THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 17, pp. 11807–11815, April 25, 2014

Thioredoxin-interacting Protein Promotes Islet Amyloid Polypeptide Expression through miR-124a and FoxA2*

Received for publication, October 8, 2013, and in revised form, March 7, 2014 Published, JBC Papers in Press, March 13, 2014, DOI 10.1074/jbc.M113.525022

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Background: Islet amyloid polypeptide (IAPP) plays an important role in beta-cell biology, but its regulation is not fully understood.
Results: Thioredoxin-interacting protein (TXNIP) induces IAPP by inhibiting miR-124a and promoting FoxA2-mediated transcription.
Conclusion: The critical beta-cell signaling pathways of TXNIP and IAPP are linked.
Significance: Identification of this novel TXNIP/miR-124a/FoxA2/IAPP signaling pathway provides new insight into an important aspect of transcriptional regulation and beta-cell biology.

Thioredoxin-interacting protein (TXNIP) is up-regulated by glucose and diabetes and plays a critical role in glucotoxicity, inflammation, and beta-cell apoptosis, whereas we have found that TXNIP deficiency protects against diabetes. Interestingly, human islet amyloid polypeptide (IAPP) is also induced by glucose, aggregates into insoluble amyloid fibrils found in islets of most individuals with type 2 diabetes and promotes inflammation and beta-cell cytotoxicity. However, so far no connection between TXNIP and IAPP signaling had been reported. Using TXNIP gain and loss of function experiments, INS-1 beta-cells and beta-cell-specific *Txnip* knock-out mice, we now found that TXNIP regulates IAPP expression. Promoter analyses and chromatin-immunoprecipitation assays further demonstrated that TXNIP increases IAPP expression at the transcriptional level, and we discovered that TXNIP-induced FoxA2 (forkhead box A2) transcription factor expression was conferring this effect by promoting FoxA2 enrichment at the proximal FoxA2 site in the IAPP promoter. Moreover, we found that TXNIP down-regulates miR-124a expression, a microRNA known to directly target *FoxA2*. Indeed, miR-124a overexpression led to decreased *FoxA2* expression and IAPP promoter occupancy and to a significant reduction in IAPP mRNA and protein expression and also effectively inhibited TXNIP-induced IAPP expression. Thus, our studies have identified a novel TXNIP/miR-124a/FoxA2/IAPP signaling cascade linking the critical beta-cell signaling pathways of TXNIP and IAPP and thereby provide new mechanistic insight into an important aspect of transcriptional regulation and beta-cell biology.

Islet amyloid polypeptide (IAPP),[2] also known as amylin, is a regulatory peptide that is co-secreted with insulin by beta-cells (1, 2). As the major component of islet amyloid, it tends to aggregate into insoluble amyloid fibrils that can be found in islets of most patients with type 2 diabetes (2, 3). IAPP deposits are often co-localized with cellular degeneration and apoptosis and have therefore been strongly associated with the progressive loss of pancreatic beta-cell mass in diabetes (3). Aside from these extracellular amyloid fibrils detected by light microscopy, more recent studies have suggested that smaller non-fibrillar oligomers contribute to the toxic effects of IAPP (2, 4, 5).

Initially, we identified thioredoxin-interacting protein (*TXNIP*) as the strongest glucose-induced gene (>10-fold increase) in our human islet oligonucleotide microarray analysis (6). Follow-up studies revealed that these glucose effects are mediated by enhanced binding of carbohydrate response element-binding protein to a unique non-palindromic E-box motif within the proximal *TXNIP* promoter, which induces *TXNIP* transcription (7, 8). Moreover, we found that TXNIP levels are dramatically increased in pancreatic islets of diabetic mice, suggesting that it might be involved in the disease pathology (9, 10). TXNIP has been known as an inhibitor of thioredoxin function and a regulator of the cellular redox state (11) and was found to be involved in inflammation and ER stress (12). We discovered that TXNIP induces beta-cell apoptosis (8) and represents a critical link between glucose toxicity and beta-cell death (9). In contrast, we found that TXNIP deficiency promotes endogenous beta-cell survival and prevents streptozotocin- and obesity-induced diabetes (8, 10, 13). Most recently, we discovered that TXNIP is a potent regulator of beta-cell microRNA expression and thereby also inhibits insulin transcription and controls beta-cell function (14). MicroRNAs are small, ~22 nucleotide, non-coding regulatory RNAs that bind to the 3′-UTR of target mRNAs leading to degradation or translational inhibition of the target mRNA and down-regulation of target gene expression (15–17). Based on these combined find-

* This work was supported by grants to A.S. from the National Institutes of Health (R01DK-078752), the American Diabetes Association (7-12-BS-167) and the Juvenile Diabetes Research Foundation and JNJSI (40-2011-1).

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[2] The abbreviation used is: IAPP, islet amyloid polypeptide.

ings, TXNIP is currently being pursued as a therapeutic target for diabetes, but the full extent of TXNIP signaling in the beta-cell and its effects on metabolism remain to be determined.

Interestingly, the dramatic increase in *TXNIP* expression in response to glucose in our human islet microarray analysis was also associated with a marked induction of IAPP (4-fold) (6). Although this could be explained by the well established glucose effects on IAPP induction (18–20), it also raised the question of whether TXNIP may be involved in the observed up-regulation of IAPP. The present studies were therefore aimed at determining the effects of TXNIP on beta-cell IAPP and uncovered a novel gene regulatory system linking the critical beta-cell signaling pathways of TXNIP and IAPP and elucidated the transcriptional mechanisms involved.

EXPERIMENTAL PROCEDURES

Cell Culture—INS-1 cells were grown in RPMI 1640 (Invitrogen) with 11.1 mM glucose, 10% FBS, 1% penicillin/streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, 10 mM HEPES, and 0.05 mM 2-mercaptoethanol. Stably transfected INS-1 cells with constitutive TXNIP overexpression (INS-TXNIP) and control cells overexpressing LacZ (INS-LacZ) were generated and selected as described previously (8).

The beta-cell specific *Txnip* knock-out mice (bTKO) have been described previously (10, 21) as have the leptin-deficient obese and diabetic C57BL/6 *lep*$^{ob/ob}$ (ob/ob) mice (JAX) (14). Mouse islets were isolated by collagenase digestion (22). All mouse studies were approved by the University of Alabama at Birmingham Animal Care and Use Committee. Human islets were obtained through the Integrated Islet Distribution Program, and islets from the same donor were used as control.

Plasmid Construction and Transient Transfection Assays—The human IAPP promoter region was amplified from genomic DNA with primers from Table 1 and subcloned into the MluI and HindIII restriction sites of the pGL3 enhancer vector (Promega) providing the IAPP-Luc reporter plasmid. For luciferase assays, transfections were performed as described previously (23), and luciferase activity was determined by Dual-Luciferase assay kit (Promega). For siRNA knockdown experiments, INS-1 cells were grown in six-well plates and transfected with specific siRNA oligonucleotides for rat *Txnip* (Dharmacon; siGENOME SMARTpool gene ID 117514), rat *FoxA2* (Dharmacon; ON-TARGETplus SMARTpool gene ID 25099) or with scrambled oligonucleotide (0.1 μM; Dharmacon D-001810-01-20) using DharmaFECT 1 transfection reagent (5 μl/well). The final concentration of oligonucleotides used was 25 nM. Cells were harvested 48 h after transfection. For microRNA overexpression experiments, INS-1 cells were grown in six-well plates and transfected with hsa-miR-124a precursor or pre-miR negative control 2 (Applied Biosystem) at a final concentration of 25 nM using the DharmaFECT1 transfection reagent. For miR-124a knockdown, cells were transfected with miRIDIAN hairpin inhibitor rno-miR-124 or miRIDIAN microRNA hairpin inhibitor negative control at a final concentration of 25 nM (Dharmacon/Thermo Scientific) using DharmaFECT 1 transfection reagent (14).

Quantitative Real-time RT-PCR—Total RNA was extracted using miRNeasy Mini kit (Qiagen) according to the manufacturer's instructions. 1 μg of RNA was reverse-transcribed to cDNA using the first strand cDNA synthesis kit (Roche Applied Science). Quantitative real-time PCR was performed on a LightCycler 480 System (Roche Diagnostics) using Sybergreen (Applied Biosystems). qRT-PCR primers are shown in Table 1. All results were corrected for the 18 S ribosomal subunit (Applied Biosystems) run as an internal standard. Expression of miR-124a was quantified using a TaqMan microRNA Assay (Applied Biosystems), and results were corrected for U6 run as the internal standard.

Western Blotting—Whole cell protein extracts from INS-1 cells were prepared as described previously (9, 24). The following antibodies were used: rabbit anti-IAPP IgG (1:1000; Bachem), mouse anti-TXNIP IgG (JY2; 1:1000; MBL), goat anti-FoxA2 IgG (1:500; sc-6554), anti-rabbit IgG-HRP (1:5000, sc-2004), anti-goat IgG-HRP (1:5000, sc-2020), and anti-mouse IgG-HRP (1:5000, sc-2005) (Santa Cruz Biotechnology). Bands were visualized by ECL plus (Amersham Biosciences, GE Healthcare) and quantified by ImageQuant.

Chromatin Immunoprecipitation—ChIP assays were performed as detailed previously (7) using 4 μg of FoxA2 antibodies (sc-6554X) (Santa Cruz Biotechnology) and the primers listed in Table 1.

Statistical Analysis—Student's *t* tests were used to calculate the significance of a difference between two groups. For data sets of more than two groups, we performed one-way analysis of variance calculations.

RESULTS

TXNIP Induces beta-Cell IAPP Expression—To determine the effects of TXNIP on beta-cell IAPP expression, we performed gain and loss of function experiments using INS-1 cells with TXNIP overexpression (Fig. 1, *A* and *B*) and with TXNIP siRNA resulting in a ~80% TXNIP knockdown (24). Indeed, IAPP mRNA and protein expression were significantly increased in INS-1 beta-cells overexpressing TXNIP (Fig. 1, *C* and *D*), whereas TXNIP knockdown resulted in decreased IAPP expression (Fig. 1*E*). Importantly, although similarities exist between the IAPP and the insulin promoter, TXNIP does not promote insulin transcription. In fact, TXNIP overexpression led to a decrease in insulin expression (Fig. 1*F*), consistent with our recently published work and the observed increase in insulin production in islets of TXNIP-deficient mice (14). This suggested that TXNIP has divergent effects on IAPP and insulin and that the TXNIP-induced transactivation observed was specific for IAPP.

Primary islets of ob/ob mice, previously shown to have a ~4-fold elevation in TXNIP levels (14), also demonstrated a marked increase in IAPP expression (Fig. 1*G*), whereas IAPP expression was again reduced *in vivo* in primary islets of beta cell-specific *Txnip* knock-out mice (bTKO) (Fig. 1*H*), providing additional support for the notion of TXNIP-mediated regulation of IAPP expression.

Recently, we demonstrated that verapamil is a powerful tool to reduce endogenous beta-cell TXNIP levels, including in human islets (23). We therefore now investigated how modulating endogenous human TXNIP with verapamil would affect human IAPP. Interestingly, we found a dose-dependent de-

TABLE 1
Primers used in this study

Sequences (5′-3′)	Use
AAAACGCGTGTATATGAAATCAATGAACTAATG	Human IAPP promoter 5′-cloning primer
AAAAGATCTCTTTTAATGTTTCAATGTCAG	Human IAPP promoter 3′-cloning primer
TGAGAAGCAATGGGCATCCT	Human IAPP 5′-qRT-PCR primer
CAATGCAACAGAGAGCACAATG	Human IAPP 3′-qRT-PCR primer
ACAGAAAAGGATTCTGTGAAGGTGAT	Human/rat TXNIP 5′-qRT-PCR primer
GCCATTGGCAAGGTAAGTGTG	Human/rat TXNIP 3′-qRT-PCR primer
CGAGTCAAAGCCGTCAGGAT	Rat TXNIP 5′-qRT-PCR primer
TTCATAGCGCAAGTAGTCCAAGGT	Rat TXNIP 3′-qRT-PCR primer
CGAGTCAAAGCCGTCAGGAT	Mouse TXNIP 5′-qRT-PCR primer
TTCATAGCGCAAGTAGTCCAAAGT	Mouse TXNIP 3′-qRT-PCR primer
GTGCAACACAGCCACATGTG	Rat IAPP 5′-qRT-PCR primer
GCTGGAGCGAACCAAGAAGT	Rat IAPP 3′-qRT-PCR primer
CAGACCTTGGCACTGGAG	Rat insulin 5′-qRT-PCR primer
GAGCAGATGCTGGTGCAGCACT	Rat insulin 3′-qRT-PCR primer
CTCAGATGGACAAACGGAAGTG	Mouse IAPP 5′-qRT-PCR primer
AAGTTTGCCAGGCGTTGTGT	Mouse IAPP 3′-qRT-PCR primer
CTGAGGTGGGTAGCCAGAAA	Rat FoxA2 5′-qRT-PCR primer
CACGGAAGAGTAGCCCTCAG	Rat FoxA2 3′-qRT-PCR primer
GCACAAAATGGAAACTCCAAA	Rat IAPP FoxA2-site 5′-ChIP primer
CCCTCAACTTGCTTAGCTCTG	Rat IAPP FoxA2-site 3′-ChIP primer
TGTGAAAGGCAGCGAGTT	Rat PDX1 FoxA2-site 5′-ChIP primer
ACCTGCCTAACCCACACC	Rat PDX1 FoxA2-site 3′-ChIP primer
TCAATTCTAAGGTGTCCCTAGTC	Rat FOXO1 FoxA2-site 5′-ChIP primer
TGGGGCACAGCTCGTCTC	Rat FOXO1 FoxA2-site 5′-ChIP primer
ACCATGCTTCACTGACATTCTGA	Rat GADPH 5′-ChIP control primer
GGTCTGCCTCCCTGCTAACC	Rat GADPH 3′-ChIP control primer
TGGAAAAGCCAGTGGGCAGG	Rat PDX1 5′-qRT-PCR primer
GATGTGTCTCTCAGTCAAGT	Rat PDX1 3′-qRT-PCR primer
TGCGGTAATCAAATTCACGA	Rat ISL1 5′-qRT-PCR primer
GCATTTGATCCCGTACAACC	Rat ISL1 3′-qRT-PCR primer

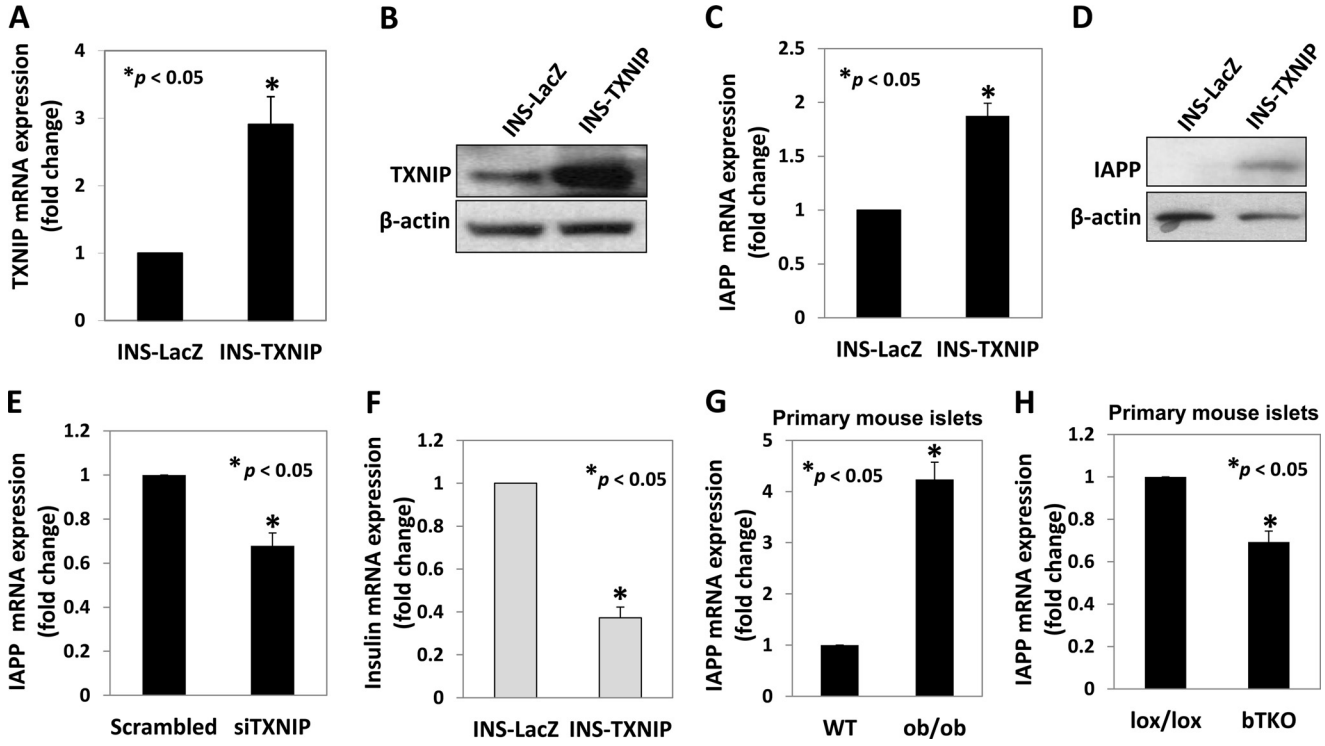

FIGURE 1. **TXNIP effects on IAPP expression in INS-1 beta cells and primary mouse islets.** *TXNIP* mRNA (*A*) and TXNIP protein (*B*) expression in INS-1 cells overexpressing TXNIP (INS-TXNIP) and control (INS-LacZ) cells. IAPP mRNA expression (*C*) and IAPP protein levels (*D*) in INS-TXNIP and INS-LacZ cells as assessed by qRT-PCR and Western blotting (protein loaded, ~20 μg/lane). *E*, IAPP expression in INS-1 cells transfected with specific siRNA oligonucleotides for rat *Txnip* or scrambled control. *F*, insulin expression in INS-LacZ and INS-TXNIP cells for comparison. IAPP expression in primary islets of wild-type (WT) or obese mice with elevated TXNIP levels (ob/ob) (*G*) or of beta-cell specific *Txnip* knock-out mice (bTKO) or control lox/lox mice (*H*). Western blots shown are representatives of three experiments. *Bars* represent means ± S.E. of at least three independent experiments.

crease in human IAPP in response to decreased *TXNIP* expression (Fig. 2, *A* and *B*), consistent with our findings in rat beta-cells and primary mouse islets. Moreover, we also generated a reporter construct driven by the human IAPP promoter and transfection into INS-TXNIP cells, and luciferase assays showed that TXNIP significantly increased human IAPP promoter activity (Fig. 2*C*), suggesting that the observed TXNIP-mediated induction of IAPP occurred at the transcriptional

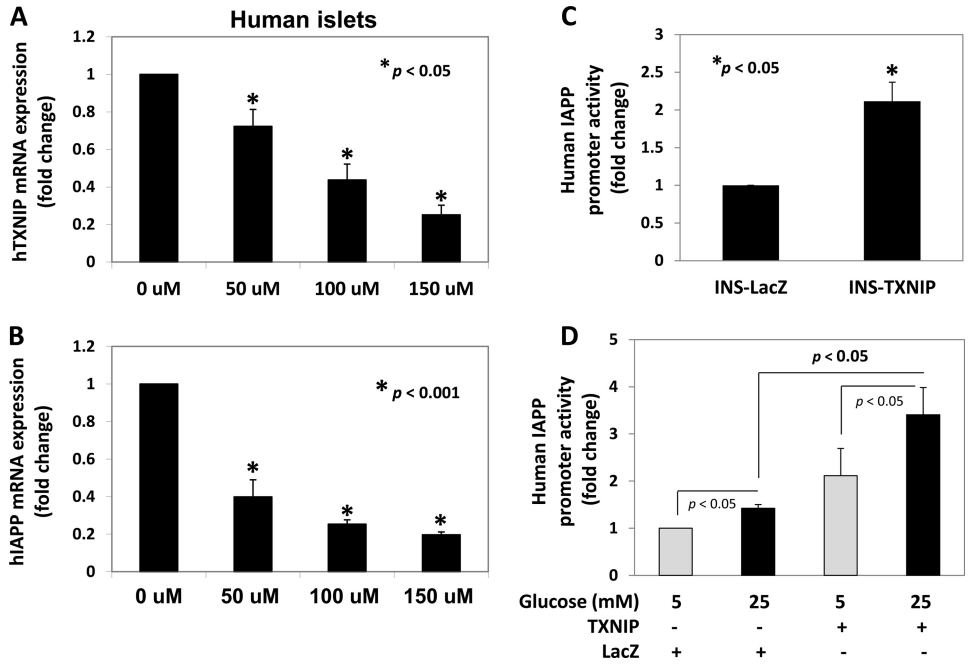

FIGURE 2. **TXNIP effects on human IAPP transcription.** *A*, endogenous TXNIP expression was inhibited in human islets using 24-h incubation with verapamil at the indicated doses and assessed by qRT-PCR. *B*, human IAPP expression in response to reduced TXNIP expression in the same human islets. Human IAPP promoter activity as assessed by luciferase activity in INS-TXNIP and INS-LacZ cells transfected with an hIAPP promoter reporter plasmid and cultured in regular growth medium (11.1 mM glucose) (*C*) or at low (5 mM glucose) or high (25 mM glucose) (*D*). *Bars* represent means ± S.E. of at least three independent experiments.

level. Furthermore, we also assessed the effects of elevated glucose on the induction of human IAPP transcription. Although glucose-induced IAPP transcription as expected, TXNIP had a strong additive effect and was able to activate the human IAPP promoter even beyond levels observed with high glucose alone (Fig. 2*D*). This suggests that TXNIP confers its effects at least in part by mechanisms that are distinct from those of glucose.

FoxA2 Mediates TXNIP-induced IAPP Transcription—To further elucidate the mechanisms by which TXNIP might regulate IAPP transcription, we analyzed the potential transcription factor binding sites in the human IAPP promoter using the MatInspector software, and this revealed two PDX1, four ISL1, and three FoxA2 sites (Fig. 3*A*). Transcription of the IAPP gene is known to be controlled by a complex promoter region and different transcription factors (25). Although PDX1 is thought to be the major transcription factor conferring the induction of IAPP by glucose (19, 26), there are also reports of ISL1 and FoxA2 being involved in the regulation of IAPP expression (19, 25, 27). We therefore next assessed whether TXNIP could alter beta-cell expression of any of these transcription factors. Interestingly, only *FoxA2* mRNA expression was significantly increased by TXNIP (Fig. 3*B*), and this was associated with a similar increase in FoxA2 protein levels (Fig. 3*C*), suggesting that FoxA2 might be a potential candidate for the regulation of IAPP by TXNIP.

To further test this possibility, we knocked down *FoxA2* using specific siRNA oligonucleotides and achieved significant inhibition of FoxA2 mRNA and protein expression (Fig. 4, *A* and *B*). This in turn resulted in completely blunted TXNIP-induced IAPP mRNA and protein expression (Fig. 4, *C* and *D*), as well as IAPP promoter activity (Fig. 4*E*), providing additional evidence for the critical role of FoxA2 in these effects. To directly evaluate whether TXNIP induces IAPP transcription by increasing FoxA2 binding to the IAPP promoter, we performed ChIP assays using FoxA2 antibodies and PCR primers flanking the three FoxA2 binding sites identified. We found that FoxA2 specifically bound to the proximal FoxA2 site but not the other two sites and that TXNIP significantly increased *in vivo* binding of FoxA2 to the IAPP promoter (Fig. 4*F*). Interestingly, alignment using ClustalW2 showed that, in contrast to the other two more distal sites, this proximal (−606 bp) FoxA2 binding site was also highly conserved across species (Fig. 3*A*). However, enrichment of FoxA2 at FoxA2 binding sites in the promoters of other known FoxA2 target genes (*i.e. Pdx1* and *FoxO1*) (28, 29) was not affected by TXNIP (Fig. 4, *G* and *H*), suggesting that the TXNIP effects were specific for IAPP. These results are consistent with the unchanged expression of *Pdx1* observed in response to TXNIP overexpression (Fig. 3*B*) and suggest that FoxA2 may be acting in concert with other regulatory factors that confer this specificity to the IAPP promoter. Together, these data suggest that TXNIP specifically induces IAPP transcription by promoting FoxA2 binding to its proximal binding site within the IAPP promoter.

TXNIP Down-regulates miR-124a Expression—Interestingly, we found that TXNIP not only enhances FoxA2 binding to the IAPP promoter but also increases the expression level of this transcription factor (Fig. 3, *B* and *C*). Taken together with our recent discovery that TXNIP regulates microRNA expression, this raised the possibility that TXNIP might do so by down-regulating a microRNA that targets *FoxA2*. In fact, *FoxA2* expression in MIN6 beta-cells has recently been found to be regulated by a specific microRNA, miR-124a (30). Moreover, these studies confirmed by luciferase reporter assays that miR-124a directly targets the *FoxA2* 3′-UTR leading to decreased expression of this transcription factor. Of note, the miR-124a sequence is 100% conserved across human, rat, and mouse, and

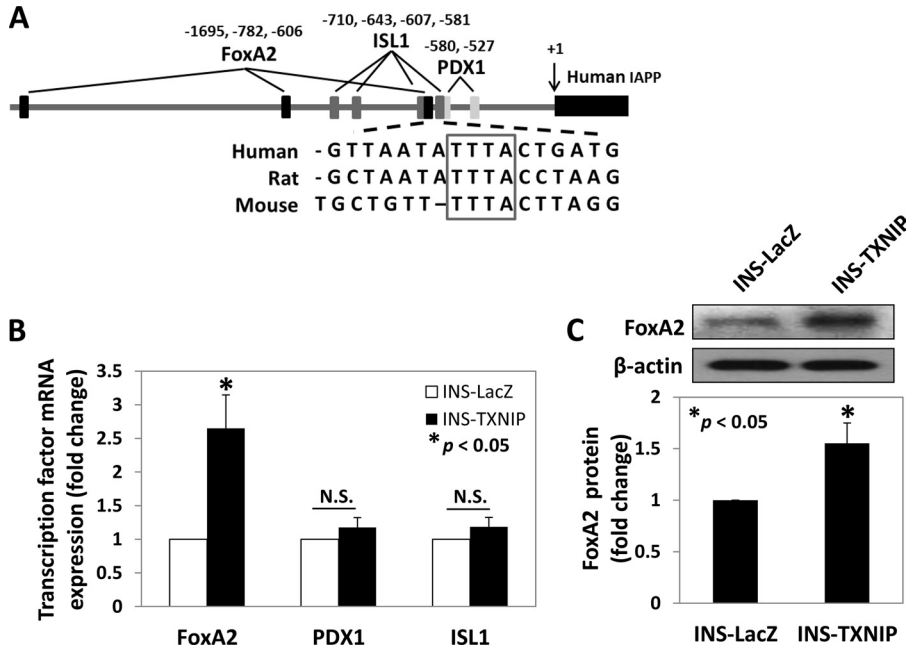

FIGURE 3. **Putative IAPP transcription factors and TXNIP effects on FoxA2.** *A*, schematic representation of the human IAPP promoter with putative transcription factor binding sites as analyzed by MatInspector software. ClustalW2 revealed a conserved FoxA2 binding site (highlighted). *B*, expression of putative transcription factors as assessed by qRT-PCR in INS-1 cells overexpressing TXNIP (INS-TXNIP). *C*, FoxA2 expression was assessed by Western blot analysis in INS-TXNIP and INS-LacZ cells.

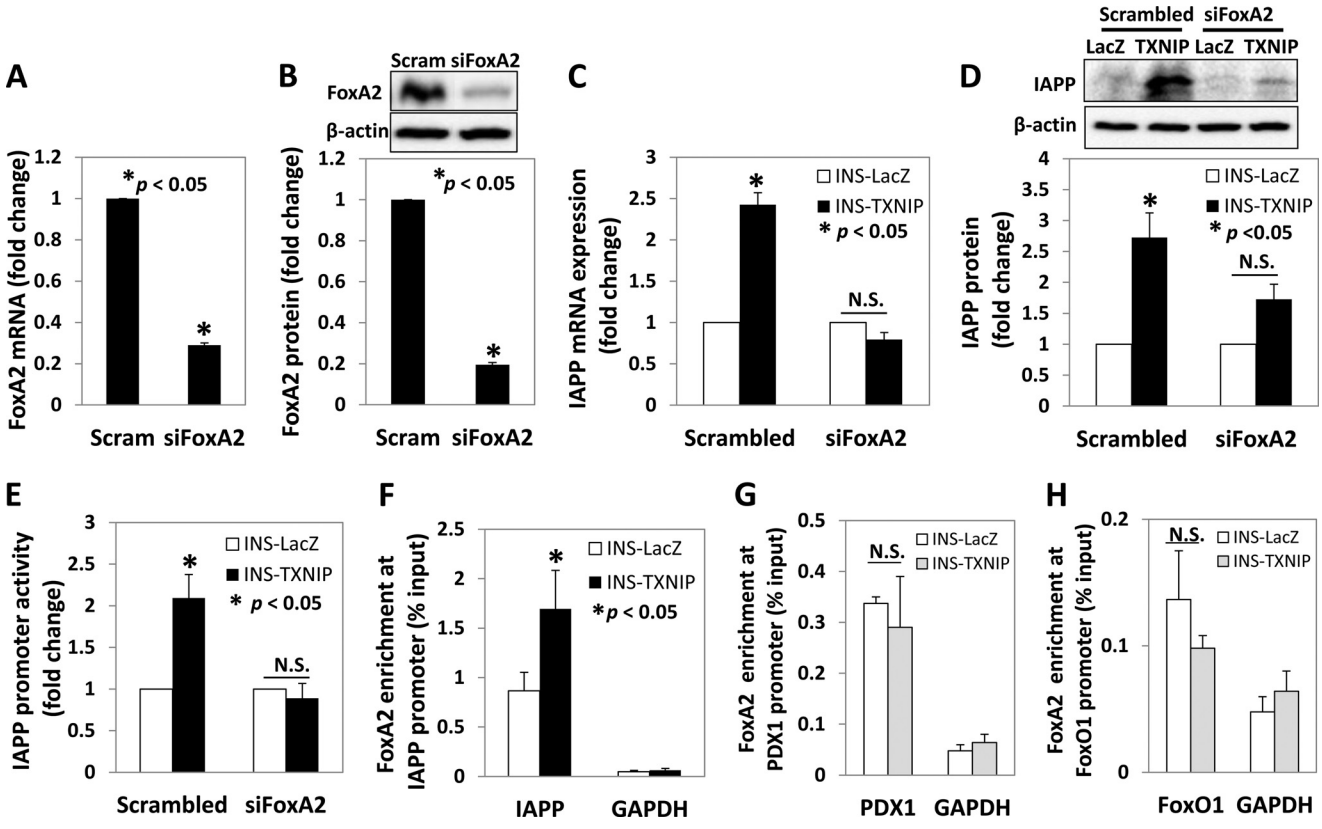

FIGURE 4. **Role of FoxA2 in TXNIP-mediated up-regulation of IAPP.** FoxA2 knockdown efficiency in response to specific siRNA oligonucleotides for rat *FoxA2* (*siFoxA2*) was assessed using qRT-PCR (*A*) and Western blotting (*B*). INS-TXNIP and INS-LacZ were transfected with siFoxA2 and IAPP expression was assessed by qRT-PCR after 48 h (*C*) and by Western blotting after 72 h (*D*). INS-TXNIP and INS-LacZ were co-transfected with siFoxA2, IAPP promoter reporter plasmid and with pRL-TK control plasmid, and 48 h after transfection, IAPP promoter activity was assessed by luciferase assays (*E*). Shown is FoxA2 occupancy of the IAPP promoter (*F*), *PDX1* promoter (*G*), and *FoxO1* promoter (*H*) as assessed by ChIP assay using a FoxA2 antibody and primers flanking the conserved FoxA2 sites. *Bars* represent means ± S.E. of at least three independent experiments; *, p = INS-TXNIP *versus* INS-LacZ control; INS-TXNIP siFoxA2 *versus* scrambled, $p < 0.05$.

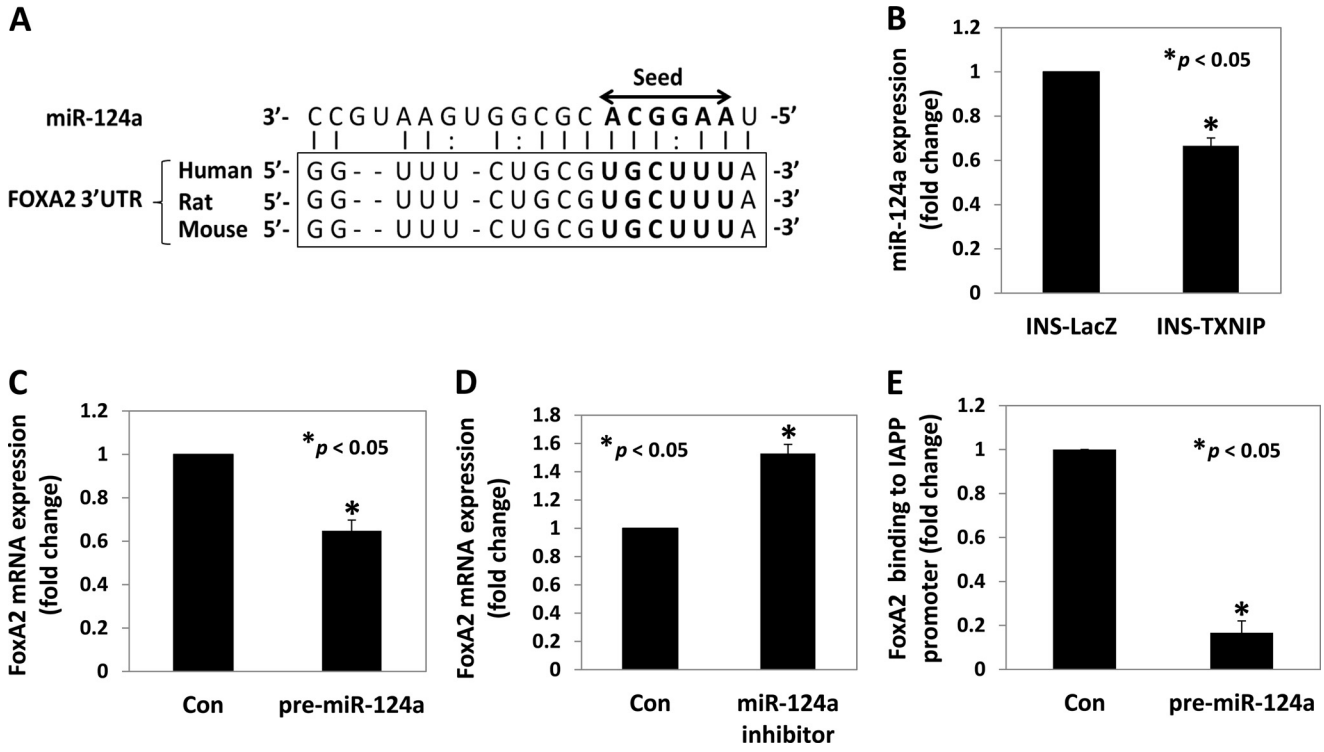

FIGURE 5. **Effects of miR-124a on FoxA2.** *A*, sequence alignment of mature miR-124a and its binding site in the human, rat, and mouse 3′-UTR of *FOXA2*; *arrow* marks seed sequence. *B*, expression of miR-124a in INS-1 cells overexpressing TXNIP (INS-TXNIP) and control cells (INS-LacZ) using qRT-PCR. Shown is *FoxA2* mRNA expression, determined by qRT-PCR, in INS-1 cells transfected with miR-124a precursor (pre-miR-124a) or scrambled control microRNA (*C*) or with a miR-124a inhibitor or control oligonucleotide (*D*). *E*, FoxA2 binding to the IAPP promoter as assessed by ChIP assay in INS-1 cells transfected with pre-miR-124a or scrambled control microRNA using FoxA2 antibody and primers flanking the conserved FoxA2 site. All *bars* represent means ± S.E. of at least three independent experiments.

the FoxA2 3′-UTR binding sites are also highly conserved, providing a perfect match for the critical seed sequence as shown by our alignment (Fig. 5*A*). Intriguingly, data from our microRNA microarray study also revealed that TXNIP led to a mild decrease in miR-124 expression (14). To now determine whether TXNIP was truly regulating miR-124a expression, we performed quantitative real-time RT-PCR experiments and indeed found significantly lower miR-124a levels in TXNIP overexpressing INS-1 beta-cells as compared with control cells overexpressing LacZ (Fig. 5*B*), suggesting that miR-124a might be involved in the observed regulation of FoxA2 and IAPP by TXNIP.

miR-124a Targets FOXA2 and Inhibits IAPP Transcription—To further determine whether miR-124a can control FoxA2-induced IAPP transcription, we first confirmed the down-regulation of *FoxA2* by miR-124a (Fig. 5*C*). Of note, inhibition of miR-124a led to the opposite effect and resulted in up-regulation of *FoxA2* providing additional evidence for the specific effects of miR-124a (Fig. 5*D*). Next, we tested the effects of miR-124a overexpression on the *in vivo* binding of FoxA2 to the IAPP promoter. Results of these studies revealed a dramatic down-regulation of FoxA2 occupancy at the IAPP promoter in response to miR-124a (Fig. 5*E*). Consistent with this finding, endogenous IAPP mRNA and protein levels were also significantly decreased by miR-124a (Fig. 6, *A* and *B*). In contrast, miR-124a had no significant effect on insulin expression (Fig. 6*C*). Furthermore, miR-124a overexpression also effectively blunted TXNIP-induced IAPP expression (Fig. 6*D*), suggesting that miR-124a indeed plays a critical role in this novel TXNIP/FoxA2/IAPP signaling pathway. Finally, although most studies presented were performed at 11.1 mM glucose, we also assessed TXNIP effects and miR-124a, FoxA2, and IAPP expression at low (5 mM) as well as high (25 mM) glucose concentrations (Table 2) demonstrating that the TXNIP/miR-124a/FoxA2/IAPP signaling cascade was still fully functioning under high glucose conditions. The TXNIP-induced changes in endogenous IAPP mRNA expression at low and high glucose presented in Table 2 are also in alignment with the TXNIP-induced changes in IAPP promoter-driven luciferase activity observed in Fig. 2*D*. In particular, both sets of experiments revealed that even in the presence of 25 mM glucose, TXNIP was still capable of significantly increasing IAPP promoter activity (Fig. 2*D*) or mRNA expression (Table 2) by ~2-fold, suggesting that TXNIP and glucose may confer their effects at least partially through different mechanisms.

Based on these combined findings, we propose a novel mechanism by which TXNIP decreases miR-124a expression, which releases *FoxA2* from the inhibition by this microRNA, resulting in increased *FoxA2* expression and FoxA2 binding to its proximal binding site in the IAPP promoter and induction of IAPP transcription (Fig. 7).

DISCUSSION

In summary, we have discovered that TXNIP induces IAPP expression and thereby reveal for the first time that the major beta-cell signaling pathways of TXNIP and IAPP are linked

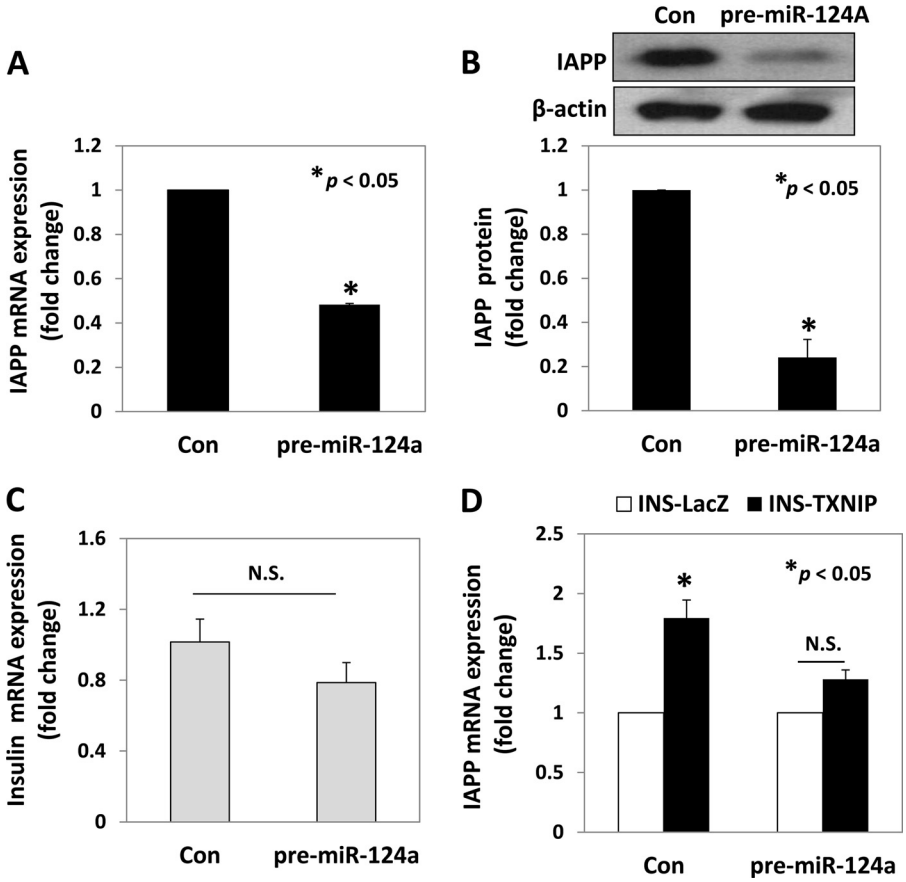

FIGURE 6. **Effects of miR-124a on IAPP expression.** IAPP mRNA expression (*A*) and IAPP protein levels (*B*) were assessed in INS-1 cells transfected with pre-miR-124a or scrambled control microRNA using qRT-PCR and Western blotting (protein loaded, ~60 μg/lane). *C*, insulin expression in response to pre-miR-124a as assessed by qRT-PCR and run as a negative control. *D*, INS-TXNIP and INS-LacZ cells were transfected with pre-miR-124a or scrambled control microRNA and harvested after 48 h, and IAPP mRNA expression was determined by qRT-PCR. *, p = INS-TXNIP *versus* INS-LacZ control; INS-TXNIP pre-miR-124a *versus* scrambled control, $p < 0.05$. All *bars* represent means ± S.E. of at least three independent experiments.

TABLE 2

TXNIP effects on the miR-124a/FoxA2/IAPP pathway at different glucose concentrations

Numbers represent fold change (± S.E.) in expression of miR-124a, FoxA2, and IAPP in INS-TXNIP *vs.* INS-LacZ incubated for 24 h at the designated glucose concentrations (n = 3).

	Low glucose (5 mM)		High glucose (25 mM)	
miR-124a	0.84 ± 0.03	$p = 0.044$	0.87 ± 0.02	$p = 0.044$
FoxA2	2.42 ± 0.33	$p = 0.071$	2.25 ± 0.31	$p = 0.083$
IAPP	2.94 ± 0.13	$p = 0.006$	2.03 ± 0.15	$p = 0.031$

intricately. Such an increase in IAPP expression has major implications for beta-cell biology as IAPP overexpression in beta cells leads to increased apoptosis (31) and decreased insulin secretion (32), whereas inhibition of IAPP expression results in enhanced beta-cell survival and glucose-stimulated insulin secretion (33).

Despite some similarities in the IAPP and insulin promoter, the TXNIP effects on IAPP transcription seem to be IAPP-specific, as we found that, in contrast to IAPP, insulin transcription is inhibited by TXNIP (14). When studying the mechanisms conferring this novel cross-talk between TXNIP and IAPP further, we also identified the transcription factor involved, FoxA2. The fact that *FoxA2* knockdown was able to completely blunt the TXNIP effects, suggests that FoxA2 signaling is a critical process in conferring the TXNIP effects on IAPP. However, other important mechanism might be involved

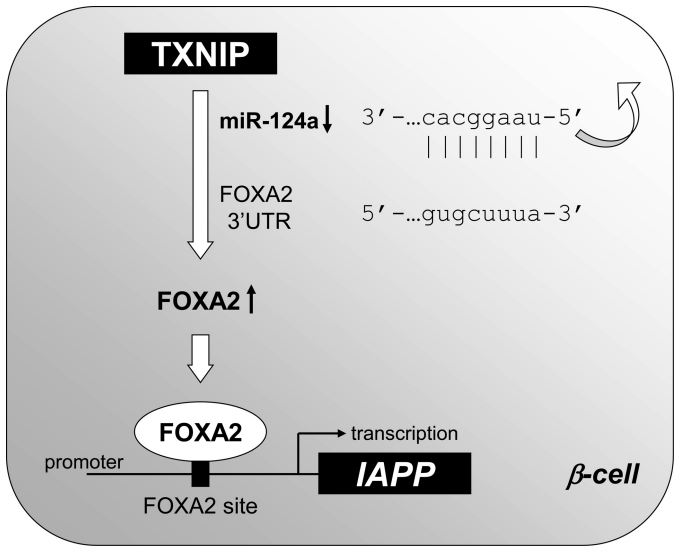

FIGURE 7. **Schematic representation of the proposed novel TXNIP/miR-124a/FOXA2/IAPP signaling pathway.**

in the regulation of IAPP expression, and the observed specificity of TXNIP-induced FoxA2 binding to the IAPP promoter is likely to be mediated by additional regulatory factors. In fact, the IAPP promoter contains well defined binding sites for PDX1 and ISL1, and there is strong evidence for the role of

these transcription factors and especially for PDX1 in the regulation of IAPP expression (19, 25–27). Although we did not observe an increase in PDX1 expression, it is still conceivable that enhanced PDX1 activity may have contributed to the increase in IAPP expression. In addition, the histone methyltransferase Set7/9 has been shown to regulate access of IAPP, but also of insulin, to the basal transcriptional machinery and to promote polymerase II occupancy (34). However, the lack of specificity makes this mechanism less likely to confer the TXNIP effects. Most recently, two microRNAs (miR-432 and miR376a) have been reported to target the 3′-UTR of IAPP (35), and we previously discovered that TXNIP regulates beta-cell microRNA expression (14). Even though our microRNA microarray did not reveal a decrease in these two microRNAs in response to TXNIP, microRNA-mediated regulation provides yet another potential level of IAPP control.

Given the wide-ranging actions of FoxA2 and the important role it has been shown to play in beta-cell development, metabolism, and survival (36, 37), the finding that it is regulated by TXNIP unravels a novel aspect of TXNIP biology. FoxA2 is a member of the FoxA subfamily of proteins that contain a centrally located DNA binding forkhead box domain (38), and it has been shown to play a pivotal role in maintaining glucose and lipid homeostasis by regulating gene expression in metabolically active tissues such as liver, fat, and pancreatic α and beta-cells (36–41). The current results seem therefore to be in alignment with this role of FoxA2 as a transcriptional activator. Intriguingly, beta-cell-specific *FoxA2* knock-out mice exhibit hyperinsulinemic hypoglycemia (39), similar to beta-cell-specific *Txnip* knock-out mice (10), whereas transgenic mice with beta-cell-specific human IAPP overexpression exhibit hyperglycemia (21). These *in vivo* findings support the idea that TXNIP, FOXA2, and IAPP could be members of a common pathway controlling beta-cell mass and glucose homeostasis. However, our current results provide the first demonstration that TXNIP, FOXA2, and IAPP are actually linked by a transcriptional activation cascade.

Interestingly, both TXNIP and aggregating human IAPP have been shown to be induced by glucose and to be involved in beta-cell apoptosis and inflammation (8, 10, 42, 43). The current new finding that TXNIP up-regulates IAPP fits therefore well with this notion of a series of detrimental processes occurring in response to elevated glucose levels or diabetes (18, 20, 44). However, the fact that TXNIP retained its effects on IAPP transcription at high glucose and that they were mediated by FoxA2 rather than Pdx1 (Pdx1 has been reported to be the main transcription factor mediating glucose-induced IAPP expression (19, 26)) also suggests that the signaling pathways by which glucose and TXNIP confer increased IAPP expression are distinct. Moreover, we found that TXNIP-induced changes in the endogenous expression of miR-124a, FoxA2, and IAPP were also maintained at high glucose. This further suggests that elevated TXNIP levels and the associated miR-124a/FoxA2/IAPP signaling may have additional detrimental effects in the context of diabetes.

The results are also in alignment with the recently discovered role of TXNIP in controlling microRNA expression in pancreatic beta-cells and its ability to thereby modulate the expression level of important beta-cell transcription factors (14). Based on findings in the MIN6 beta-cell line where miR-124a has been shown to target *FoxA2* and down-regulate its expression (30), we hypothesized that this microRNA may also contribute to the TXNIP effects on IAPP expression, which we found to be mediated by FoxA2. If so, we would expect TXNIP to down-regulate miR-124, whereas miR-124a overexpression should lead to down-regulation of FoxA2 signaling, decreased IAPP levels, and inhibition of TXNIP-induced IAPP expression. In fact, this is exactly what we observed (Figs. 5 and 6). Combined, these findings support the notion of TXNIP and IAPP being linked by a novel signaling cascade involving FOXA2 and miR-124a (Fig. 7).

Taken together, the identification of this novel TXNIP/miR-124a/FOXA2/IAPP signaling pathway provides new mechanistic insight into an important and previously unappreciated aspect of transcriptional regulation and beta-cell biology.

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