analysed by western blot. (B) Densitometric quantification of Bmi1, Ptf1a/p48, Amylase 2, and Carboxypeptidase A1 normalized to Lamin B or α-Tubulin. Data represent the average of at least three experiments and error bars correspond to the standard deviation. Statistics were performed by the Student's *T*-test. $^{***}p < 0.001$; n.s. = non-significant difference ($p > 0.5$)

acute as well as chronic pancreatic injury is associated with an induction of Bmi1 expression, which persists for months. Bmi1 expression in areas of chronic pancreatic damage was associated with acinar–ductal metaplasia, a lesion that has been proposed to have a preneoplastic nature. In these areas, increased cell proliferation has been reported in association with repression of acinar cell differentiation [40]. The role of Bmi1 in cancer is thought to involve escape from senescence or quiescence of premalignant cells by repression of $p16^{Ink4a}$ and/or induction of telomerase [23,41]. Importantly, *p16 INK4a* is one of the major genes involved in PDAC, where it is almost universally inactivated by a variety of genetic mechanisms [42]. There is some evidence that some PanINs arising in chronic pancreatitis show loss of $p16^{INK4a}$ expression [43], suggesting that Bmi1 could play a role in this process. Additionally, Bmi1 could also enhance acinar cell proliferation by repressing Ptf1a/p48 expression, which displays growth inhibitory effects [40].

These data suggest that Bmi1 overexpression in pancreatitis might contribute to an increase in the population of cells susceptible to oncogenic transformation by blocking terminal differentiation and providing relief of cell cycle inhibition by $p16^{INK4a}$ and Ptf1a/p48. This possibility is further supported by the loss of Amy2 expression in Bmi1-positive cells and by the observation that Bmi1 levels specifically impinge on Cpa1 expression in an acinar cell line. The latter result is especially intriguing since Cpa1 has recently been reported to be expressed, together with Pdx1, Ptf1a/p48, and c-Myc, by pancreatic progenitors at E9.5 of mouse development, a stage in which differentiated lineage markers are not yet present [36].

Ring1B, the other Polycomb protein studied, functions as an E3 ubiquitin ligase, while Bmi1 cooperates

in Ring1B-mediated H2A ubiquitylation [44,45]. Our results indicate that both proteins are independently regulated in inflammatory and neoplastic conditions in the pancreas, but could act in concerted action in high-grade PanINs and in PDAC: Bmi1 expression is maintained during pancreatitis, PanINs, and PDAC, whereas Ring1B expression is substantially activated later on in the course of tumour development. Since PanIN1 lesions entail little risk to invasive PDAC, the existence of barriers that prevents them from progressing to PanIN2, the postulated starting point of progressive neoplastic changes that lead to invasive PDAC has been proposed [2,46]. Our results suggest that Ring1B up-regulation in PanIN2/3 and PDAC could be involved in bypassing one or more barriers to neoplasia, such as the oncogene-induced senescence [47].

In conclusion, our work suggests an important role for Polycomb in both early and late steps of pancreatic carcinogenesis. In addition, we propose that these proteins could be crucial for inflammatory preneoplastic conditions. A better understanding of the mechanisms involved therein may provide clues for PDAC prevention in patients at risk, particularly in individuals with sporadic or hereditary pancreatitis.

Acknowledgements

This work should be attributed to Programa de Recerca en Càncer, Institut Municipal d'Investigació Mèdica (IMIM-Hospital del Mar), Parc de Recerca Biomèdica de Barcelona, Doctor Aiguader 88, 08003-Barcelona, Spain. We thank Xavier Sanjuan for microscopy technical support and Sergi Mojal for statistical analysis of the data. IH-M is supported by Instituto de Salud Carlos III. Work in our laboratories is supported by grants from Plan Nacional de I+D, Ministerio de Educación y Ciencia (SAF2007-60860 to FXR); Biomed Programme (LSHB-CT-2006-018771 MOLDIAG-PaCa) to FXR; and Instituto de Salud Carlos III (02/3053 and PI05/2738 to AS; CP04/00292 and PI05/1912 to IH-M). CMR is a recipient of a graduate fellowship from the Ministry of Education and Science. IR is supported for this work by a Marie Curie Intra-European Fellowship (MEIF-CT-023281) and a Marie Curie Reintegration Grant (MERG-CT-2007-204582), as well as a post-doctoral fellowship from the Fund of Scientific Research — Flanders.

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Note: Reference 48 is cited in the Supporting information to this article.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article.

Supporting Materials and Methods.

Figure S1. Bmi1 expression in a normal duct in chronic pancreatic injury-associated PDAC.

Figure S2. Negative control for immunohistochemical analysis.