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Two conserved domains in PCIF1 mediate interaction with pancreatic transcription factor PDX-1

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Abstract PCIF1 is a TRAF and POZ domain containing nuclear factor that interacts with and inhibits transactivation of pancreatic homeodomain transcription factor PDX-1. Here, we demonstrate interaction of endogenous PDX-1 and PCIF1 in MIN6 insulinoma cells. Within PCIF1, the TRAF and POZ domains are both required for physical and functional interaction with the C-terminus of PDX-1, whereas the C-terminal domain of PCIF1 directs its nuclear localization. A human PDX-1 mutation associated with diabetes, E224K, disrupts the ability of PCIF1 to inhibit PDX-1 transactivation, suggesting that the interaction between PDX-1 and PCIF1 is required for normal glucose homeostasis. Inhibition of transactivation occurs by a mechanism distinct from the classical role of POZ domains to recruit co-repressors and histone deacetylases. Understanding the functional roles of PCIF1 domains may have application to therapeutic β-cell replacement strategies involving PDX-1 for the treatment of diabetes.

Keywords: Diabetes; MODY; Pancreas; Islet; Development; Insulin

1. Introduction

PDX-1 (pancreas and duodenum homeobox-1) is a *Hox* type homeodomain-transcription factor that is pivotally positioned in the transcriptional hierarchy governing pancreatic β-cell development. PDX-1 has two critical roles, first in the early development of both the endocrine and exocrine pancreas, and then in the later differentiation of the β-cell (reviewed in [1,2]). Mice and humans deficient for Pdx1 have pancreatic agenesis. Heterozygous null Pdx1 mutation leads to the development of an initially normal morphological mass of β-cells but impaired β-cell function and impaired cell survival leading to abnormal glucose tolerance in mice and early-(MODY4) and late-onset forms of type 2 diabetes in humans.

In contrast to the N-terminal transactivation domain and the DNA binding homeodomain, the role of the C-terminus of PDX-1 is poorly understood. Human mutations in the regions are associated with adult onset type 2 diabetes and MODY, emphasizing its functional importance in vivo [3–5]. Some data support an inhibitory role [6–9], yet other data indicate that the C-terminus is required for full transactivation [3,4]. Recently, the concept that the insulin gene is actively repressed under low glucose conditions has been put forth [10], mediated by recruitment of histone deacetylases (HDAC) 1 and 2 to PDX-1 amino acids 79–284, suggestive of a context-dependent repressive role of either the homeodomain or the C-terminus [11].

We previously identified PCIF1 (PDX C-terminus Interacting Factor-1) as a partner for PDX-1 in a yeast two-hybrid screen [12]. PCIF1 encodes a 374 amino acid protein with an N-terminal TNF receptor associated factor (TRAF) domain and a typical poxvirus and zinc finger (POZ) domain. The physical interaction between PCIF1 and PDX-1 was confirmed in vitro by GST interaction assay and in vivo by co-immunoprecipitation of over-expressed proteins. Functionally, PCIF1 inhibits PDX-1 transactivation of target gene promoters in a specific and dose-dependent manner that requires an intact PDX-1 C-terminus. PCIF1 mRNA is enriched in adult pancreas, and PCIF1 protein is expressed in adult pancreatic insulin-producing β-cells. The co-expression of PCIF1 with PDX-1 in β-cells and its ability to inhibit PDX-1 transactivation suggest that PCIF1 modulation of PDX-1 function may regulate β-cell differentiation.

2. Materials and methods

2.1. Constructs

Expression vectors were full length mouse PDX-1, mouse PDX-1(1-210), pGBKT7-PDX-1(144–283) and GST-PDX-1(206–283) [12], rat PDX-1 [13], human PDX-1 [14], zebrafish PDX-1 [15], Flag-PCIF1 [12], Gal-4 PDX-1 [6], Gal-4 BCL6 POZ and Gal-4 PLZF POZ [16], human PDX-1 wild-type and E224K [3] and GST-TRAF and GST-POZ [17]. Reporters were the PDX-1-responsive somatostatin promoter reporter $(TAAT)_5$-65 SMS-CAT [13] and the Gal4 responsive reporters G51bCAT [12] and Gal4SV40Luc. Flag-tagged mutants of PCIF1 and Gal4-PCIF1 constructs were created by polymerase chain reaction (PCR). Primers and details of cloning strategies are available upon request. All constructs were confirmed by sequencing.

2.2. Immunoprecipitations and Western blot analysis

Mouse insulinoma cell line 6 (MIN6) cells were lysed in 150 mM NaCl, 40 mM Tris–HCl, pH 7.6, 10% glycerol and 0.3% NP-40 and immunoprecipitations were carried out as described [12]. Primary

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Abbreviations: PDX-1, pancreas and duodenum homeobox-1; MODY, maturity onset diabetes of youth; PCIF1, PDX-1 C-terminus interacting factor; MIN6, mouse insulinoma cell line 6; TRAF, TNF-receptor associated factor domain; POZ, poxvirus and zinc finger domain; BTB, broad-complex, tramtrack, and bric-a-brac domain; HDAC, histone deacetylase; PCR, polymerase chain reaction

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antisera were: HDAC3 (mouse, gift from M. Lazar) and HDAC3 (Santa Cruz #sc-11417); HDAC2 (Santa Cruz #sc-7899); SMRT (mouse, gift from Mitch Lazar) and SMRT (ABR, # PA1-842). PCIF1 and PDX-1 antisera were previously described [12,14].

2.3. Transfections

HeLa cells were transfected with expression and reporter vectors and an internal control cmv-β galactosidase expression vector. Chloramphenicol acetyl transferase, luciferase and β-galactosidase activities were measured as described [12].

2.4. GST interaction assays

Bacterially expressed GST fusion proteins were incubated with in vitro-transcribed and -translated ^{35}S-labelled full length PDX1, PDX1(1–210), PDX1(Δ210–238), PDX1(144–283) [12]. After washing, proteins bound to glutathione beads were analyzed by SDS–PAGE.

2.5. Immunofluorescence

Transfected cells were stained with a Flag monoclonal antibody as described [12].

3. Results

3.1. In vivo association of endogenous PDX-1 and PCIF1

PDX-1 and PCIF1 are associated when over-expressed in HeLa cells [12]. To rule out artifactual association due to high-level over-expression, we determined whether endogenous PDX-1 and PCIF1 associate in vivo. Using extracts prepared from MIN6 insulinoma cells that express both PDX-1 and PCIF1, immunoprecipitation with α-PDX1 resulted in the co-immunoprecipitation of PCIF1, whereas control immunoglobulin did not (Fig. 1), indicating a specific interaction between endogenous PDX-1 and PCIF1.

3.2. An evolutionarily conserved motif in the PDX-1 C-terminus mediates the physical and functional interaction with PCIF1

We previously localized the functional ability of PCIF1 to inhibit Gal4-PDX1 fusion protein transactivation to amino acids (AA) 210–238 of the PDX-1 C-terminus. Here we show that the functional interaction of PCIF1 with PDX-1 is evolutionarily conserved. Transactivation by mouse, rat, human and zebrafish PDX-1 is similarly inhibited by mouse PCIF1 (Fig. 2A), indicating that PCIF1 interacts with a conserved motif in PDX-1. Comparison of the C-terminus from multiple species reveals a highly conserved 14 AA motif (Fig. 2B). In agreement with our previous findings in a series of Gal4 PDX-1 truncations, internal deletion of AA 210–238 from full length PDX-1, including the conserved motif, abrogated the ability of PCIF1 to inhibit PDX-1 (Fig. 2C). This is due to the requirement of AA 210–238 for co-immunoprecipitation of PDX-1 and PCIF1 (Fig. 2D), indicating that these residues are essential for both the physical and functional interaction of PDX-1 and PCIF1.

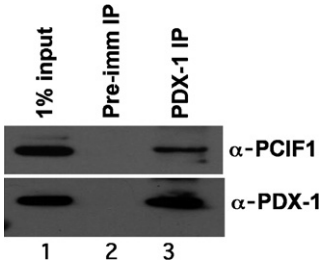

Fig. 1. Interaction of endogenous PDX-1 and PCIF1 in MIN6 insulinoma cells. Cell extracts were immunoprecipitated with α-PDX1 and immunoblotted with α-PDX1 and α-PCIF1. Lane 1, 2% of input extract; lane 2, pre-immune IP; lane 3, α-PDX-1 IP.

3.3. Diabetogenic E224K PDX-1 mutation impairs the functional effect of PCIF1

A recent genetic report described a novel diabetes-associated mutation, E224K, in the PDX-1 C-terminus in two diabetic subjects from Trinidad [3]. Within one large family the mutation co-segregated with early onset diabetes, suggesting a MODY4 form of diabetes. The E224K mutation results in the replacement of a negative charge with a positively charged side chain, and it occurs within the conserved motif that mediates physical and functional interaction with PCIF1 (Fig. 2B). Therefore, we examined the ability of PCIF1 to inhibit the E224K variant in transient transfection assays. PCIF1 does not inhibit transactivation of the PDX-1 E224K mutant protein, in agreement with the above data indicating the critical importance of this motif and in contrast to the effect of PCIF1 on wild-type human PDX-1 (Fig. 3).

3.4. Both POZ and TRAF domains mediate the physical interaction with PDX-1

To map the critical motifs in PCIF1 that mediate interaction with PDX-1 we created deletion mutants of PCIF1 in which the TRAF, POZ or C-terminal domains were removed (Fig. 4A). GST interaction assays demonstrated the interaction of GST-PDX1 (206–283) with full length PCIF1 as previously reported (Fig. 4B) [12]. PCIF1 (1–161)(m1) and (1–298)(m4) showed the strongest signal, suggesting that the N-terminus containing the TRAF domain mediates the physical interaction with PDX-1. A weak interaction with PCIF1 (Δ162–298)(m3) was also observed, consistent with a TRAF domain-mediated interaction.

The requirement for the TRAF domain was directly tested in GST interaction assays utilizing recombinant GST-TRAF and GST-POZ fusion proteins (Fig. 4C). A strong specific interaction between GST-TRAF and full length PDX-1 was observed. A weaker but specific interaction with GST-POZ was also observed. Similarly, both GST-TRAF and GST-POZ specifically pulled down PDX-1 (144–283), encoding the homeodomain and C-terminus of PDX-1. The interaction with both GST-TRAF and GST-POZ was abrogated with the truncation of the PDX-1 C-terminus at amino acid 210 [PDX1(1–210)]. Thus, both the TRAF and POZ domains mediate physical interaction of PCIF1 with the C-terminus of PDX-1. Of note, internal deletion of residues 210–238 [PDX1(Δ210–238)] abrogated interaction with the TRAF domain but not the POZ domain, indicating that the POZ domain has additional contacts with the PDX-1 C-terminus outside of the conserved motif required for functional inhibition by PCIF1.

3.5. Critical roles of TRAF, POZ and C-terminal domains in PCIF1 inhibition of PDX-1 transactivation

The domain requirements for PCIF1 inhibition of PDX-1 were assessed in over-expression reporter assays (Fig. 4D). Deletion of the TRAF, POZ or C-terminal domains individually each abrogated the inhibitory effect of PCIF1, indicating

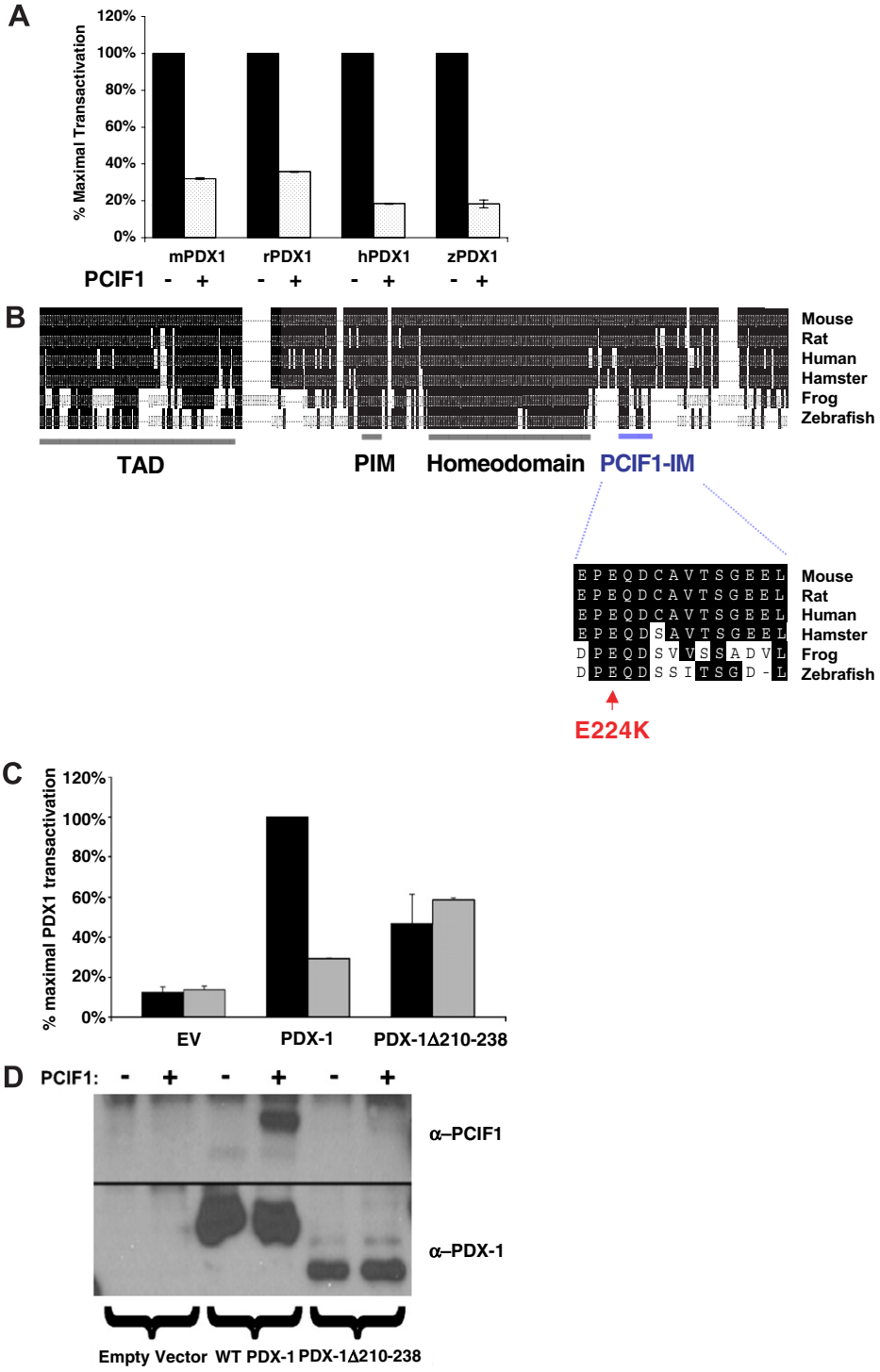

Fig. 2. Evolutionarily conserved PDX-1 amino acids 210–238 are critically required for physical and functional interaction with PCIF1. (A) Evolutionary conservation of PCIF1 function. Transactivation tested in transient transfection assays in HeLa cells using the SMS(TAAT1)$_5$CAT reporter. The activity of each PDX ortholog (mouse, rat, human and zebrafish) in the absence of PCIF1 was set to 100% (black boxes) for comparison to activity in the presence of PCIF1 (shaded boxes). (B) Evolutionary comparison of the amino acid sequence of the PDX-1 proximal C-terminus. The entire coding sequences of mouse, rat, human, hamster, frog and zebrafish PDX-1 are indicated in compressed form, with residues identical to the mouse sequence shaded in black. Regions of high identity include the transactivation domain (TAD), the Pbx interaction motif (PIM), the homeodomain and a 14 amino acid peptide in the C-terminus, the proposed PCIF1-Interaction Module (PCIF1-IM; in blue). The PCIF1-IM sequence is expanded below and the location of the human diabetes mutation, E224K, is indicated by an asterisk (*). (C) and (D) Internal deletion of evolutionarily conserved AA 210–238 abrogates (C) the ability of PCIF1 to inhibit PDX-1 transactivation and (D) the PCIF1 interaction with PDX-1. (C) The activity of PDX-1Δ210–238 was compared to PDX-1 in the absence (black boxes; empty vector) and presence (shaded boxes) of co-transfected PCIF1 expression vector. Transfections were carried out in HeLa cells, using the SMS(TAAT1)$_5$ CAT reporter. (D) Empty vector, PDX-1 and PDX-1Δ210–238 were co-transfected with empty vector or PCIF1 into HeLa cells and subjected to immunoprecipitation with α-PDX-1 antiserum (lower panel). PCIF1 was detected in association with full length PDX-1 but not PDX-1Δ210–238 (upper panel).

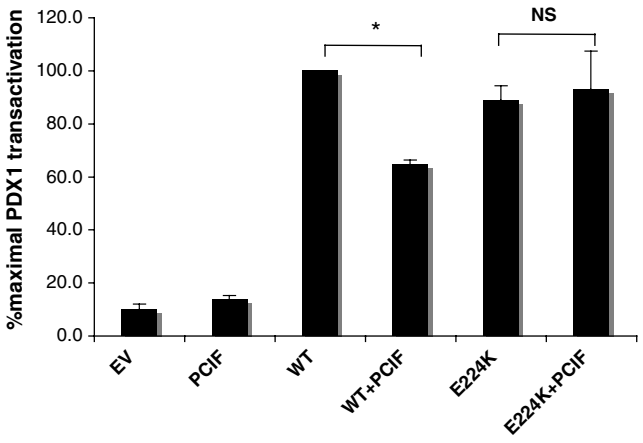

Fig. 3. Diabetogenic E224K mutation in human PDX-1 disrupts the ability of PCIF1 to inhibit PDX-1 transactivation. Transactivation was tested in transient transfection assays in HeLa cells using the SMS(TAAT1)$_5$CAT reporter. The activity of wild-type (WT) and E224K mutated human PDX-1 (E224K) was assessed in the presence and absence of PCIF1. Activity of wild-type human PDX-1 in the absence of PCIF1 was set to 100%. $^*P < 0.001$. NS, not significant. EV, empty vector.

the requirements for all three domains for the ability of PCIF1 to inhibit PDX-1 transactivation.

3.6. The PCIF1 POZ domain is not an autonomous repression domain

The most well characterized POZ domain transcription factors are transcriptional repressors in which the POZ domain mediates direct interactions with co-repressors and HDACs [18,19]. To determine whether the PCIF1 POZ domain mediates inhibition of PDX-1 by a similar mechanism, we assessed the activity of Gal4-PCIF1 and Gal4-POZ fusion proteins. In this assay, transcriptional repressors and repression domains cloned in-frame with the Gal4 DNA binding domain repress the high basal activity of the Gal4SV40 luciferase reporter [20]. Surprisingly, basal reporter activity was not inhibited by Gal4-PCIF1 or Gal4-POZ (Fig. 5A), although the established repressive POZ domains from PLZF and BCL6 did inhibit in this assay. Further, HDAC inhibitors (trichostatin A, sodium butyrate and nicotinamide) had no effect on the ability of PCIF1 to inhibit PDX-1 transactivation (Fig. 5B). We examined whether PCIF1 interacts with HDAC3 and co-repressor SMRT in MIN6 cells (Fig. 5C). Although we detected the interaction between HDAC3 and SMRT, we observed no interaction between PCIF1 and either HDAC3 or SMRT. Similarly we did not detect interaction between PCIF1 and HDAC2 (not shown). Altogether, these data strongly indicate that the PCIF1 POZ domain is not an autonomous repression domain and that inhibition of PDX-1 transactivation does not involve the recruitment of HDACs.

3.7. The PCIF1 C-terminus directs its nuclear localization

HeLa cells were transfected with wild-type and mutant PCIF1 and expression was determined by immunofluorescence detection of the Flag epitope (Fig. 6). Consistent with a previous report [21], the TRAF domain directs PCIF1 localization to nuclear speckles, as deletion of the TRAF domain in the PC mutant led to a diffuse nuclear localization. Of note, deletion

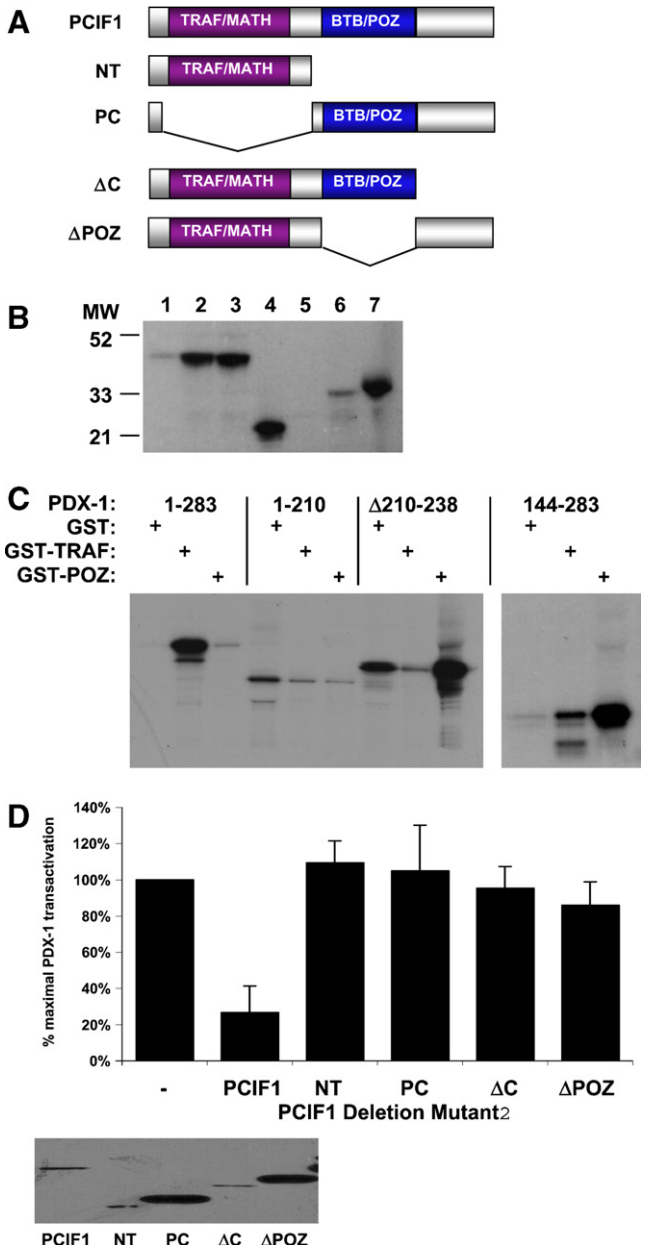

Fig. 4. Critical role of the BTB/POZ and TRAF domains in the physical and functional interaction of PCIF1 with PDX-1. (A) Flag tagged PCIF1 deletion mutants are depicted in schematic form. (B) GST-PDX-1 206–283 interaction assays with ^{35}S-labelled in vitro transcribed and translated PCIF1 and PCIF1 deletion mutants. Lane 1 GST only; lanes 2–7 GST-PDX-1 206-283; lanes 1–3 PCIF1 (wt); lane 4 NT (m1); lane 5 PC (m2); lane 6 ΔPOZ (m3); lane 7 ΔC (m4). (C) GST TRAF and GST POZ interaction assays with ^{35}S-labelled in vitro transcribed and translated PDX-1 and PDX-1 mutants 1–210, Δ210–238 and 144–283. Western blots shown are representative of three independent experiments. (D) Upper panel, Full-length PCIF1 and PCIF1 deletion mutants tested in transient transfection assays for the ability to inhibit PDX-1 transactivation of the SMS(TAAT1)$_5$CAT reporter. Transactivation of PDX-1 in the absence of PCIF1 is set to 100%. Lower panel, Western blot demonstrating expression of PCIF1 and PCIF1 deletion mutants in transfected HeLa cells.

of the C-terminus resulted in cytoplasmic expression, indicating that the C-terminus mediates nuclear localization of PCIF1.

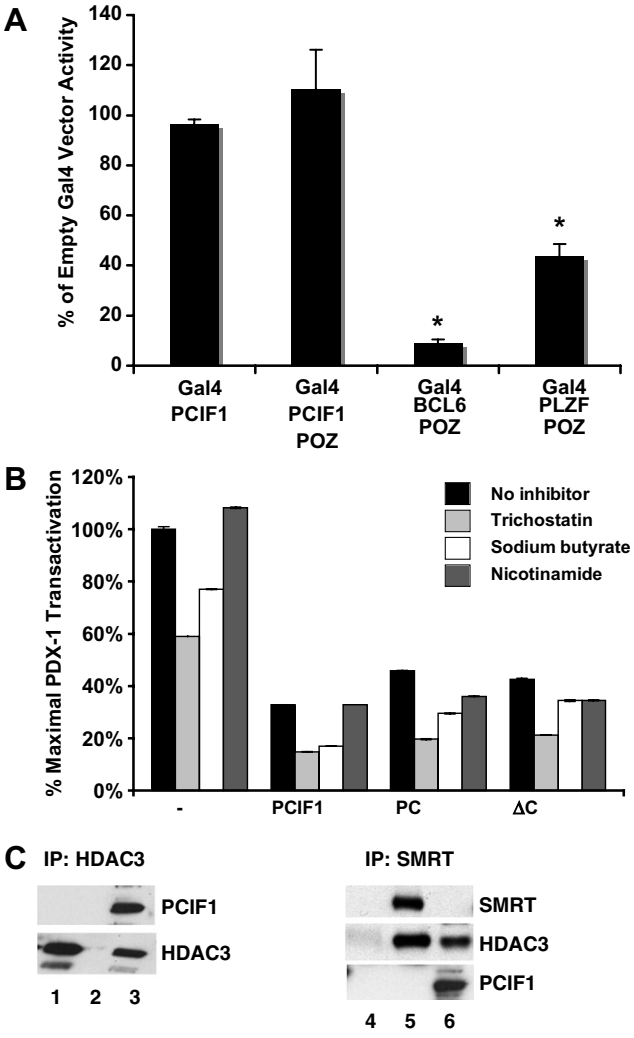

Fig. 5. The PCIF1 POZ domain does not mediate transcriptional repression. (A) Gal4-PCIF1 and Gal4-POZ domain fusion proteins do not repress basal Gal4 transactivation in HeLa cells co-transfected with a Gal4-SV40 Luciferase reporter. Gal4-PLZF POZ and Gal4-Bcl6 POZ both repress basal Gal4 transactivation. $^{*}P < 0.001$ compared with empty vector Gal4. (B) The HDAC inhibitors trichostatin A (300 nM), sodium butyrate (5 mM) and nicotinamide (5 mM) do not relieve the inhibition of Gal4-PDX1 transactivation caused by PCIF1, PC or DC. (C) PCIF1 does not interact with SMRT or HDAC3. Left panel, HeLa cell lysates were immunoprecipitated with non-immune IgG or HDAC3 antibody. Western blot analysis for HDAC3 and PCIF1 was performed. Right panel, HeLa cell lysates were immunoprecipitated with non-immune IgG or SMRT antiserum. Western blot analysis was performed for SMRT, HDAC3 and PCIF1. The low level of endogenous SMRT expression precluded detection in the input sample. Lanes 1 and 5, immune precipitation; lanes 2 and 4, non-immune control precipitation; lanes 3 and 6, 1% input lysate.

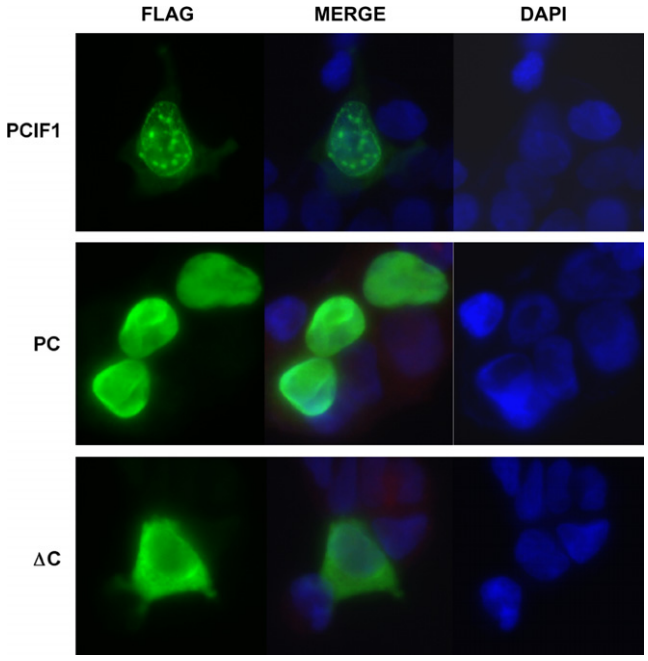

Fig. 6. Conserved PCIF1 TRAF and C terminal domains mediate proper nuclear localization of PCIF1. Full-length PCIF1 and PCIF1 deletion mutants PC and ΔC (see Fig. 4A) were transfected into HeLa cells and visualized by immunofluorescence for the Flag epitope. Images with (middle panel) and without (left panel) DAPI nuclear counterstain are shown. DAPI only counterstain is shown in the right panel.

4. Discussion

PCIF1 is a novel regulatory molecule that interacts with the homeodomain transcription factor PDX-1. Here, we determine the critical motifs in both PDX-1 and PCIF1 that are required for this interaction. Within the PDX-1 C-terminus we demonstrate an evolutionarily conserved region that mediates both physical and functional interaction with PCIF1. A recently reported human diabetes causing mutation, E224K [3], located within this conserved motif disrupts the functional interaction with PCIF1, suggesting that interaction between PDX-1 and PCIF1 is required for normal glucose homeostasis. Other PDX-1 C-terminal variants associated with human diabetes are located outside the conserved motif and are not impaired in the functional interaction with PCIF1 in over-expression reporter assays (Liu and Stoffers, unpublished observations). These mutations may impair PDX-1 function via a PCIF1 independent mechanism; however the observation from this study that the POZ domain interacts with residues in the PDX-1 C-terminus outside the conserved motif suggests that these mutations could also modify the interaction with PCIF1.

The requirement for the POZ domain suggested recruitment of a repressor complex as a potential molecular mechanism. The most-well characterized POZ domain transcription factors recruit repressor complexes through direct interactions with co-repressors and HDACs [18,19]. However, we found no evidence for PCIF1 POZ domain as a repression domain, nor could we demonstrate any effect of multiple HDAC inhibitors or direct interactions with the co-repressor SMRT and HDACs 2 and 3. These results support a distinct molecular mechanism for the inhibition of PDX-1 by PCIF1 that does not involve the classical paradigm of transcriptional repression.

We identify roles for the TRAF and C-terminal domains in the subcellular targeting of PCIF1. Consistent with a previous report, the TRAF domain is required for proper sub-nuclear localization to nuclear speckles [21], dynamic subnuclear organelles that may regulate pools of factors required for transcription and splicing and are often observed near sites of active transcription (reviewed in [22]). Previously, we observed that PDX-1 redirected PCIF1 from nuclear speckles to a

diffuse nuclear distribution [12]. Taken together with the direct interaction of PDX-1 with the TRAF domain, the results suggest that PDX-1 redirects PCIF1 localization within the nucleus by masking a motif within the TRAF domain required for localization to speckles. The PCIF1 C-terminus is also required for nuclear localization. The mechanism for nuclear import is generally unknown for nuclear POZ domain proteins. Recently, a conserved basic motif mediating nuclear localization that did not conform to consensus nuclear import signals was identified for the nuclear POZ domain protein Kaiso [23]. Interestingly, this motif bears high homology to a motif in the C-terminus of PCIF1. Future experiments will determine whether nuclear import of PCIF1 is mediated by this conserved motif.

Current research on PDX-1 is unequivocal with regard to its pivotal position in the transcriptional hierarchy regulating both early and late pancreatic islet development, critically underscored by the identification of PDX-1 as a human diabetes gene (MODY4); yet many important mechanistic questions remain unanswered. The identification of critical domains mediating the interaction of PDX-1 and PCIF1 may have potential application to therapeutic β-cell replacement strategies in type 1 and type 2 diabetes, as PDX-1 is explored as a target for induction of β-cell neogenesis, transdifferentiation of non-β-cell types into insulin producing cells, and gene therapy approaches to modify embryonic and other stem cells for transplantation.

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