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Mist1 Regulates Pancreatic Acinar Cell Proliferation through p21$^{CIP1/WAF1}$

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Abstract

Background & Aims—Mist1 is a basic helix-loop-helix (bHLH) transcription factor that is important to the proper development of the exocrine pancreas. The aim of this study was to investigate the role of Mist1 in modulating acinar cell proliferation.

Methods—Ductal and acinar pancreatic cell lines were engineered to express an inducible *Mist1* cDNA or to express an shRNA that targeted endogenous *Mist1*. Alterations in RNA and protein levels were detected by real-time RT-PCR and immunoblots. Chromatin immunoprecipitation and reporter gene assays were performed to map Mist1-responsive elements on target genes; the overall proliferation index of acinar cells from *Mist1* null pancreata was evaluated by immunohistochemistry.

Results—Expression of *Mist1* resulted in a significant decrease in the proliferative potential of cells that was associated with induced expression of $p21^{CIP1/WAF1}$. shRNA-directed knock-down of $p21^{CIP1/WAF1}$ generated cells that were refractory to *Mist1* expression wheras knockdown of *Mist1* transcripts or deletion of *Mist1* from the mouse genome led to increased cell proliferation and a concomitant decrease in $p21^{CIP1/WAF1}$ protein levels. Surprisingly, Mist1-dependent activation of the $p21^{CIP1/WAF1}$ promoter was independent of classic bHLH protein binding sites. Instead, Sp1 binding sites were essential for Mist1-dependent transcription, suggesting that Mist1 activates $p21^{CIP1/WAF1}$ expression through a unique Sp1 pathway. Indeed, coimmunoprecipitation studies demonstrated that Mist1 and Sp1 were found within the same transcription complex.

Conclusions—Our results show that Mist1 has a dual role in the development of the exocrine pancreas - controlling cell proliferation and promoting terminal differentiation.

INTRODUCTION

Mouse models of pancreas development have provided important molecular insights into how individual cell lineages are specified and the role that key regulatory factors have in the genesis of pancreatic diseases including diabetes mellitus, pancreatitis, and pancreatic cancer. The pancreas consists of three major cell types: endocrine cells, acinar cells, and duct cells. The endocrine cells are organized into the Islets of Langerhans, which are responsible for producing hormones that regulate blood glucose levels. Acinar cells synthesize and secrete large amounts

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of digestive enzymes that are transported via an elaborate ductal system to the small intestine to aid in digestion [1, 2]. Development of the three main pancreatic cell types is tightly controlled by a regulatory network of transcription factors that modulate gene expression [3]. Included in this network are homeobox transcription factors (PDX1, Pbx1, HB9), paired-box homeoprotein transcription factors (Pax4, Pax6), Forkhead box transcription factors (Foxa1, Foxa2), and basic helix-loop-helix (bHLH) transcription factors (ptf1a/p48, Mist1, neurogenin3, NeuroD) [2, 4–6]. Loss of these individual regulators often results in complete or partial pancreas agenesis that leads to embryonic or early postnatal lethality [4, 7–9]. Although the importance of each factor in establishing pancreatic cell lineages and regulating terminal differentiation events is well documented, little is understood about how these regulatory pathways maintain proper cell numbers and cellular identity in the adult.

The bHLH transcription factors are particularly critical to development and differentiation events due to the combinatorial nature of these proteins. bHLH factors are classified into two main groups - Class A proteins that include the widely expressed E12/E47/HEB and Class B proteins that display a tissue-restricted expression pattern. In most cases, the preferred bHLH complex is a heterodimer consisting of a Class A member and a Class B member. These heterodimers bind to E-box sites found in the promoter and enhancer regions of target genes to regulate their transcription [10]. Four Class B bHLH proteins have been shown to exhibit a pancreas restricted expression pattern (Neurogenin3, NeuroD, ptf1a/p48, Mist1) [4]. The *Neurogenin3* gene is a downstream target of Notch signaling and is required for the development of all pancreas endocrine cell lineages [9, 11]. *NeuroD*, a downstream target gene of Neurogenin3, serves as a key regulator of *insulin* gene transcription in β cells [12]. In contrast to these endocrine-restricted bHLH factors, *ptf1a/p48* is expressed in early pancreatic progenitor cells but becomes restricted to acinar cells in late embryogenesis and in the adult [8, 13]. Together with the Class A bHLH factor HEB and the vertebrate Suppressor of Hairless factor RBPJL, ptf1a/p48 forms a unique trimeric protein complex that initiates transcription of acinar-specific genes [14–16].

Another bHLH transcription factor that is expressed to high levels in pancreatic acinar cells is Mist1 (also called bhlhb8) [17]. Deletion of the *Mist1* gene ($Mist1^{KO}$) leads to disorganization of the exocrine pancreas, including loss of cellular polarity and defective exocytosis [18]. $Mist1^{KO}$ mice also exhibit an altered stress response and increased sensitivity to caerulein-induced pancreatitis [19]. Unlike most Class B bHLH proteins, Mist1 preferentially forms homodimer complexes that activate gene transcription [20, 21]. The altered acinar cell phenotype in $Mist1^{KO}$ pancreata suggested that Mist1 is critical to the maintenance of the acinar cell lineage. This function could influence a variety of intracellular processes, such as regulated exocytosis, but also might be essential for controlling cell growth decisions that are instrumental in maintaining proper cell numbers in the mature organ. To determine if Mist1 has a role in controlling acinar cell growth, we examined the effects of ectopic Mist1 expression in pancreatic cells. Our results show that expression of Mist1 inhibits cellular proliferation through induction of $p21^{CIP1/WAF1}$. Target gene analysis confirmed that Mist1 regulates $p21^{CIP1/WAF1}$ gene expression and the activation is dependent on specific Sp1 binding sites located within the $p21^{CIP1/WAF1}$ proximal promoter. Analysis of intact pancreata also revealed that $Mist1^{KO}$ acinar cells exhibit a higher proliferative index when compared to control littermates. Importantly, this phenotype could be rescued by ectopic *Mist1* expression. These findings uncover a novel role for Mist1 - serving as a key regulator of terminal differentiation and controlling acinar cell proliferation events in the exocrine pancreas.

MATERIALS AND METHODS

Mouse strains

Control wild type, *Mist1*^{KO} and *elastase*$_{pr}$-*Mist1*^{myc} (*El*$_{pr}$-*Mist1*^{myc}) transgenic mice were maintained on a C57Bl/6 background. All studies were conducted in compliance with NIH and the Purdue University IACUC guidelines.

Cell culture, transfections and viral infections

ARIP and AR42J cells were cultured in 40% F-12 Kaighn's nutrient mixture, 25% F-12 nutrient mixture, 25% high glucose Dulbecco's modified Eagle's medium, 10% fetal bovine serum. To generate the ARIP^{tet}-Mist1 cell line, ARIP cells were electroporated with 18 μg pcDNA6-TR (Invitrogen) and 3 μg pcDNA4-TO-Mist1-Myc/His (Invitrogen). Transfected cells were selected in complete medium containing 5 μg/ml Blasticidin, 200 μg/ml Zeocin. The ARIP^{tet}-Mist1-p21^{RNAi} cell lines were generated by transfecting ARIP^{tet}-Mist1 cells with 20 μg pSuper-p21^{RNAi} constructs and selected in complete medium containing 260 μg/ml G418. The AR42J-Mist1^{RNAi} and control cell lines were generated by electroporating AR42J cells with 20 μg pSuper-Mist1^{RNAi} or pSuper-Control^{RNAi} and selected in complete medium containing 260 μg/ml G418. To restore Mist1 expression, AR42J-Mist1^{RNAi} cells were infected with pBrit or pBrit-Mist1^{ER} viruses in the presence of 8 μg/ml polybrene and selected in complete medium containing 0.8 μg/ml puromycin.

Plasmid constructs

The 2.4 kb *p21*$^{CIP1/WAF1}$ promoter construct was a gift from Dimitris Kardassis [22]. Truncated p21 promoter constructs were cloned into the pGL2 vector (Promega) by standard PCR procedures. Point mutations within the Sp1 sites of the p21 −124/+3 construct were generated using the QuickChange Site-Directed Mutagenesis Kit from Stratagene. *Mist1* and *p21*$^{CIP1/WAF1}$ RNAi sequences that specifically targeted the rat *Mist1* and *p21*$^{CIP1/WAF1}$ transcripts were designed using the Oligoengine program and cloned into the pSuper vector (Oligoengine).

Immunohistochemistry

Paraffin-embedded pancreas sections were processed as previously described [20] and incubated with primary antibodies at 4°C overnight followed by biotinylated secondary antibodies. Primary antibodies included rabbit anti-Mist1 (1:2000), rabbit anti-Myc (Santa Cruz A-14, 1:100), mouse anti-β-gal (Developmental Studies Hybridoma Bank, 1:100), and rabbit anti-phospho-H3 (Upstate 06–570, 1:200).

RNA isolation and real-time RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen). Reverse transcription assays were performed using the Iscript cDNA synthesis kit (Bio-Rad). PCR reactions (20 μl) included SYBR green PCR master mix (Applied Biosystems, 10 μl), 0.5 μM of each PCR primer, 1 μl of cDNA template, and 8 μl of distilled dH_2O. Thermocycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of amplification at 94°C for 15s, 60°C or 63°C for 30s, and 72°C for 30s. Primer sequences for each gene target are available upon request.

Chromatin immunoprecipitation assays

The generation and characterization of the AR42J^{tet}-Mist1~IRES~BirA cell line will be described elsewhere (Jia and Konieczny, in preparation). Cells were transfected with the WT p21-124/+3 or mut 1–4 p21-124/+3 genes and Mist1 expression was induced with 1 μg/ml tetracycline. 48 hours following transfection, cells were subjected to cross-linking using 1% formaldehyde and incubated at room temperature for 10 min with shaking. Cross-link reactions

were stopped by addition of 0.125 M glycine. Nuclear extractions were performed by adding 1 ml of cell lysis buffer (50 mM HEPES, pH 7.9, 1 mM EDTA, 140 mM NaCl, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100) on ice for 30 min. Nuclei were collected and resuspended in 750 µl nuclear lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS). Nuclei were then sonicated three times and soluble nuclear fractions were used for chromatin immunoprecipitation. Briefly, lysates were precleared by incubating 100 µl Protein G Sepharose in binding buffer (0.01% SDS, 1.1% Triton-X 100, 167 mM NaCl, 16.7 mM Tris-HCl, pH 8.1) for 1 hour at 4°C. Precleared lysates were incubated with 20 µl anti-Myc at 4°C overnight. 100 µl of Protein G Sepharose was then added and the incubation continued for 1 hour. Bound protein/DNA complexes were washed and resuspended in TE buffer supplemented with 0.195 M NaCl. Samples were reverse-crosslinked at 65°C overnight and digested with protease K at 56°C for 4 hours. DNA was purified using a Qiaquick PCR purification kit (Qiagen) and used for gene specific PCR.

Pull-down assays

AR42J^{tet}-BT/Mist1~IRES~BirA and AR42J^{tet}-Mist1~IRES~BirA cells were induced with 1 µg/ml tetracycline for 48 hours and nuclear extracts were harvested as previously described [20]. 100 µg nuclear protein was incubated with 10 µl streptavidin-conjugated Dynal beads (Invitrogen) in TBS-N buffer (10 mM Tris-HCl, 150 mM NaCl, 0.3% NP-40) at 4°C for 2 hours. Samples were washed six times with TBS-N buffer and analyzed by SDS-PAGE, followed by immunoblotting using Mist1 and Sp1 (Santa Cruz, 1:1000) antibodies.

RESULTS

Ectopic expression of Mist1 leads to growth inhibition

Previous studies have shown that Mist1 accumulates in pancreatic acinar cells where it controls several acinar-specific functions [18, 23–26]. To examine the effects of mis-expressing Mist1 in non-acinar cells, a pancreatic ductal cell line (ARIP) was used to generate stable clones (ARIP^{tet}-Mist1) that expressed Mist1 upon tetracycline (tet) induction. ARIP^{tet}-Mist1 cells did not express endogenous Mist1 (data not shown) or the Mist1^{myc} transgene when maintained in the absence of tet. However, following tet treatment, Mist1^{myc} was detected by day 1 (Figure 1A). Induction of Mist1 led to a 30% reduction in colony forming efficiency and a 65% decrease in overall colony size when compared to uninduced ARIP^{tet}-Mist1 or control ARIP cells (Figure 1B,C). Mist1 induction also produced a significant decrease in the growth rate of ARIP^{tet}-Mist1 cells (Figure 1D).

To explore the mechanism by which Mist1 exerts its anti-proliferative effects, we next analyzed the expression of key cell cycle regulators in cells treated with or without tet. Although p27, cyclin D, and cyclin A levels remained constant, p21$^{CIP1/WAF1}$ protein levels increased approximately 3-fold in the tet treated cells (Figure 2A,B). Real-time RT-PCR confirmed that *p21$^{CIP1/WAF1}$* transcript levels were elevated in Mist1-expressing cells, whereas no change was observed in *cyclin D*, *cyclin E*, *p16*, *p18*, *p27*, or *p57* transcript levels (Figure 2C). To establish if elevated p21$^{CIP1/WAF1}$ levels were required for Mist1-induced growth inhibition, we generated ARIP^{tet}-Mist1-p21^{RNAi} cell lines in which the endogenous *p21$^{CIP1/WAF1}$* levels were "knocked-down" by *p21$^{CIP1/WAF1}$* shRNA (Figure 2D). Although tet treatment led to the predicted induction of Mist1 expression, p21$^{CIP1/WAF1}$ levels remained undetectable in the p21$^{CIP1/WAF1}$ shRNA lines (Figure 2D). Interestingly, induced Mist1 expression did not affect the growth properties of the ARIP^{tet}-Mist1-p21^{RNAi} cells (Figure 2E), confirming that p21$^{CIP1/WAF1}$ expression was required for Mist1 to induce growth inhibition.

Knock-down of endogenous Mist1 leads to increased cell proliferation and decreased p21$^{CIP1/WAF1}$ levels

The coordinated induction of *p21$^{CIP1/WAF1}$* with *Mist1* expression suggested that Mist1 negatively influenced cell proliferation by transcriptionally activating the *p21$^{CIP1/WAF1}$* gene. To test this hypothesis, we employed a second pancreatic cell line, AR42J, which possesses acinar cell characteristics and accumulates endogenous Mist1 protein (Figure 3A). An AR42J-Mist1^{RNAi} stable cell line was generated in which the endogenous *Mist1* transcript levels were knocked-down by an shRNA transcript that specifically targeted the rat *Mist1* gene. As shown in Figure 3A, expression of *Mist1* RNAi produced an 80% reduction in Mist1 protein levels, whereas nonspecific control RNAi had no effect. Importantly, *Mist1* RNAi also generated a reduction in p21$^{CIP1/WAF1}$ protein levels (Figure 3A). As expected, reduced Mist1 and p21$^{CIP1/WAF1}$ levels permitted a higher proliferation rate and much shorter cell cycle times when compared to the control RNAi cells (Figure 3B; supplemental Table I). Knock-down of endogenous Mist1 led to a significant reduction in the length of G1, indicating that *Mist1* expression primarily impacts the activity of G1 cyclin/CDK complexes. As a final test, a non-targeted form of Mist1 (mouse Mist1^{ER}) was introduced into AR42J-Mist1^{RNAi} cells by viral infection (Figure 3C). As predicted, ectopic *Mist1* expression restored growth restriction to AR42J-Mist1^{RNAi} cells (Figure 3D), confirming that hyper-proliferation of AR42J-Mist1^{RNAi} cells was due to the reduction in Mist1 levels.

Sp1 sites are essential for Mist1-induced p21$^{CIP1/WAF1}$ transcription

The observation that Mist1 influenced pancreatic cell growth and *p21$^{CIP1/WAF1}$* gene expression suggested that Mist1 binds to the regulatory region(s) of target genes to control their transcriptional expression. To test this hypothesis, we examined if Mist1 could activate a p21-luc reporter gene containing −2.4 kb of the *p21$^{CIP1/WAF1}$* promoter (Figure 4A). As shown in Figure 4B, co-transfection of Mist1 and p21-luc produced a large increase in *p21$^{CIP1/WAF1}$* promoter activity. Sequence analysis revealed a total of 16 E-box regulatory elements within the −2.4 kb promoter which could serve as potential Mist1 binding targets (Figure 4A). To eliminate most sites, we next generated the −194/+3 construct containing 1 E-box (E3) and the −124/+3 and −44/+3 constructs containing no E-boxes (Figure 4A). Expression of the −194/+3 and −124/+3 genes was fully induced by Mist1, whereas the −44/+3 promoter region remained transcriptionally inactive (Figure 4B). Thus, the necessary regulatory elements required for Mist1-induced expression lie within the −124 and −44 proximal region. Surprisingly, this 80 bp sequence lacks a putative Mist1 binding site (E-box).

Further analysis of the −124/+3 *p21$^{CIP1/WAF1}$* promoter region revealed 6 Sp1 binding sites that have been implicated in *p21$^{CIP1/WAF1}$* transcriptional expression [22]. To determine their importance for Mist1-induced transcription, point mutations were introduced into each Sp1 binding site (Figure 4C). Luciferase assays revealed that mutations within Sp1 sites 1–4 significantly diminished Mist1-induced expression (Figure 4D). When sites 1–4 were simultaneously mutated (mut 1–4), Mist1-induced activity was completely abolished. As a further test, Sp1 and Mist1 were tested independently and together with the −124/+3 p21-luc reporter gene. As expected, Mist1 and Sp1 individually induced expression of the truncated *p21$^{CIP1/WAF1}$* promoter (Figure 4E), although the response to Sp1 was minimal due to the low Sp1 levels obtained from this expression plasmid. Nonetheless, cotransfection of Mist1 and Sp1 resulted in synergistic activation (Figure 4E), demonstrating that together both transcription factors significantly elevate *p21$^{CIP1/WAF1}$* expression.

Mist1:Sp1 complexes bind to the p21$^{CIP1/WAF1}$ proximal promoter

Our studies support a model where Mist1 interacts with Sp1 to elicit transcriptional activation of *p21$^{CIP1/WAF1}$*. To examine this directly, we performed modified co-immunoprecipitation assays using AR42J cells that contained a tet-inducible Mist1^{myc} or a tet-inducible biotinylated

Mist1^{myc} (BT-Mist1^{myc}) protein. Following tet-induction, nuclear extracts were harvested and subjected to "pull-down" assays using streptavidin-conjugated beads (Figure 5A). As expected, endogenous Mist1 protein co-purified with BT-Mist1^{myc}, confirming that Mist1 forms homodimers *in vivo* [20, 21]. Interestingly, endogenous Sp1 also co-purified with BT-Mist1^{myc}, revealing for the first time the existence of a Mist1:Sp1 complex in pancreatic acinar cells.

The ability of Mist1 to interact with Sp1 and activate *p21$^{CIP1/WAF1}$* gene expression suggested that the Mist1:Sp1 protein complex must associate with the endogenous *p21$^{CIP1/WAF1}$* gene promoter. To test this hypothesis, we next performed chromatin immunoprecipitation (ChIP) assays using our tet-inducible Mist1 cell lines and gene-specific primers. As shown in Figure 5B, binding of Mist1 to the proximal AR42J *p21$^{CIP1/WAF1}$* promoter was enhanced ~3-fold in the +tet group compared to the −tet samples. In contrast, control genes (*actin*, *tubulin*) were not significantly enriched. Importantly, the association of Mist1 with the *p21$^{CIP1/WAF1}$* promoter was completely dependent on intact Sp1 binding sites. Whereas *p21$^{CIP1/WAF1}$* promoter-Mist1 complexes were readily detected by ChIP, no complexes were immunoprecipitated when the Sp1 sites were destroyed (Figure 5C). We conclude that *p21$^{CIP1/WAF1}$* is a direct target of Mist1 and that Sp1 sites are required for interaction with the proximal promoter.

Loss of Mist1 leads to increased acinar cell proliferation in the exocrine pancreas

Our studies have shown that Mist1 regulates cell growth of cultured pancreatic cell lines through induction of *p21$^{CIP1/WAF1}$*. Thus, one would predict that p21$^{CIP1/WAF1}$ levels and cell numbers would be similarly influenced in intact pancreata by the presence or absence of Mist1. To examine this, we first analyzed protein levels of p21$^{CIP1/WAF1}$ in wild type (WT) and *Mist1^{KO}* pancreata [18]. As expected, deletion of *Mist1* resulted in decreased levels of p21$^{CIP1/WAF1}$ (Supplemental Figure 1). Quantification of pancreatic acinar cells in WT and *Mist1^{KO}* mice revealed that adult *Mist1^{KO}* pancreata always contained a 2-fold higher acinar cell content when compared to control WT littermates (Figure 6A,B). Detailed analysis revealed a slight increase in the number of *Mist1^{KO}* acinar cells at 3 weeks of age, with a KO/WT cell ratio of 1.2 (Supplemental Table II). However, as animals aged, the KO/WT ratio increased so that by 3 months *Mist1^{KO}* pancreata had twice the number of acinar cells as control samples. The acinar cell increase in *Mist1^{KO}* pancreata was due to an increased proliferation rate and not due to loss of cells in WT samples (data not shown). This was confirmed by anti-phospho-Histone 3 (p-H3) immunohistochemistry in which *Mist1^{KO}* pancreata exhibited an elevated acinar cell proliferation index (Figure 6C–F; Supplemental Table II). Interestingly, *p21$^{CIP1/WAF1}$* null pancreata exhibited a similar increase in acinar cell numbers (Supplemental Figure 2), supporting the hypothesis that Mist1 and p21$^{CIP1/WAF1}$ are critical regulators of acinar cell growth.

Expression of Mist1^{myc} in the exocrine pancreas leads to lower acinar cell proliferation indices

Our *in vitro* experiments demonstrated that Mist1 controlled the growth potential of pancreatic cells. To test if a similar phenomenon could be observed *in vivo*, we took advantage of *El$_{pr}$-Mist1^{myc}* mice in which acinar cells expressed a *Mist1^{myc}* transgene from the *elastase* promoter (Figure 7A,B). Crossing the *El$_{pr}$-Mist1^{myc}* transgene into the *Mist1^{KO}* line restored the normal acinar-specific growth inhibition that is a hallmark of WT pancreata (Figure 7C). Similarly, WT mice expressing *Mist1^{myc}* exhibited even lower proliferation indices than control littermates lacking the transgene (Figure 7C). These results, combined with our *in vitro* studies, support the hypothesis that Mist1 negatively regulates acinar cell proliferation potency through induction of *p21$^{CIP1/WAF1}$*.

DISCUSSION

Specification of pancreas cell lineages is orchestrated by key transcription factors that play important roles in promoting differentiation and in controlling cell growth. Cell numbers of the endocrine and exocrine compartments must be carefully regulated to insure the proper function of the organ, since misregulation of cell mass often results in pancreatic diseases. Defects in maintenance or expansion of β-cell mass can lead to impaired glucose metabolism and diabetes [27], while improper growth control of the exocrine pancreas is often associated with pancreatitis and pancreatic cancer [28, 29]. Although recent studies suggest that the overall size of the pancreas is controlled by the initial number of embryonic progenitor cells [30], little is known about how cellular proliferation events are regulated in the adult exocrine pancreas.

In this study, we addressed whether Mist1 contributes to maintaining a proper acinar cell population. We found that *Mist1*^{KO} pancreata exhibited elevated acinar cell numbers as well as a higher acinar cell proliferation index. Expression of an acinar specific *Mist1*^{myc} transgene led to decreased proliferation potency. As predicted, ectopic expression of Mist1 in pancreatic cell lines also led to a lower proliferation rate that was coupled to induced *p21*$^{CIP1/WAF1}$ gene expression. The growth inhibition effect of Mist1 was solely dependent on the induction of *p21*$^{CIP1/WAF1}$, suggesting that Mist1 controls *p21*$^{CIP1/WAF1}$ transcription. Indeed, chromatin immunoprecipitation and reporter gene assays confirmed that Mist1 transcriptionally activated *p21*$^{CIP1/WAF1}$ gene expression. Since p21$^{CIP1/WAF1}$ is a cyclin dependent kinase inhibitor and serves to arrest the cell cycle [31], we propose that Mist1 controls acinar cell proliferation through the induction of p21$^{CIP1/WAF1}$.

The classic mechanism by which bHLH transcription factors activate gene transcription is through binding to E-box sequences located in the proximal regions of target promoters. Our results reveal that transcriptional activation of the *p21*$^{CIP1/WAF1}$ promoter by Mist1 is independent of E-box sequences. Indeed, mutations within the proximal Ebox sites E1–E3 resulted in no significant alteration in Mist1-dependent promoter activity (data not shown). Instead, our analysis defined four proximal Sp1 binding sites that were essential for *p21*$^{CIP1/WAF1}$ expression. Previous studies have shown that Sp1 serves as a key regulator of *p21*$^{CIP1/WAF1}$ transcription, not only by activating *p21*$^{CIP1/WAF1}$ gene transcription, but also by functioning as a docking site for additional transcriptional activators and/or repressors including Smad proteins [32], c-Jun [33], and c-Myc [34]. In this study, we showed that a similar mechanism operates in acinar cells in concert with Mist1. Indeed, co-immunoprecipitation and ChIP experiments revealed a common Mist1:Sp1 complex bound to the *p21*$^{CIP1/WAF1}$ promoter, supporting the hypothesis that Mist1 activates *p21*$^{CIP1/WAF1}$ transcription through Sp1. These results offer a unique glimpse into how a tissue-restricted factor (Mist1) influences the activation of the ubiquitous cell cycle machinery in pancreatic acinar cells.

The ability of Mist1 to regulate *p21*$^{CIP1/WAF1}$ expression and cell proliferation is reminiscent of activities reported for other bHLH transcription factors, such as ptf1a/p48 and MyoD [35, 36]. Aside from their essential role in pancreas and muscle development, respectively, these proteins also function to inhibit cell proliferation through activation of the *p21*$^{CIP1/WAF1}$ gene. Additional studies have shown that other bHLH transcription factors, including E12 in osteoblasts [37], NeuroD in neuronal cells [38], and myogenin in muscle cells [39], participate in regulating cell growth through activating transcription of cyclin-dependent kinase inhibitor genes. Thus, members of the bHLH superfamily often control terminal differentiation events by inhibiting cell proliferation. However, Mist1 regulates *p21*$^{CIP1/WAF1}$ gene expression by a mechanism that is distinct from other bHLH proteins. Instead of direct binding to E-box sequences, Mist1 controls *p21*$^{CIP1/WAF1}$ transcription through binding to the ubiquitously expressed transcription factor Sp1.

The antiproliferative activity of Mist1 correlates with its function to serve as a gate keeper to the development of exocrine pancreas diseases such as pancreatitis and pancreatic cancer. For instance, *Mist1*^{KO} mice are hypersensitive to caerulein-induced pancreatitis [19] and recent studies have shown that Mist1 also may be critical to controlling the development of pancreatic ductal adenocarcinoma (PDA). Although PDAs are thought to originate from duct cells upon activation of the *Kras* oncogene [40], several studies have shown that acinar cells can also contribute to the precursor lesions of PDA, termed pancreatic intraepithelial neoplasia (PanIN) [41–43]. This process involves silencing acinar-specific gene expression (*amylase, Mist1*), turning on duct-specific genes (*keratin 19*), and activating the cell cycle machinery. In heterogeneous acinar-ductal metaplastic units, acinar cells that remain Mist1 positive are growth arrested, while cells exhibiting a ductal phenotype are Mist1 negative and highly proliferative [42]. Activating the Kras pathway within Mist1 expressing cells leads to the rapid formation of PanINs, but this process is dramatically accelerated when tested in a *Mist1*^{KO} background (L. Zhu, G. Shi, and S. Konieczny, unpublished data). These findings suggest that Mist1 also may function as a key anti-cancer factor. Future studies will be designed to elucidate the regulatory pathways that impinge on Mist1 activity in normal pancreas function as well as in disease states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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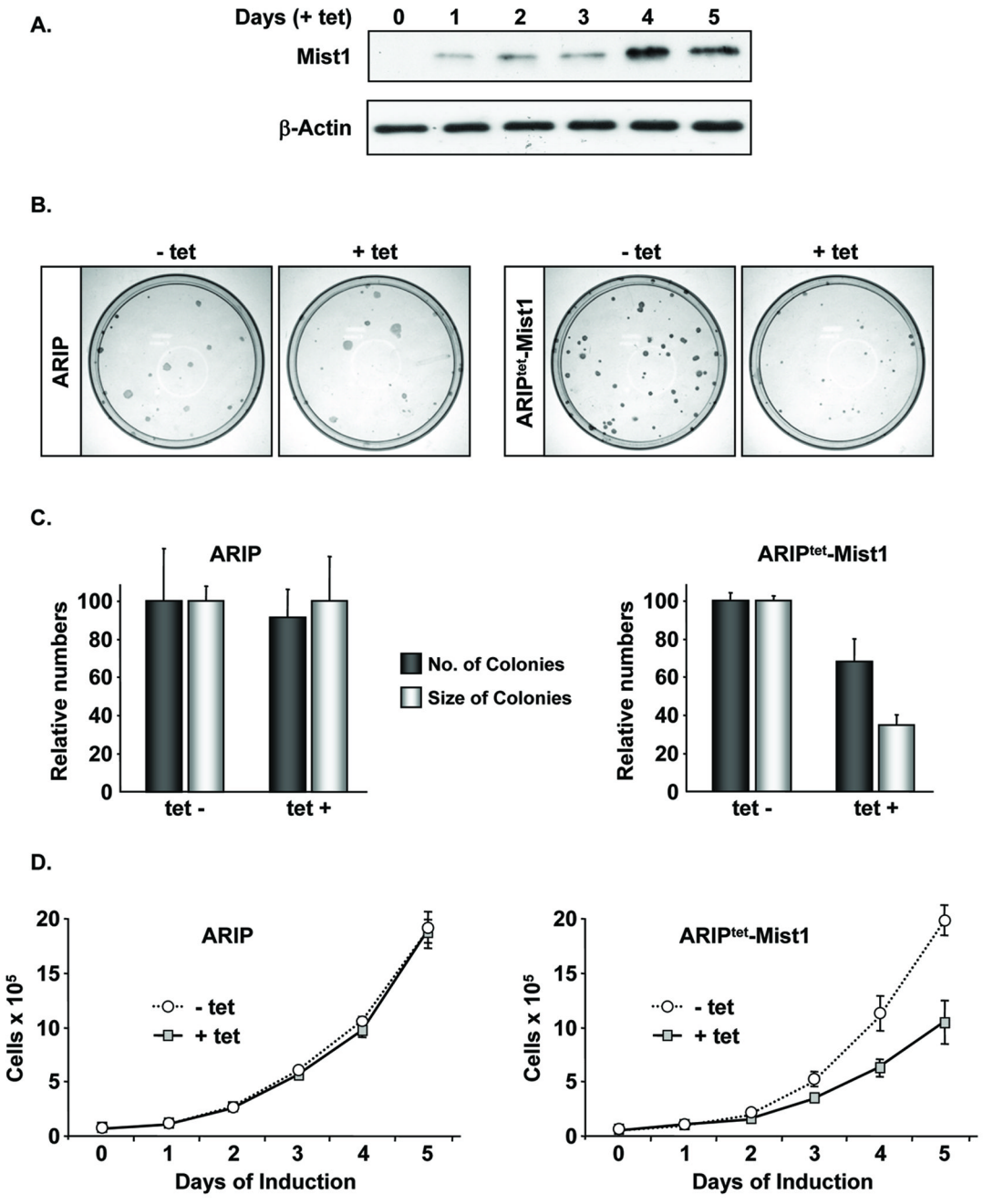
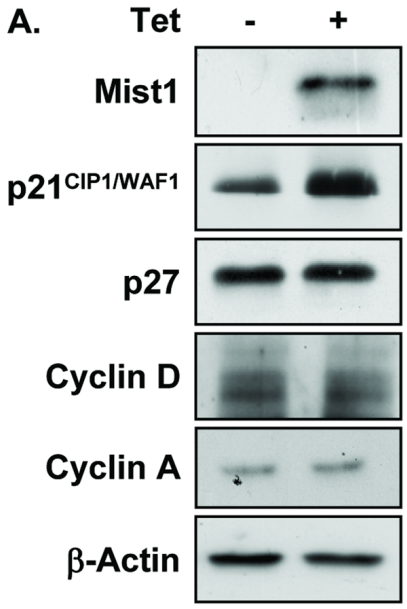
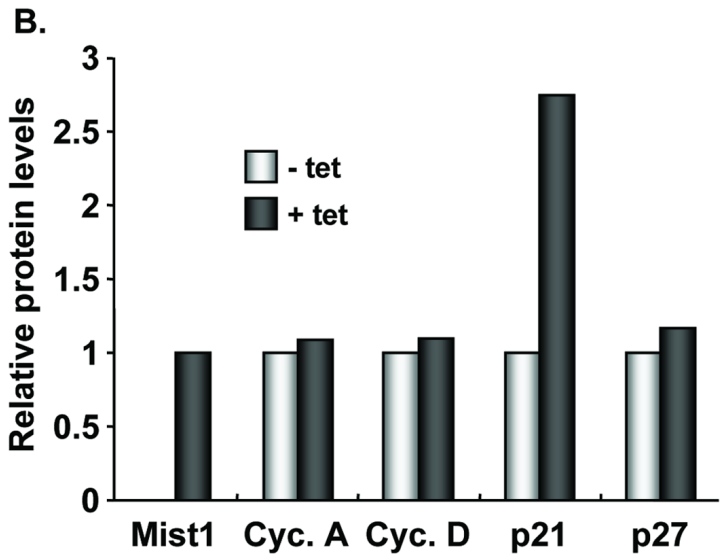
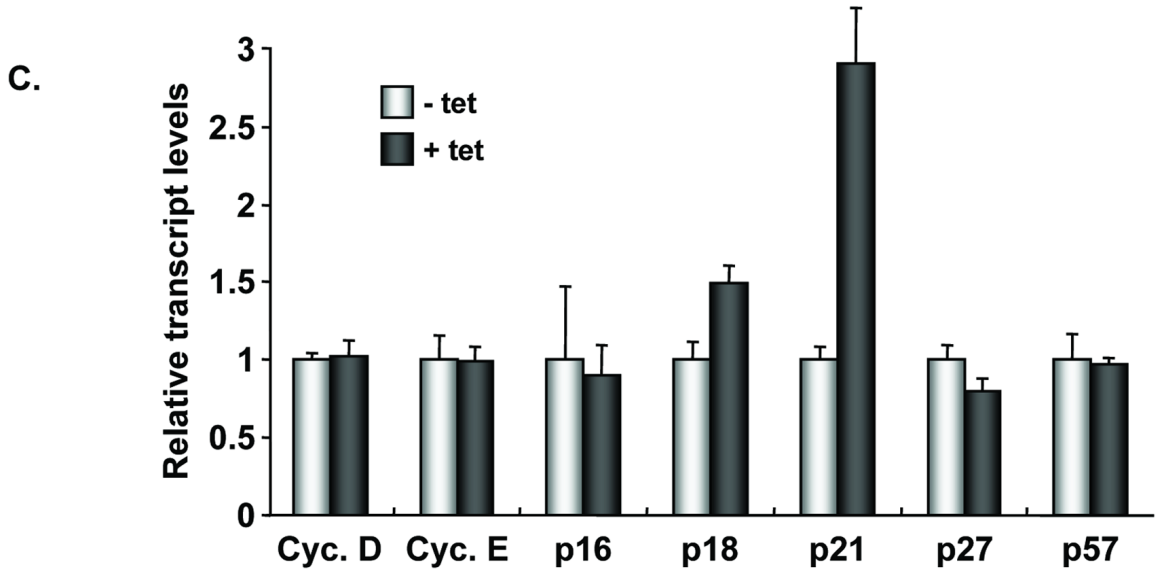


Figure 1. Cell growth inhibition of pancreatic cells by ectopic expression of Mist1

(**A**) ARIP^{tet}-Mist1 cells were induced on day 0 by tetracycline (tet) addition and whole cell extracts were harvested daily. Immunoblots were performed using anti-myc to detect Mist1^{myc}. (**B,C**) Parental ARIP and ARIP^{tet}-Mist1 cells were cultured +/− tet for 2 weeks and colonies were stained and quantified. Values represent the average of 3 independent experiments ± s.d. (**D**) ARIP or ARIP^{tet}-Mist1 cells were treated +/− tet as indicated and cell numbers were determined over a 5-day period. Each time point corresponds to the average of 3 independent experiments (± s.d.).







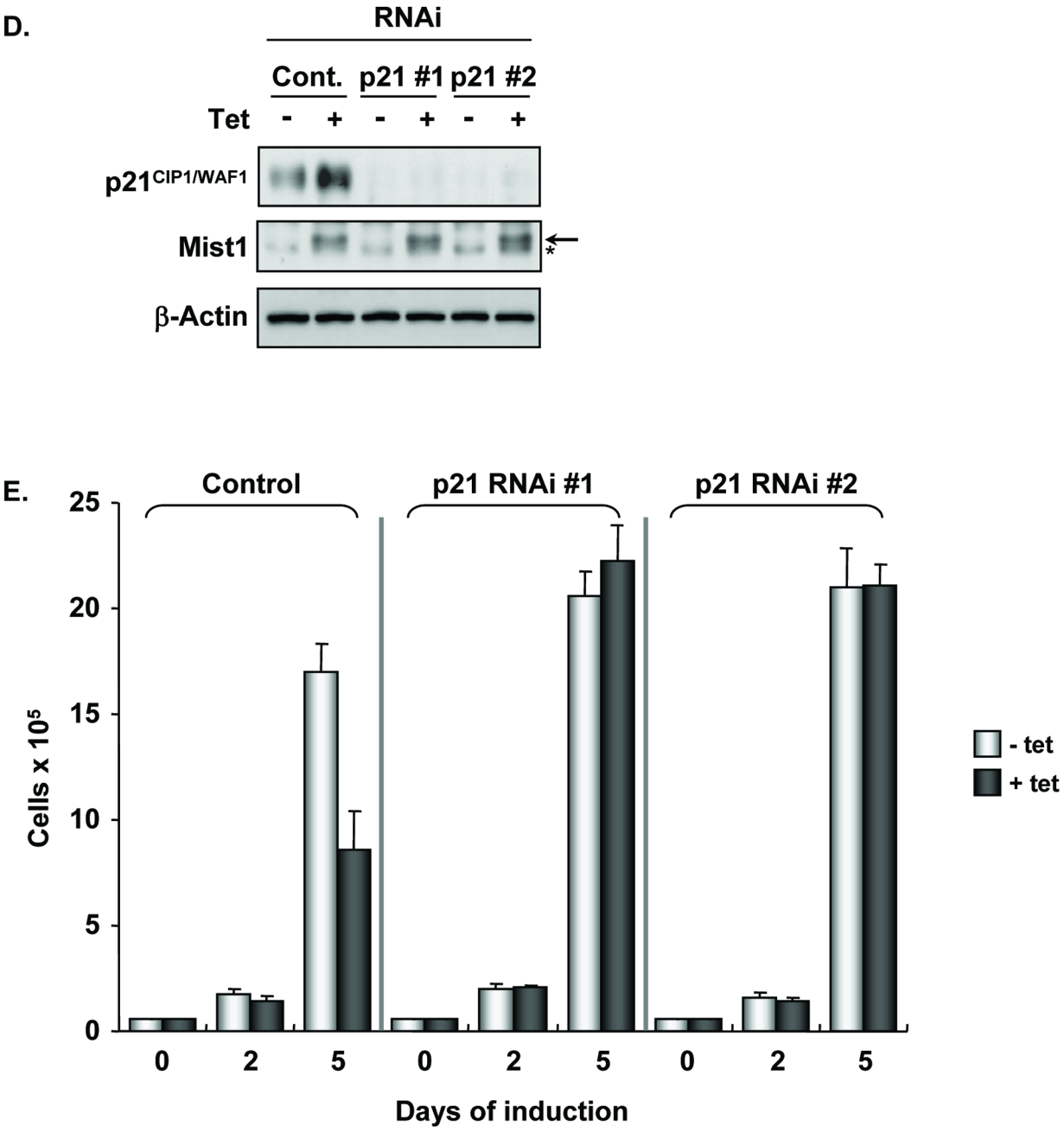


Figure 2. Mist1 inhibits cell proliferation through induction of p21$^{CIP1/WAF1}$

(**A,B**) Immunoblots of cell cycle regulators from ARIP^{tet}-Mist1 cells +/− tet. (**C**) Real-time RT-PCR analysis of ARIP^{tet}-Mist1 cells treated +/− tet. (**D**) Immunoblots of ARIP^{tet}-Mist1-p21^{RNAi} cell lines showing that p21$^{CIP1/WAF1}$ protein levels are undetectable in the presence or absence of tet (arrow, Mist1 protein; asterisk, nonspecific band). (**E**) Control and ARIP^{tet}-Mist1-p21^{RNAi} cell lines were treated +/− tet and cell numbers were determined over a 5-day period. Each time point corresponds to the average of 3 independent experiments (± s.d.).

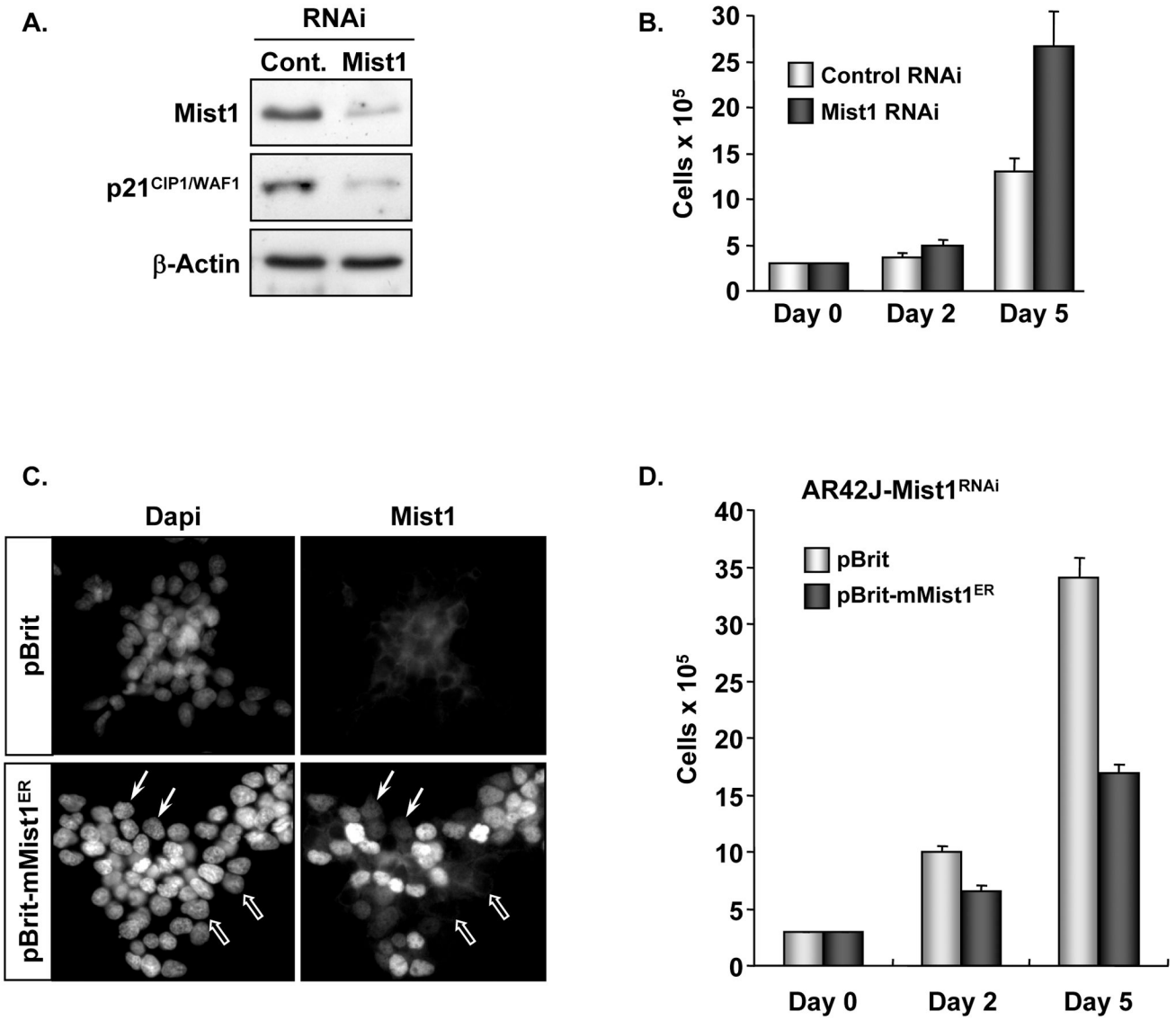
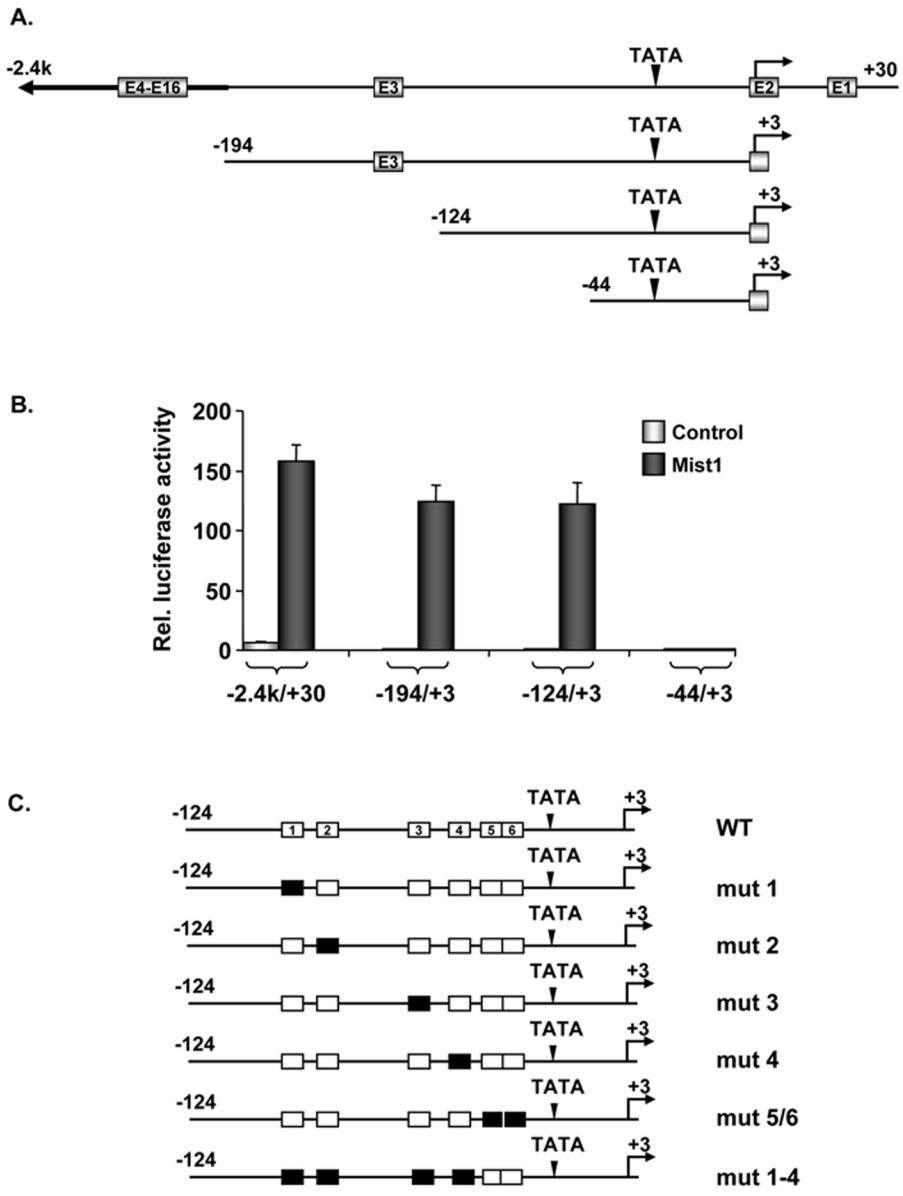


Figure 3. shRNA-directed knock-down of endogenous Mist1 leads to decreased p21$^{CIP1/WAF1}$ levels and increased proliferation

(**A**) Immunoblots of AR42J-Control^{RNAi} and AR42J-Mist1^{RNAi} cell lines showing that "knock-down" of endogenous *Mist1* transcripts results in decreased *p21$^{CIP1/WAF1}$* levels. (**B**) Growth analysis of AR42J-Control^{RNAi} and AR42J-Mist1^{RNAi} cells. (**C**) Immunofluorescence of AR42J-Mist1^{RNAi} cells infected with pBrit or pBrit-mMist1^{ER} virus. 90% of pBrit-Mist1^{ER} cells were strongly or weakly (closed arrows) positive for Mist1^{ER} while a small number of cells remained Mist1^{ER} negative (open arrows). (D) Growth analysis of AR42J-Mist1^{RNAi} cells infected with pBrit or pBrit-mMist1^{ER} virus. Each time point corresponds to the average of 3 independent experiments (± s.d.).



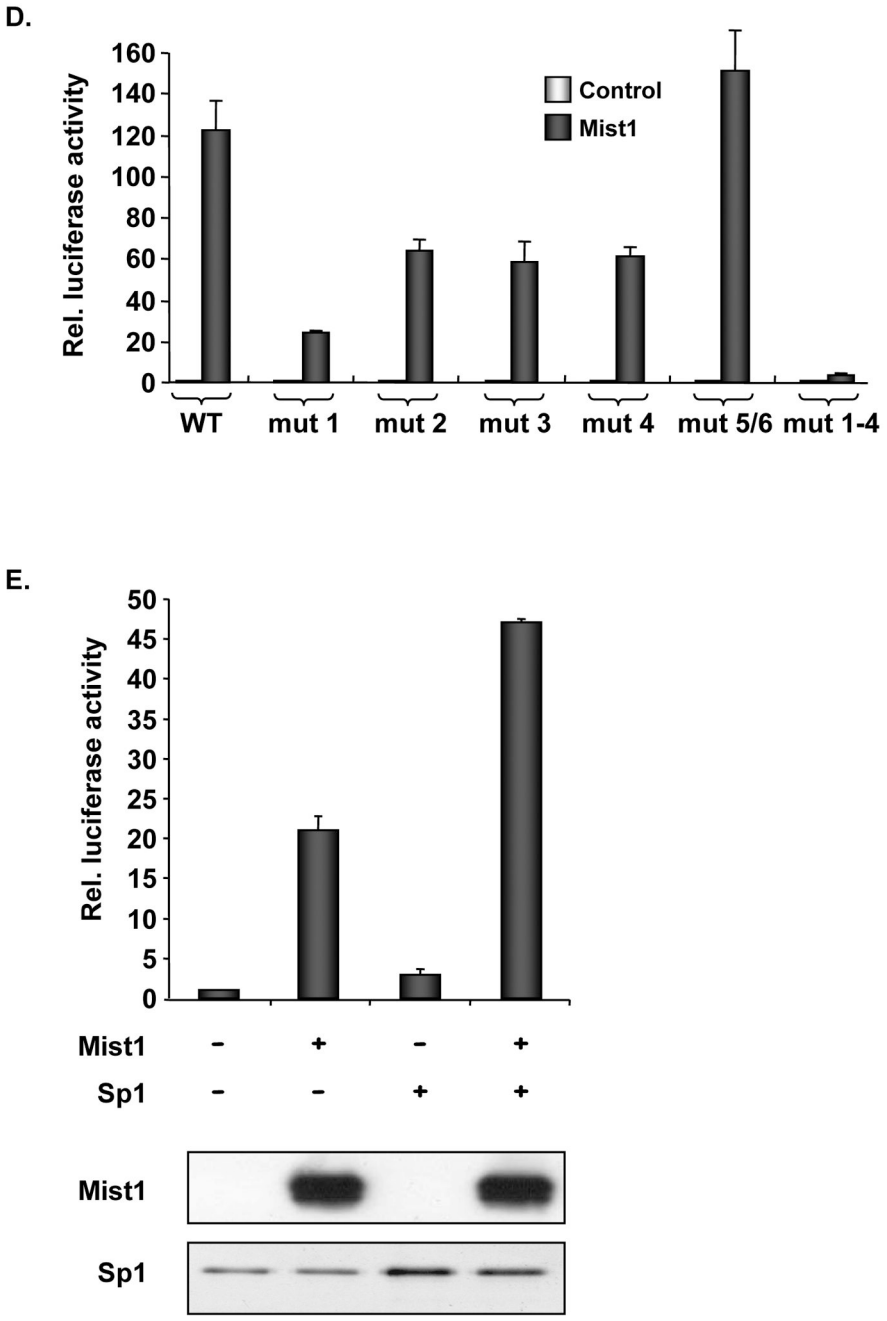


Figure 4. Sp1 binding sites are essential for Mist1-induced p21$^{CIP1/WAF1}$ expression

(**A**) Schematic representation of the full-length and truncated *p21$^{CIP1/WAF1}$* promoter luciferase gene constructs. Boxes (E1–E16) represent E-box sites. (**B**) Control pcDNA or pcDNA-Mist1 expression plasmids were co-transfected with the indicated p21-luc promoter genes into AR42J cells and p21-luc activity was quantified. (**C**) Schematic representation of WT and mutated −124/+3 p21-luc constructs. Open boxes: WT Sp1 binding sites (1–6). Solid boxes: mutated Sp1 binding sites. (**D**) Sp1 binding sites 1-4 are essential for Mist1 activity. Control pcDNA or pcDNA-Mist1 expression plasmids were co-transfected with the indicated −124/+3 p21-luc promoter constructs into AR42J cells and p21-luc activity was quantified. (**E**) Cells were transfected with the corresponding plasmids and luciferase assays were performed.

Immunoblots reveal Mist1 and Sp1 expression levels. Mist1 and Sp1 synergistically activated the −124/+3 p21-luc reporter gene. Values in **B**, **D**, **E** are the average of 3 or more independent experiments (± s.d.).

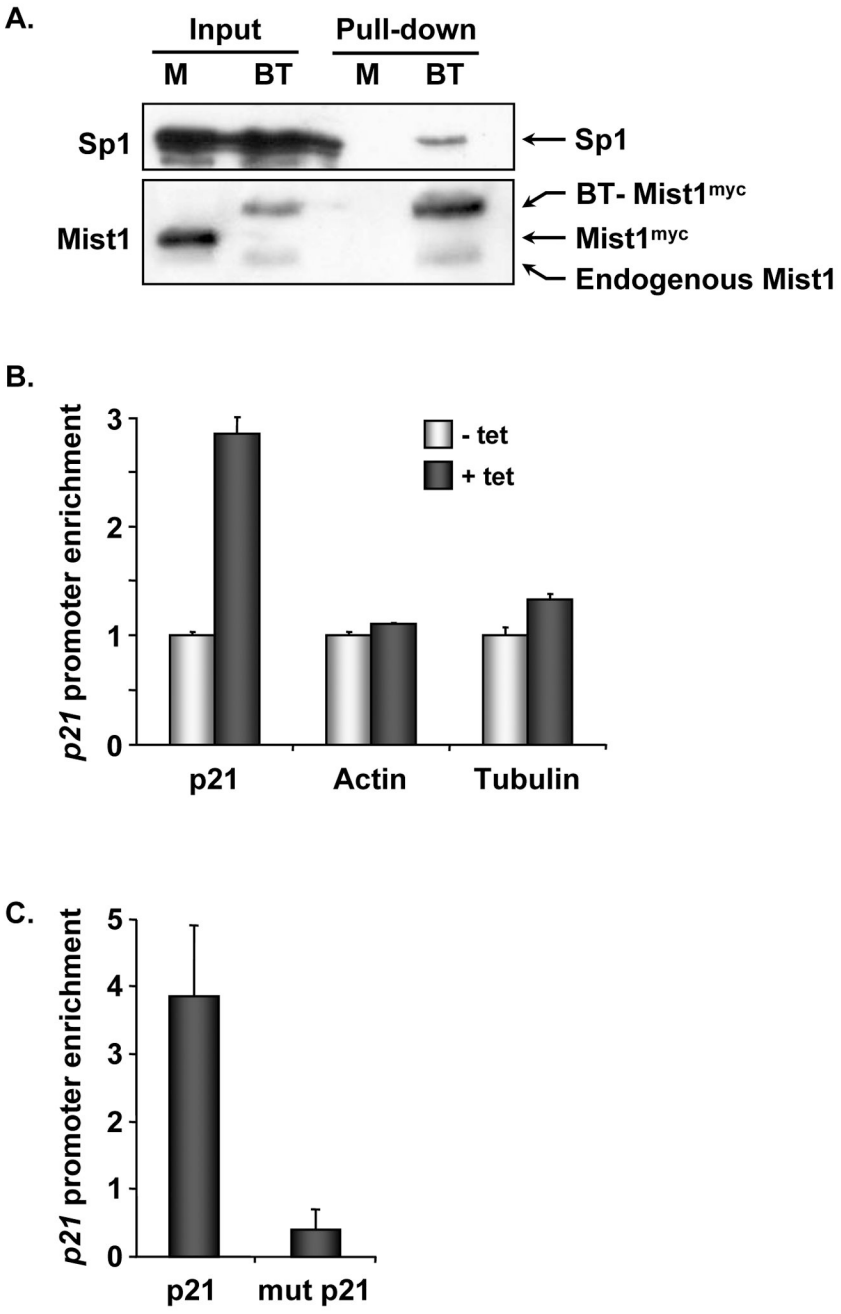


Figure 5. Mist1:Sp1 complexes are associated with the p21$^{CIP1/WAF1}$ promoter

(**A**) AR42J cells that expressed tet-inducible Mist1^{myc} (M) or biotinylated Mist1^{myc} (BT) were induced with tetracycline and nuclear extracts were subjected to pull-down assays using streptavidin conjugated beads. Sp1 co-purifies with BT-Mist1^{myc}. **(B)** AR42J^{tet}-Mist1 cells were treated +/− tet and real-time PCR was performed on Mist1 ChIP products using primers specific to the endogenous *p21$^{CIP1/WAF1}$*, β-*actin* and *tubulin* promoters. Values are the average of 3 or more independent experiments (± s.d.). (**C**) AR42J^{tet}-Mist1 cells were used to introduce the WT −124/+3 p21-luc or mut 1–4 −124/+3 p21-luc gene construct into the cells. Real-time PCR was performed on Mist1 ChIP products using primers specific to the flanking region of the −124/+3 *p21$^{CIP1/WAF1}$* promoter. Mist1 specifically bound the WT *p21$^{CIP1/WAF1}$* promoter

but not the mutated *p21*$^{CIP1/WAF1}$ promoter. Values are the average of 3 independent experiments (± s.d.).

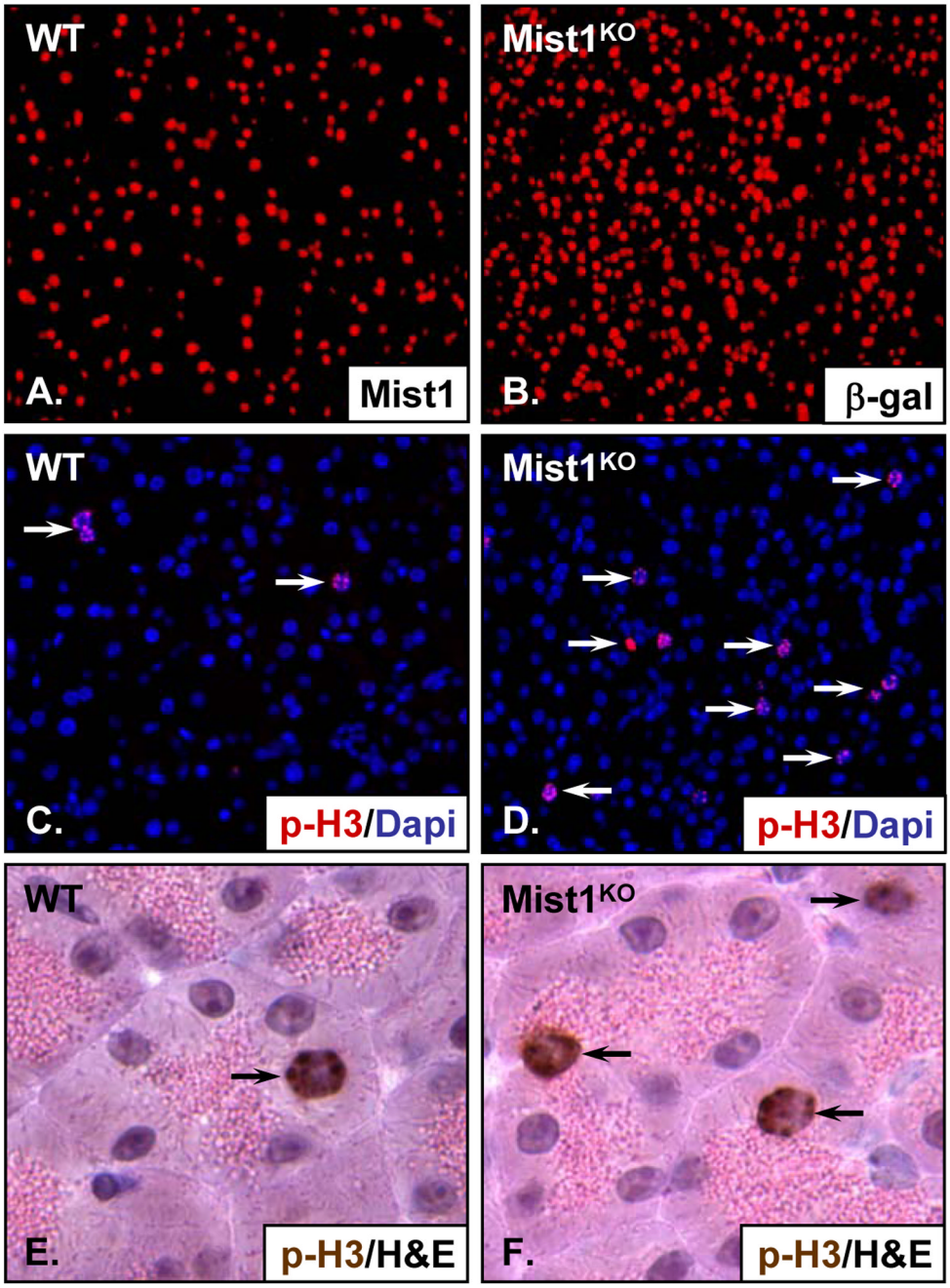


Figure 6. The Mist1^{KO} pancreas has an increased acinar cell proliferation index

(**A,B**) Pancreas sections from WT and *Mist1^{KO}* littermates were subjected to IHC using Mist1 or β-gal antibodies to detect acinar cells. (**C,D**) Anti-p-H3 IHC showing that *Mist1^{KO}* acinar cells exhibit a higher cell proliferation index when compared to WT samples. (**E,F**) Pancreas sections from WT and *Mist1^{KO}* littermates were subjected to IHC using the p-H3 antibody and hematoxylin and eosin staining to identify acinar cells containing zymogen granules.

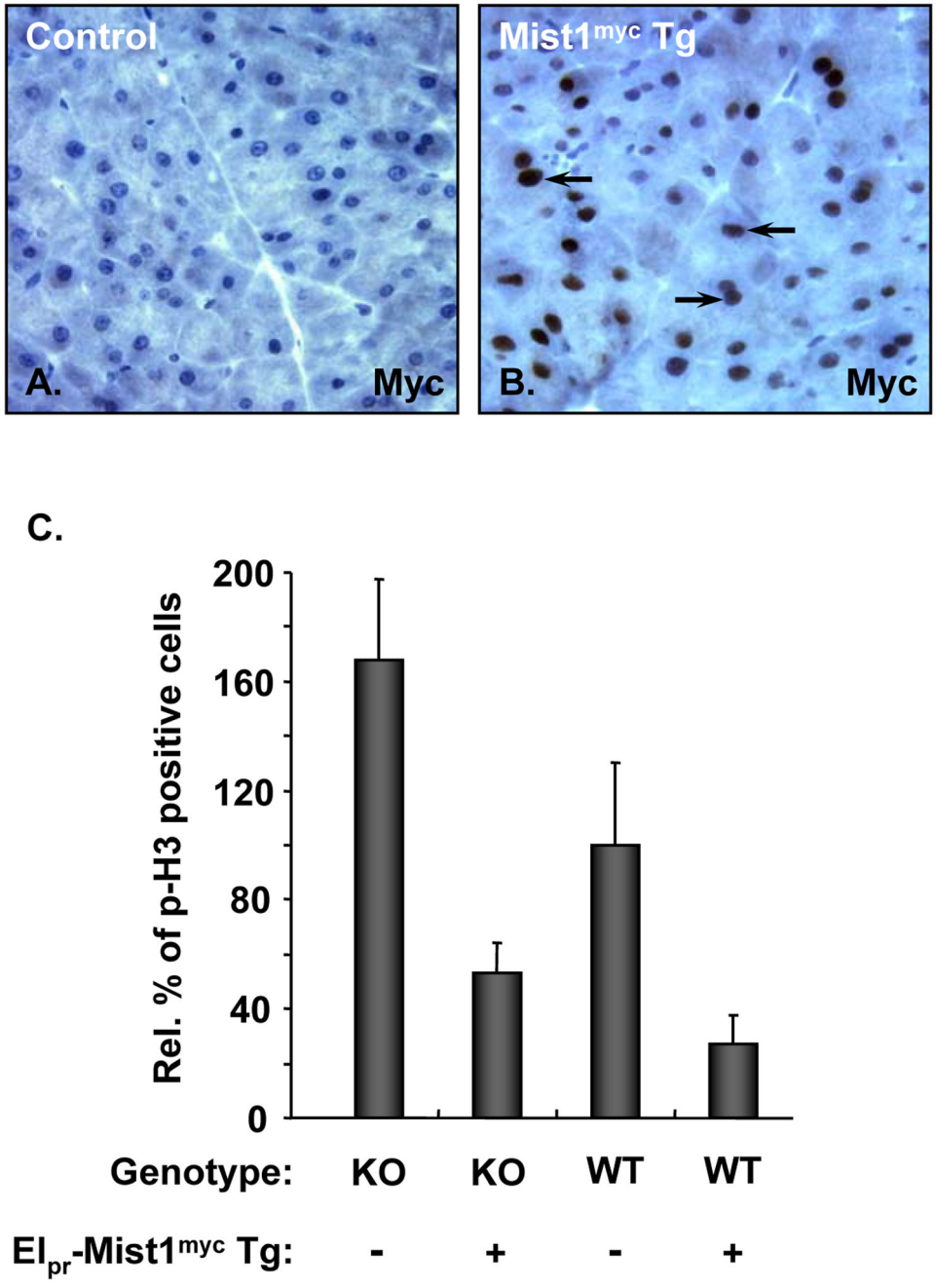


Figure 7. Expression of a Mist1^{myc} transgene results in decreased acinar cell proliferation

(**A,B**) Pancreas sections from WT and *El$_{pr}$-Mist1^{myc}* transgenic littermates were subjected to IHC to detect *Mist1^{myc}* protein (arrows). (**C**) Pancreas sections from *Mist1^{KO}*, *Mist1^{KO}/El$_{pr}$-Mist1^{myc}*, WT and WT/*El$_{pr}$-Mist1^{myc}* littermates were subjected to IHC using anti-p-H3. Each value corresponds to the average of 10 or more sections.