Tead1 reciprocally regulates adult β-cell proliferation and function

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Summary

Diabetes ensues when there is a net decrease in functional β-cell mass. Efforts to increase β-cell mass are limited by a concurrent loss of mature function. The molecular mechanisms underlying the reciprocal regulation between β-cell proliferation and mature function are unclear. Here, we demonstrate that constitutive and inducible genetic deletion of Tead1 in mouse β-cells leads to diabetes. Tead1, the transcription factor downstream of the mammalian-hippo pathway, has a developmental stage-specific critical function in β-cells and is required for maintenance of mature β-cell functional competence by direct transcriptional regulation of a network of critical β-cell transcription factors, including, *Pdx1, Nkx6.1 and MafA.* Concurrently, Tead1 directly activates *Cdkn2a* transcription in adult β-cells to inhibit proliferation. Studies in human β-cells demonstrate that Tead1 plays a similar regulatory role. Taken together, our findings uncover the non-redundant role of Tead1 in modulating the balance between proliferative capacity and functional competence.

Introduction

Diabetes has assumed epidemic proportions with over 422 million people affected by it worldwide (Roglic and World Health Organization, 2016). A reduction of functional β-cell mass underlies all forms of diabetes. In type 1 diabetes, there is an autoimmune destruction of insulin-producing β-cells, while in type 2 diabetes (T2D) there is, often, significant insulin resistance with varying degrees of β-cell dysfunction, accompanied by a reduction in β-cell mass (Butler et al., 2003) . Current strategies to promote proliferative capacity in adult β-cells are limited by concurrent loss of mature function (Bensellam et al., 2018; Efrat, 2008; Puri et al., 2018). Hence, understanding the interacting mechanisms regulating maintenance of functional competence, proliferation, and quiescence in adult β-cells is critical to enhancing functional β-cell mass.

Neonatal β-cells proliferate at a much higher rate than mature adult β-cells. However, these neonatal β-cells are functionally immature with limited glucose stimulated insulin secretion (GSIS) and display a lower expression of many genes associated with mature function, while expressing many genes normally 'disallowed' in mature β-cells (Lemaire et al., 2016; Pullen et al., 2010; Pullen and Rutter, 2013; Thorrez et al., 2011). Unlike their neonatal predecessors, adult β-cells, under basal physiological conditions, are mostly in proliferative quiescence, self-renewing at low rates that decline with aging (Dor et al., 2004; Meier et al., 2008; Teta et al., 2005). However, they do retain the ability to proliferate, under specific circumstances when faced with increasing metabolic demand, such as with pregnancy and peripheral insulin resistance. This ability, to proliferate on demand, declines dramatically with aging (Dhawan et al., 2016; Wong et al., 2009). While many intracellular signaling pathways have been linked with this differential response, their regulation remain poorly understood (Bernal-Mizrachi et al., 2014; Kulkarni et al., 2012; Stewart et al., 2015). Interestingly, many of these pathways converge on the expression of $p16^{INK4a}$ (*Cdkn2a*), an important cell cycle inhibitor that prevents progression from G1 to S phase of the cell cycle. p16 has been implicated to be central in maintaining this proliferative quiescence in mature β-cells (Chen et al., 2009; Dhawan et al., 2009; Krishnamurthy et al., 2006; Krishnamurthy et al., 2004). While recent studies have explored molecular pathways that regulate proliferation and the differential expression of p16 in adult and neonatal β-cells (Chen et al., 2011; Chen et al., 2009; Zhou et al., 2013), mechanisms that evolved to coordinate proliferative capacity and functional competence remain unknown. In this study, we identify the transcription factor, Tead1, as a regulator of this reciprocal regulation of proliferation and functional competence in β-cells.

The mammalian-hippo-Tead1 pathway regulates cell proliferation in many tissues (Dong et al., 2007; Heallen et al., 2011; Karpowicz et al., 2010; Lee et al., 2010; Liu et al., 2019; Lu et al., 2010; Yu et al., 2015). It consists of a core kinase cascade involving Mst1&2 kinases that

phosphorylate Lats1&2 kinases, which in turn phosphorylate coactivators, Yap and Taz. Phosphorylated Yap/Taz are retained in the cytoplasm and subsequently targeted for degradation. In the absence of this kinase signaling, Yap and Taz remain dephosphorylated and translocate to the nucleus, bind to various transcription factors, chief among them being the Tead family (Tead1-4), to regulate transcription, including enhancing cell proliferation (Meng et al., 2016; Yu et al., 2015). However, the role of the hippo-Tead1 pathway in β-cells is unclear. Deletion of Mst1/2 in mouse pancreas, leading to activation of Yap/Taz in Pdx1 positive-pancreatic progenitors, led to significant pancreatitis and ductal metaplasia, but no significant changes in β-cell proliferation or function were observed (Gao et al., 2013; George et al., 2012). However, overexpression of Yap has been shown to induce proliferation in β-cells, ex vivo (George et al., 2015b; Yuan et al., 2016), though Yap itself is not expressed in mature β-cells (Ardestani and Maedler, 2018; Gao et al., 2013; George et al., 2012). In addition, Tead1 and Yap have recently been implicated to regulate human embryonic pancreatic progenitors, with Yap serving as an inhibitor of endocrine lineage specification (Cebola et al., 2015; Mamidi et al., 2018; Zhang et al., 2013). Despite the recent increase in attention to the role of the hippo-Tead pathway in β-cells and in islets (Ardestani and Maedler, 2018), the function of the hippo-effector Tead1, the canonic downstream transcription factor of the mammalian-hippo pathway, in adult β-cell function and proliferation has not been addressed.

Here, by selectively deleting Tead1, in a cell-specific manner, at distinct stages of development, we dissect the function of Tead1 in β-cells. Our findings from two independent lines of mouse models uncover a surprising essential non-redundant function of Tead1 in mature islet glucose-stimulated insulin secretion (GSIS). Mechanistically, we identify Tead1 as a direct transcriptional activator of a network of genes, including, *Pdx1, Nkx6.1* and *Mafa*, that confer β-cells functional competence. Notably, contrary to its expected role in promoting proliferation, Tead1, is critical for maintaining proliferative quiescence in mature β-cells through direct transcriptional control of the cell cycle inhibitor, *Cdkn2a* ($p16^{INK4A}$). Consequently, deletion of Tead1, leads to a loss of insulin secretion and diabetes, despite an increase in β-cell proliferation. We demonstrate that this regulation is conserved in human β-cells. In conclusion, our finding of Tead1 in maintaining mature β-cell function and proliferative quiescence highlight its role in adult islet function and demonstrates its developmental context-dependent actions. Modulation of these activities may allow us to increase functional β-cell mass, without a loss of critical glucose homeostatic function, as a therapeutic approach.

Results

Tead1 is expressed in embryonic and adult beta cells

The mammalian Hippo-Tead pathway regulates proliferative pathways and determines organ size in many tissues during development (Yu et al., 2015; Zheng and Pan, 2019). However, its role in pancreatic and specifically β-cell development and function remains unclear. While loss of Yap expression during development is required for endocrine specification (Mamidi et al., 2018), deletion of the upstream kinases Mst1&2 did not significantly affect islet and β-cell development or function (Gao et al., 2013; George et al., 2012). This suggested that many of the regulatory functions of hippo-Tead pathway in islets and β-cells may be developmental-context specific. To address this, and determine the function of Tead1, the canonical transcriptional effector of the Hippo pathway, in β-cell proliferation and function during development and in the adult state, we examined the expression dynamics during islet and β-cell development and maturation. During mouse islet development, immunostaining revealed Tead1 protein was abundant at e15.5 in all pancreatic tissues, but become more restricted to islets around weaning and persists at a high level in adult β-cells (**Figure. 1a**), in contrast to the diminished but persistent expression in the exocrine pancreas. While Taz was robustly expressed throughout, from e15.5 into adult stage, Yap was absent in the islets at all stages (**Figure. 1a**).We then examined Hippo pathway gene expression in isolated adult islets. Among the Tead family members, we found that Tead1 - was preferentially expressed in adult mouse islets, whereas other members of the Tead family are present at 10-fold lower level (Tead2) or not detected (Tead 3 &4) (**Figure. 1b**). Of the two canonical Tead1 co-activators Yap and Taz, only Taz was expressed at moderate level (**Figure. 1b**). Most of upstream signaling components of Hippo pathway (including Mst1&2, Lats1&2) are expressed, suggesting this pathway exists functionally in islets.

Deletion of Tead1 in β-cells leads to diabetes

Tead1 floxed ($Tead1^{F/F}$) mice, carrying a floxed exon 3 that encodes the DNA binding TEA domain (**Fig. S1a-c**) (Liu et al., 2017), were crossed with two lines of Cre-deletor mice (Rip-Cre, two-Cre, Mip-CreERT) to specifically determine the role of Tead1 in β-cell proliferation, and function at distinct developmental-stages. Rip-β-$Tead1^{-/-}$ ($Tead1^{F/F}$ mice crossed with Rip-Cre mice) mice were generated to test Tead1 function in β-cells through early constitutive deletion at e15.5. The deletion Tead1 was evident in whole islets as indicated by >80% reduction in transcript (**Fig. 1c**) and near absence of detectable protein levels (**Fig. 1d**). Tead1 immunostaining was lost in β-cells (**Fig. 1e**), but not in other hormone producing cells in the islet **(Fig. S1d**) in these mice. Strikingly, Rip-β-$Tead1^{-/-}$ mice develop diabetes early, at 5 weeks of age, with steady increase of fasting and fed glucose levels that reaches >500 mg/dl at 2 month. (**Fig. 1f**). To test whether these mice develop diabetes due to impaired islet function, we examined first phase insulin secretion during the first 10 min after a glucose challenge. Near complete loss of glucose-induced first-phase insulin secretion peak at 2min, with significantly diminished insulin levels - less than 1/3 of the controls (**Fig. 1g**), was observed in these mice.

First-phase insulin secretory response - release of insulin from preformed primed granules into the circulation within few minutes of a glucose load, represents mature β-cell function, which is lost early in type 2 diabetes (Cerasi et al., 1972; Davies et al., 1994). Consistent with this finding of severe loss of islet secretory function in adult β-cells with Tead1 inactivation, Rip-β-Tead1$^{-/-}$ mice displayed significant glucose intolerance during an intraperitoneal glucose tolerance testing (GTT) accompanied by substantially blunted GSIS (**Fig. 1h-i**). These effects were not secondary to changes in body weight (**Fig. S1e**), food intake, whole body energy expenditure, VC02, V02, and RER (**Fig. S1f-j**), as they are similar in Rip-β-Tead1$^{-/-}$ mice, as compared to floxed littermate controls. While these results demonstrate that Tead1 plays a critical role in maintaining β-cell insulin secretory function, deletion of Tead1 early in embryonic β-cell development, as in Rip-β-Tead1$^{-/-}$ mice, precludes us from determining the absolute requirement of Tead1 in adult β-cells, without the confounding effects from potential developmental stage defects.

Tead1 is required to maintain mature β-cell function in adult mice

To determine the requirement of Tead1 in adult β-cell function and proliferation and distinguish it from its role in embryonic β-cell development, we used an inducible line, the Mip-CreERT deletor mice (Tamarina et al., 2014) to generate Mip-β-Tead1$^{F/F}$ mice, in which tamoxifen (TM) administration can achieve developmental stage-specific deletion of Tead1 in β-cells (Mip-β-Tead1$^{-/-}$ mice). Upon tamoxifen administration to 5 month Mip-β-Tead1$^{F/F}$ mice, near total loss of Tead1 protein was detected in whole islet lysates, as compared to all the controls tested (**Fig. 2a**), along with an absence of Tead1 nuclear staining in large majority (~90%) of β-cells (**Fig. 2b**).

Strikingly, with deletion of Tead1 in β-cells of adult 5month old males (**Fig. 2c**) or 11month old females (**Fig. 2d**), Mip-β-Tead1$^{-/-}$ mice became glucose intolerant within 2 months post-TM treatment. Similar to the Rip-β-Tead1$^{-/-}$ mice, the Mip-β-Tead1$^{-/-}$ mice also exhibited a severe blunting of in vivo GSIS during the GTT (**Fig. 2e**), indicative of significant loss of β-cell function.

Demonstration of the loss of β-cell functional competence in Mip-β-Tead1$^{-/-}$ model excludes limitations of hypothalamic expression and potential secretory dysfunction due to the Cre-transgene reported in the Rip-Cre models (Lee et al., 2006; Tamarina et al., 2014). Taken together, both models with Tead1 deletion, in embryonic (Rip-β-Tead1$^{-/-}$) or adult (Mip-β-Tead1$^{-/-}$) β-cells, demonstrate the non-redundant required function of Tead1 in maintaining mature β-cell function and whole body glucose homeostasis.

Tead1 controls the gene regulatory network required for maintaining functional competence in adult β-cells

To decipher mechanisms underlying how Tead1 regulates β-cell function, we undertook a detailed analysis of the islets in Rip-β-Tead1$^{-/-}$ mice. Isolated islets from Rip-β-Tead1$^{-/-}$ mice displayed impaired GSIS, with a significantly lower insulin secretion index (**Fig. 3a**), despite normal basal insulin secretion (1.625 vs. 1.616ng insulin/μg DNA in Rip-β-Tead1$^{-/-}$ and Tead1$^{F/F}$ controls respectively, p-not significant). Knockdown of Tead1 in Ins-1 (832/13) cells yielded a similar defect in GSIS (**Fig. S2a-b**), consistent with the cell-autonomous requirement of Tead1 for GSIS, seen in Rip-β-Tead1$^{-/-}$ mice. In addition to the impairment in GSIS, Rip-β-Tead1$^{-/-}$ mice had a significant reduction in insulin content, in the whole pancreas, per equally sized islet and per cell, as compared to floxed controls, (**Fig. 3b-d**).

To determine the mechanisms underlying the impairment of GSIS with Tead1-loss-of-function, we compared the transcriptomes of Tead1-deficient islets with those of littermate floxed controls (see Methods). Global transcriptome analysis by RNA-seq and validation by qPCR, revealed that transcripts of many genes, critical for GSIS, insulin gene expression, and maintenance of mature β-cell function, were significantly decreased in Tead1-deficient islets (**Fig. S3a and Fig. 3e**). We then compared the global transcriptome profile of Tead1-deficient islets to data from publicly available transcriptome datasets from neonatal and adult wild type islets (GSE47174) (Martens et al., 2014). Gene Set Enrichment Analysis (GSEA) revealed that genes that were upregulated in adult β-cells as compared to neonatal β-cells were significantly downregulated in the Tead1-deficient islets (**Fig. 3f**). However, many of the disallowed genes, such as *ldha* that are downregulated in normal adult β-cells, as compared to neonatal β-cells, were not significantly different in Tead1-deficient islets (**Fig. S3b**). Pathways that are normally active and enriched with differentiation and acquisition of mature secretory function in the β-cell, such as OXPHOS, substrate metabolism, protein secretion and export, vesicular transport and UPR were significantly decreased with loss of Tead1 (GSEA, q<0.25) **(Fig. S3c**). This was consistent with the phenotypic data, shown above, and expression data that demonstrated the loss of expression of many genes associated with β-cell maturity in Tead1-deficient β-cells. Specifically the transcript/protein levels of Ins1, Glut2, Pdx1, NeuroD, MafA and Nkx6.1 were all decreased in β-Tead1$^{-/-}$ islets, along with Ucn3, a marker of mature β-cells (Blum et al., 2012; van der Meulen et al., 2012) (**Fig. 3g-i**). These data demonstrated that Tead1 controlled, directly or indirectly, the gene regulatory network critical to maintain β-cell functional competence and its loss-of-function results in impaired GSIS.

Tead1 is a direct transcriptional regulator of critical β-cell transcription factors

While Tead1 has been shown to be a component of a combination of transcription factors, including Pdx1, that activates pancreatic progenitor enhancers during embryonic pancreatic differentiation (Cebola et al., 2015); the molecular function of Tead1 in adult β-cells is

unknown. To determine if Tead1 was a direct transcriptional regulator of critical genes that regulate β-cell function and identity, we analyzed the occupancy of Tead1 on the promoter elements of transcription factors critical for adult β-cell function. ChIP-qPCR with pull-down of either endogenous Tead1 (**Fig. 4a**) or overexpressed Myc-Tead1 (**Fig. S4a**), in mouse insulinoma cell line (Ins-2 cells – See Methods) demonstrated that Tead1 occupied cis-promoter elements of these genes, suggesting direct transcriptional regulation. Interestingly, there were multiple Tead1 responsive elements in the Pdx1 promoter region (**Fig. 4b**), including one in Area IV region, critical to β-cell specific Pdx1 expression and postnatal mature β-cell function (Spaeth et al., 2017), which displayed the highest enrichment in ChIP pull down studies with Myc-Tead1 overexpression in Ins-2 cells (**Fig. 4c**). Tead1, when co-expressed with its co-activators, Yap or Taz, activated transcription from this native Pdx1-promoter carrying the upstream Tead1 response elements (**Fig. 4d**) in an *in vitro* promoter luciferase assay in Ins-2 cells, demonstrating functional regulation.

Cell autonomous regulation of Pdx1 was also confirmed in Ins-2 cells, as Tead1-knockdown decreased Pdx1 protein level (**Fig. 4e**). Similarly, Tead1 overexpression, by adenoviral vector, in human β-cell line (EndoC-βh2) or in cadaveric islets led to an increase in expression of PDX1 (**Fig. 4f-g)**. Consistent with this, inhibition of Tead1 activity in human islets, with Verteporfin (VP) that prevents the binding of TEAD1 to its co-activators (Liu-Chittenden et al., 2012), caused a significant reduction in PDX1 protein (**Fig. 4h**), indicating TEAD1 regulation of *PDX1* is conserved in human β-cells. This demonstrated that Tead1 occupies upstream cis-promoter regions and as a functional direct transcriptional regulator orchestrates the gene regulatory network of transcription factors, including Pdx1, Nkx6.1, MafA, critical to acquiring and maintaining mature β-cell identity and functional competence.

Tead1 is required to maintain proliferative quiescence in adult β-cells

Since Tead1 enhances cell proliferation in many tissues (Liu et al., 2019; Meng et al., 2016; Yu et al., 2015), the expectation was that early during development at the secondary transition e15.5, when β-cell proliferation is high, Tead1 deletion would cause a reduction in β-cell proliferation and number, with consequent diabetes. Contrary to this expectation, Rip-β-Tead1$^{-/-}$ mice, surprisingly, did not have a decrease in β-cell area (**Fig. 5a**). In fact, Rip-β-Tead1$^{-/-}$ mice had more β-cells per islet, reflected by increased DNA content in equally sized islets (**Fig. 5b**), and theseβ-cells were smaller in size (**Fig. 5c** and **Fig. S5a**). This was also demonstrable in TM-treated Mip- β-Tead1$^{-/-}$ mice, wherein, inducible adult β-cell Tead1 deletion did not result in a change in β-cell area, nor a change in size of individual islets, as compared to controls (**Fig. S5b-c**). This increase in β-cell number was not due to a change in apoptosis (**Fig. S5d-e**), but to an increase in proliferation, reflected by a significant increase in staining for Ki67, an indicator of

cells in active cell cycle (G1/S/G2/M), in 3 month old Rip-β-Tead1$^{-/-}$ mice (2% vs. 0.62% in Rip-β-Tead1$^{-/-}$ and Tead1$^{F/F}$ controls, respectively; p<0.01) (**Fig. 5d-e**).

Embryonic and neonatal β-cells have a high proliferation rate which drops dramatically to adult levels after weaning (Teta et al., 2007), as they acquire mature GSIS function. Since the data above demonstrated an increased proliferation in adult β-cells with Tead1 loss-of-function, we proceeded to test if Tead1 has a developmental stage-specific regulation of proliferation in β-cells. Analysis of BrdU incorporation into β-cells, in vivo, over a 2 hour period, in neonatal (postnatal day 12), post-weaning (4 weeks) and adult (12 weeks) mice revealed that, while there was no significant difference in proliferation in postnatal day 12 (4.4% vs. 4.1%, in Rip-β-Tead1$^{-/-}$ and Tead1$^{F/F}$ littermate controls, respectively; p-not significant), there was a significant increase in BrdU incorporation in Tead1 deficient β-cells in Rip-β-Tead1$^{-/-}$ mice, compared to Tead1$^{F/F}$ controls, at 4 weeks (5.31% vs. 4.08% respectively; p<0.03) and 12 weeks of age (0.5% vs. 0.2%, respectively; p<0.05) (**Fig. 5f-g)**. It is noteworthy that the increase in β-cell proliferation at 4 week old Rip-β-Tead1$^{-/-}$ mice precedes the hyperglycemia seen at 5 weeks of age (Fig. 1F), which suggests that the increase in proliferation is not secondary to hyperglycemia-induced compensation. This data demonstrated that Tead1 does not modulate β-cell proliferation during physiologic neonatal expansion when the proliferative drive is high, but is required for attaining proliferative quiescence as β-cells mature in adult mice.

While the β-cell incorporation of BrdU, over a 2 hour period, indicated that more Tead1-null β-cells were in S-phase of the cell cycle in 12 week old Rip-β-Tead1$^{-/-}$ mice, labelling all β-cells going through S1 phase over a 6 day period, by administering BrdU via drinking water, revealed that the pool of β-cells that were actively proliferating during this period was ~4 fold higher in Tead1-deficient β-cells as compared to controls (17.1% vs. 4.46% respectively; p≤0.01) (**Fig. 5h-i**). Interestingly, the fraction of proliferating β-cells in S-phase (2hr labelling BrdU positive) to proliferating β-cells in all phases (Ki67 positive cells) was unchanged between the Rip-β-Tead1$^{-/-}$ and floxed controls (**Fig. S5f**), suggesting that cell cycle kinetics, *in vivo*, were not significantly altered, once Tead1-deficient β-cells entered cell cycle. Furthermore, while Tead1 depletion in Ins-2 cells led to an increased fraction of cells in S+G2/M phases, when compared to controls, it did not alter the ratio of cells in S and G2/M phases (**Fig. S5g-h**). This supports the assertion that the increased proliferation in Tead1-deficient β-cells was secondary to more β-cells moving from the quiescent (G0) state into active cell cycle (G1). β-cells from TM-treated Mip-β-Tead1$^{-/-}$ mice also exhibited similar findings of a 2 fold increase in proliferation (**Fig. 5j and Fig. S5i**), even when Tead1 was deleted at 9 months of age, when most β-cells are normally quiescent and resistant to proliferative signals (Zhou et al., 2013). This data demonstrates that Tead1 was required to maintain proliferative quiescence in adult β-cells, and deletion of Tead1 led to a loss

of quiescence with an increase in the number of β-cells in the active phases of cell cycle. To conclusively test if this is secondary to a cell-autonomous requirement of Tead1 to maintain proliferative quiescence, we established a mosaic β-cell Tead1-deletion model. We developed a TM administration protocol, wherein we obtained ~ 50% deletion of Tead1 in β-cells (see methods, Refs: (Tamarina et al., 2014; Wicksteed et al., 2010) and **Fig. S5i**). Using this model, we asked if proliferation, as assessed by BrdU incorporation, is different in adult β-cells, with (Tead1 positive) and without (Tead1-negative) functional Tead1, in the same mouse, thus excluding any secondary non-cell-autonomous effects (such as hyperglycemia) on proliferation. Consistent with the findings in the Rip- β-Tead1$^{-/-}$ mice, double staining for BrdU and Tead1 revealed that proliferation was 5 fold higher in Tead1-negative β-cells as compared to Tead1-positive β-cells (0.58% vs. 0.11%; $p<0.05$), within the same Mip-β-Tead1$^{-/-}$ mouse (**Fig. 5k**). This confirmed cell cycle regulation by Tead1 was cell-autonomous and not secondary to changes in glucose homeostasis or other non-cell autonomous factors.

Tead1 is a direct transcriptional activator of p16^{INK4a} to regulate β-cell proliferation

While the increased proliferation of Tead1-deficient β-cells was surprising and contrary to the current paradigm of Tead1 inducing cellular proliferation, global transcriptome analysis of Tead1-deficient islets revealed significant upregulation of genes that were related to cell proliferation, including those involved in G1-S checkpoint (**Fig. 6a**). Expression of Cyclin A2, a G1-S phase transition-promoting gene critical in commitment to proliferation and promoting the proliferation of most cells (Bertoli et al., 2013) including β-cells (Song et al., 2008), was significantly increased (**Fig. 6b**). Though Tead1 deletion led to a mild decrease in Cyclin D1 transcript expression, there was no significant decrease in protein levels (data not shown). However, there was a significant decrease in expression of p16^{INK4a} and p19^{ARF}, critical cell cycle inhibitory genes (**Fig. 6b**). p16^{INK4a} protein, responsible for limiting β-cell proliferation in adult mice (Chen et al., 2009; Dhawan et al., 2009; Krishnamurthy et al., 2006), was decreased in Tead1-deificent islets (**Fig. 6c**). Tead1-knockdown in Ins-2 cells, led to a decrease, while overexpression led to an increase, in p16^{INK4a} protein levels (**Fig. 6d-e**). *Cdkn2a* locus generates both of these proteins (p16^{INK4a} and p16^{ARF}) from alternate start sites/alternate splicing and, interestingly, has several Tead1 binding consensus motifs (**Fig. 6f**). A chromatin immunoprecipitation assay revealed that Tead1 occupied these motifs in the promoter of p16^{INK4a} in the *Cdkn2a* locus (**Fig. 6g**), and activated the native mouse promoter of *Cdkn2a* encoding p16^{INK4a} and p16^{ARF} in Ins-2 cells, when co-expressed with its co-activators, Yap or Taz, in a promoter luciferase reporter assay (**Fig. 6h**). These data demonstrated that Tead1 exerts cell-autonomous, direct transcriptional activation of p16^{INK4a} in adult β-cells and thus repress proliferation to maintain proliferative quiescence.

Taken together, these data indicate that Tead1 is a critical β-cell transcription factor that directly activates the transcription of critical genes required for maintaining mature functional competence in β-cells of adult islets, while restraining their proliferation to maintain proliferative quiescence.

Discussion

Acquisition of mature function is often accompanied by proliferative quiescence in many cell types, including β-cells (Puri et al., 2018). The molecular mechanisms underlying this reciprocal regulation has been poorly understood and this knowledge gap limits many therapeutic approaches in regenerative medicine. Here, we place Tead1 as a novel transcriptional factor required to maintain adult β-cell mature identity and functional competence, as well as proliferative quiescence, by direct transcriptional activation of critical β-cell genes and cell cycle inhibitors (schematically shown in **Fig. S6**). Our data demonstrates that Tead1 is required for normal adult β-cell function and proliferative quiescence, but not for β-cell proliferation during the neonatal period. We have confirmed this regulation using multiple transgenic lines, to exclude confounding factors stemming from the individual Cre-lines and in ex vivo human and mouse islet models to demonstrate the conserved nature of this regulation.

The hippo-Tead pathway has been extensively studied in development and ontogenesis, though its role in β-cell function has received scant attention. Two groups, independently, reported that deletion of upstream kinases of the hippo pathway (Mst1&2), using the Pdx1-Cre driver, in Pdx1-positive pancreatic progenitors, led to activation of Yap in the pancreatic progenitors and significant changes in ductal and exocrine lineages with severe ductal metaplasia and pancreatitis. Strikingly, the endocrine lineage was minimally affected with normal functional β-cells, indicated by preserved glucose homeostasis (Gao et al., 2013; George et al., 2012). In other studies, Yap-Tead1 biding motifs were shown to be enriched in stage specific enhancers enriched in multipotent pancreatic progenitors in developing human pancreas (Cebola et al., 2015), while a recent study demonstrated that Yap functioned to limit endocrine and promote duct/exocrine lineages in Pdx1-positive progenitors and that loss of Yap expression, possibly regulated by mechanotransduction from extracellular matrix cues, was required for Ngn3-positive cells to develop into the endocrine lineage (Mamidi et al., 2018). Similar findings of the inhibitory effects of Yap on iPS-derived insulin producing β-cell differentiation were also recently reported (Rosado-Olivieri et al., 2019). Interestingly and in contrast to this, Yap overexpression has been shown experimentally to promote human β-cell proliferation, ex vivo (George et al., 2015a; Yuan et al., 2016), though its physiological role is undetermined. These studies strongly suggest that Yap-Tead1 axis regulates endocrinogenesis and that this

regulation may be independent of upstream canonical hippo kinase signaling. Yet, the function of Tead1 in β-cell function has remained unknown thus far.

β-cells are highly proliferative before weaning, with proliferative rates as high as 4%. However, as they gain mature function, like many highly specialized post-mitotic cells, β-cells lose their high basal proliferative rate, while still retaining their capacity to re-acquire it under specific conditions of high metabolic demand, such as with pregnancy and exposure to high fat diet (Dhawan et al., 2016; Stewart et al., 2015). In the mature adult state, the proliferative quiescence is enforced primarily by the increasing expression of the cell cycle inhibitor $p16^{INK4A}$ (encoded by *CDKN2A*), while the polycomb repressive complex proteins, including Ezh2 and Bmi1, regulate $p16^{INK4a}$, to permit proliferation in immature β-cells, but restrict proliferation in β-cells in an age-dependent manner (Chen et al., 2011; Chen et al., 2009; Dhawan et al., 2009; Tschen et al., 2009; Zhou et al., 2013). In this study, we demonstrate that Tead1 is a novel transcriptional regulator of p16 and expression of Tead1 is required for continued expression of p16 and proliferative quiescence in mature β-cells. A loss of this regulation leads to a reacquisition of a high proliferative rate. While we demonstrate Tead1 as a direct transcriptional regulator of p16, it is possible that it also interacts with other regulators of p16 including Ezh2, Bmi1 and others, especially in age-related restriction of β-cell proliferation and how this can be modulated to enhance functional β-cell mass will be a subject for future studies.

It has been well established that immature β-cells, as in neonatal mice, express many proteins encoded by genes that are disallowed in mature β-cells, including hexokinase1 (HK1), AldolaseB, Ldha, Acot7, MCT1 and many others (Dhawan et al., 2015; Lemaire et al., 2016; Pullen et al., 2010; Thorrez et al., 2011). Many of these have been associated with the poor GSIS associated with fetal/immature beta cells. In addition, in rodent β-cells, there is a transition from high MafB/low MafA expression to low MafB/high MAfA expression, with acquisition of mature function (Martens et al., 2014; Nishimura et al., 2006), exemplified by robust GSIS along with a high expression of genes associated with mature function including Pdx1, Neurod1, MafA, Nkx6.1, Glut2, Glucokinase, Ucn3 and others. A loss of function of these genes is associated with a loss of mature function accompanied by a fetal/immature gene expression phenotype with expression of disallowed genes with high MafB expression and loss of MafA expression. In contrast to this de-differentiation phenotype, that is seen in many adult β-cell proliferative phenotypes, such as with activation of c-Myc (Puri et al., 2018) or Notch (Bartolome et al., 2019), loss of Tead1 function is not associated with re-expression of many of the disallowed genes, such as *Ldha*, nor a re-expression of MafB, markers of immature mouse β-cells (Nishimura et al., 2006). Interestingly a change in methylation status of the promoters of the genes has been associated with acquisition of mature β-cell function, specifically demethylation of the promoters of genes associated with maintenance of mature β-cell

function and methylation of disallowed gene promoters (Dhawan et al., 2015). This finding was consistent with a methylation drift in aged mouse islets as compared to juvenile islets wherein increased DNA methylation of genes involved in proliferation along with a decrease in methylation of maturity/metabolism related genes, correlating with the decreased and increased expression of these genes respectively (Avrahami et al., 2015). These are similar to the changes seen in Tead1-deficient islets raising the possibility that Tead1 may regulate these methylation/demethylation enzymes to reciprocally regulate proliferation and maturity genes in β-cells, a possibility that needs further investigation. Thus, Tead1 function appears to be required for maintaining the mature identity genes, but not a repression of the fetal/immature genes.

While the requirement of Tead1 for normal β-cell function is demonstrable in these studies, it would be interesting to speculate how this could be regulated in physiology and in disease states. While factors that regulate upstream hippo kinase cascade could certainly play a role, other stimuli may play a direct role in regulating Tead1 function, independent of the hippo kinase cascade. Interestingly, recent studies have shown that Tead1 subcellular localization could be altered by p38 Map kinase activation and glucose itself (Lin et al., 2017) or its stability of co-factor binding can be regulated by palmitoylation (Chan et al., 2016; Kim and Gumbiner, 2019; Noland et al., 2016). This raises the intriguing possibility that stress pathways and glucolipotoxicity could affect β-cell function via changes in Tead1 localization and activity. Could then glucotoxicity, lipotoxicity or other stress-induced induced β-cell dysfunction be modulated by regulation of Tead1 activity and provide novel therapeutic targets? Answers to these questions in future studies could uncover new druggable targets for therapy of diabetes.

This reciprocal regulation of mature function and proliferative quiescence enforced in β-cells, by Tead1, could also potentially be the basis of the hurdles in inducing β-cell proliferation without loss of function. However, recent studies on human islets with Yap overexpression have shown that activation of this pathway could lead to proliferation without loss of function (George et al., 2015a; Yuan et al., 2016), suggesting that there are as yet undetermined pathways that are regulated by Tead1. While the regulators of Tead1 action in β-cells remain unknown, identification of these would allow therapeutic modulation to enhance β-cell replacement therapy.

Methods

Methods and any associated references are appended below.

Expression data have been deposited in GEO: 12 weeks (GSE139228) and 1 year (GSE139152).

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Author Contributions

J.L. designed and conducted the experiments, performed data analyses, interpreted data and helped write the manuscript; R.L., B.S.K. helped with specific experiments and data analysis. P.K.S., O.S., M.O.H., F.L., R.J., P.Y., V.N., E.M.P-G., R.B., Y.Z., C.C., C.J.C., K.M., and M.M. provided critical scientific input, data analysis, and logistical support. R.L., M.O.H, K.M. and M.M. provided critical input into editing the manuscript. V.K.Y supervised the project, designed experiments, interpreted data and wrote the manuscript.

Competing Financial interests

The authors declare no competing financial interests.

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Methods

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine and the University of Pittsburgh. Tead1 floxed ES cells were obtained from KOMP (Clone Name: EPDO207_2_H11), wherein the DNA binding domain coding Exon 3 is floxed. Targeted ES cells were injected into blastocysts to generate chimeric mice and those that demonstrated germline transmission were crossed with mice expressing FLPe recombinase to remove the neomycin resistance cassette to generate Tead1 loxP alleles [floxed exon: ENSMUSE00000203030]. The homologous recombination was verified by long range PCR using primer set #1 (forward: GCAGTGCTCTCAGGAGTGCTGAGTGCGAC; reverse: AGAAGCCACAGTGCCCTGGAAGTGT) and primer set #2 (forward: GCCTTCTGAGTGCTGGCATTAAAGG; reverse: CACTGCAGCGCGCATAGCCTATGCTCCTTC) (**Extended Fig. 1**). Mice were genotyped using *Tead1*-flox-forward (GCCTTCTGAGTGCTGGCATTAAAGG) and reverse (AAGGCAGACTCCTTCATTGGAATGG) primers. Floxed Tead1 ($Tead1^{F/F}$) mice were crossed with Rip-Cre transgenic mice to generate β-cell-specific Tead1 deletion during embryonic development. For adult β-cell inducible deletion, Tead1 $^{F/F}$ mice were crossed with Mip-CreERT transgenic mouse (Ins1 promoter driven Cre-ER, courtesy of Dr. Louis Philipson) to generate Tead1 $^{F/F}$ Mip-CreERT^{+} mice that when treated with Tamoxifen (TM) 100 mg/ kg every other day by gavage for 5 doses induced β -cell-specific Tead1 deletion (Mip-β-$Tead1^{-/-}$). Mice were maintained under standard 12-h light-dark cycles with ad lib access to food and water, unless specified otherwise.

In vivo experiments

Glucose tolerance tests (GTTs) and acute insulin secretion tests were performed in 16 hour overnight fasted mice administrated 1.5 g/kg or 3 g/kg D-glucose i.p. respectively.

Metabolic cage measurements

Metabolic cage measurements were performed as described previously (Lee et al., 2013). Oxygen consumption (VO_2), energy expenditure, carbon dioxide production (VCO2), and the respiratory exchange ratio (RER) were measured with the comprehensive laboratory animal monitoring system (Columbus Instruments) in individual cages without bedding. Mice were acclimated to the metabolic cages for 4 days prior to the start of data collection. Data was collected for 72 h. All data was analyzed and averaged for the dark and light cycles separately.

Islet isolation and *ex vivo* insulin secretion

Islets were isolated as previously described (Lee et al., 2011; Lee et al., 2013). Briefly, 1mg/ml collagenase P (Roche) dissolved in HBSS was injected into the common bile duct of anesthetized mice and the pancreas was removed, minced and incubated in collagenase solution at 37°C for 17 min. After three washes with 10% FBS, the islets were pelleted at 200 g and purified on Histopaque (Sigma) gradient. Islets from each mouse were individually purified by handpick for

all experiments. The islets were incubated in 11.1 mM RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin, L-Glutamine overnight. GSIS studies were performed as described previously (Lee et al., 2011). Ten similar sized islets from individual mice were used, with at least three mice from each genotype, under each experimental condition. Incubation times for ex vivo insulin secretion studies were for 30 min for each condition and the results were normalized to the DNA content or to the total insulin content of the islets measured after acid ethanol extraction at the end of the experiment. All insulin assays were performed using mouse insulin ELISA kit (Mercodia). Insulin content was assayed after acid-ethanol extraction from whole pancreas and isolated islets and normalized to DNA and protein measured from the same extract. Similar protocol was used for the GSIS experiments in 832/13 cells.

Gene and protein expression

Quantitative PCR (qPCR) from cDNA made from DNase-digested RNA was performed with gene-specific primers with SYBR green mix and normalized to the expression of housekeeping genes (*Topl, Actb,* and *Ppia*). The sequences of the primers used are available in extended information table.

Islets were isolated from each mouse individually as described above and 150–200 islets were snap frozen immediately. Western blotting was performed, as described before[31]. Briefly, total protein was extracted from 150-200 islets mice using a protein lysis buffer with a cocktail of protease inhibitors and separated by a standard SDS-PAGE and transferred to a nitrocellulose membrane. Tead1 antibody (rabbit polyclonal, 1:10,000; Abcam), p16 antibody (mouse

monoclonal, 1:1,000; Abcam), Pdx1 (rabbit polyclonal, 1:10,000; BCBC), and β-actin antibody (rabbit monoclonal-horseradish peroxidase conjugate, 1:10,000; Cell Signaling), as a housekeeping control, were visualized by enhanced chemiluminescence (Pierce).

Immunostaining

Mouse pancreas were harvested, embedded in paraffin and sectioned to 5 μm thickness. Immunostaining was performed as previously described (Lee et al., 2013). Primary antibodies used were Glut2 (rabbit polyclonal, 1:200; Millipore), Insulin (guinea pig ploYclonal, 1:500; Abcam), Glucagon (rabbit polyclonal, 1:200; Dako), Tead1 (rabbit polyclonal, 1:200; Abcam), Taz (rabbit polyclonal, 1:200; Santa Curz), Yap (rabbit poly clonal 1:200; Cell signaling), p16 (mouse monoclonal, 1:1,000; Abcam), Pdx1 (guinea pig polyclonal, 1:500; Abcam), Mafa (rabbit polyclonal, 1:100; Bethyl lab), Ucn3 (rabbit polyclonal, 1:2,000; MGI), Ki67 (rabbit poly clonalm 1:50; Abcam), Brdu (rat polyclonal, 1:1,000; Abcam), Caspase-3 (rabbit polyclonal, 1:200; Cell signaling), and Cleaved Caspase-3 (rabbit monoclonal, 1:200; Cell signaling). To measure total pancreatic islet area, pancreas paraffin blocks from mice were cut 5 μm thickness sections spaced 100 μm apart between each slide. 5 pancreatic sections per mouse were stained and measured islets area using ImageJ software. For assessing number of Brdu positive and Ki67 expression cells in β-cell, 3,000 to 5,000 cells were counted from mouse pancreatic sections in 3 to 4 mice from each group.

Cell culture and human islet

832/13 (Ins-1, courtesy of Dr. Christopher Newgard) rat β-cell line was cultured as described previously (Lee et al., 2013). Ins-2 murine β-cell line (Courtesy of Akio Koizumi) (Kobayashi et al., 2013) was cultured in high glucose DMEM (4.5 g/L) containing 15% fetal bovine serum, 150 µM beta-2-mercaptoethanol, and Penicillin (100 units/ml)/Streptomycin (100 µg/ml). EndoC-β H2 (purchased from EndoCells and courtesy of Drs. Ravassard and Scharfmann) (Scharfmann et al., 2014) were cultured according to the manufacturer's instruction. EndoC-βH2 cells were cultured in DMEM (1 g/L Glucose) containing 2% BSA fraction V, 50 µoM 2-mercaptoethanol, 10 mM nicotinamide, 5.5 µg/ml transferrin, 6.7 ng/ml sodium selenite, Penicillin (100 units/ml)/Streptomycin (100 µg/ml). Tissue culture flasks and dishes were coated with DMEM (4.5 g/L Glucose) containing fibronectin (2 µg/ml), 1 % ECM, and Penicillin (100 units/ml)/Streptomycin (100 µg/ml). Human islets were obtained from human pancreas, obtained from heart-beating non-diabetic male (BMI 31.5) donor, deceased by brain death after head trauma, with informed consent for transplant or research use from relatives of the donor. All studies involving the use of human islets were approved by the Institutional Review Board of the Houston Methodist Research Institute. Received human islets were collected by centrifugation, selected by hand picking and cultured in CMRL1066 (Mediatech, Corning) supplemented with 10% human serum (Sigma), 1% P/S and 2 mM L-glutamine. Intact islets were used for Verteporfin (VP) treatment experiments. For adenovirus (AdV) infection, human islets were disassociated with 0.05% Trypsin as previously described[33] and subjected to infection at 20 MOI, the islets re-aggregated spontaneously and formed pseudoislets in 2 days. Protein samples were collected post 72hrs of AdV infection. All cell lines were initially authenticated by the donating investigator or vendor and were tested to be mycoplasma free.

In vitro experiments

Lentiviral shRNA against Tead1 and scrambled shRNA controls were obtained from Thermo Scientific and used to generate stable cell lines with mouse insulinoma (832/13 and Ins-2) cells. The following shRNAs were used: shRNA-Tead1#1: 5'-CTCGCCAATGTGTGAATATA-3', shRNA-Tead1#3: 5'-GACTGAACCTGGTTAATTTA-3'. The knockdown efficiency was confirmed by RT-qPCR and western blotting.

For cell cycle analysis, insulinoma (Ins-2) cells (scrambled control and Tead1 knockdown#1 and #3) were grown to 70% confluence. They were synchronized by culturing in serum free media overnight. They were then collected at 0 hrs and 8 hrs after serum addition, stained with propidium iodide for DNA content, and data was acquired on an LSR II (BD) and analyzed with FlowJo (Tree Star, Inc.) software.

Native *Pdx1* and *Cdkn2a* promoter containing Tead1 binding element were amplified by PCR, cloned to pGL3 basic, mouse insulinoma cell line Ins-2 was used for the promoter activity assay.

Chromatin immunoprecipitation (ChIP) was performed using standard protocols in Ins-2 cells with Tead1 and Myc antibody or control IgG for pulldown. Immunoprecipitated DNA was then used as the template for qPCR with primers flanking conserved Tead1 binding element on promoters of target genes.

Microarrays

Biotin-labeled aRNA was generated from total RNA from isolated islets from 3 mice of each genotype at 12 weeks of age and was tested for integrity on the Agilent Bioanalyzer and quantified using the NanoDrop® ND-1000 spectrophotometer. 12 µg of labeled aRNA samples were fragmented and re-checked for concentration and size. Hybridization cocktails containing Affymetrix spike-in controls, 12 µg of each fragmented labeled aRNA and the Arcturus Turbo Blocking Reagent heated to 99 °C for 5 minutes, then incubated at 45 °C for 5 minutes. The hybridization cocktails were loaded onto Affymetrix GeneChip Mouse 430 2.0 arrays. The arrays were hybridized for 16 hours at 45 °C with rotation at 60 rpm in the Affymetrix GeneChip® Hybridization Oven 640.The arrays were washed and stained with a streptavidin, R-phycoerythrin conjugate stain using the Affymetrix GeneChip® Fluidics Station 450. Signal amplification was done using biotinylated antistreptavidin.The stained arrays were scanned on the Affymetrix GeneChip® Scanner 3000. The images were analyzed and quality control metrics recorded using Affymetrix Command Console software version 3.0.0. The data was normalized by quantiles and then significant genes analyzed using multiple sample corrected t-testing and 1.5 fold change and by gene ontology terms and MSigDb searches.

Rna-Seq:

Total RNA samples with RNA integrity number (RIN) ≥8 were used for transcriptome sequencing. Total RNA (10ng) from a pooled sample from 1 year old mice (n=3) each for two independent samples for each group was used for amplified double-stranded cDNA that was sheared to 200-300bp and ligated to Illumina paired-end adaptors using the Illumina TruSeq

DNA library preparation kit according to the manufacturer's instructions (Illumina, San Diego, CA). PCR amplification was performed to obtain the final cDNA library using Illumina kits. Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) analysis was used to verify fragment size after amplification, library size and concentration before clustering. A total of 10pM of the library was then used for paired-end sequencing on the HiSeq 2500 at the Sequencing core at Brigham Young University. All further analysis were performed using the CLC genomics workbench ver 12. Raw RNA sequencing reads were mapped to the mouse reference genome build 10 (UCSCmm10/GRCm38). Mapped reads were counted using the feature counts and differential expression between the samples analyzed using multiple hypothesis testing. Pathway analysis: Gene Set Enrichment (GSEA, version 2.2.3, http://software.broadinstitute.org/gsea/) was performed on normalized count per million (CPM) or on ranked gene lists. Ranked lists were created based on the expression levels of DEGs in Rip-Tead1 KO vs. Flox controls ($q<0.05$). Significance was assessed by analyzing signal-to-noise ratio and gene permutations based on 1,000 permutations. Molecular signature database (MSigDB) 3.0 curated gene sets for hallmark and canonical pathways were used for the analysis. Significant gene sets with enrichment score and a q value cutoff of 0.05 are presented.

Statistical methods

All statistical testing was performed either by two-tailed Student's t-test, assuming unequal variance for two groups or ANOVA for multiple samples with $p \leq 0.05$ considered significant between the groups.

Primer List:

	5'	3'
Csnk1e qPCR	GTGGGGTGGACAATGACTTC	GGGTGACCACAGGTGACTCT
Fat4 qPCR	TGGGACGTACAAACTGACCA	GACTCGGTTTGGCTGAACAT
Frmd6 qPCR	TTGTTGCCTCACAGAGGTTG	CTGGGAAGGTCATGGAAGAA
Mer qPCR	ACACAGCGAGAGCTCAGACA	ACAAGCCAGCCCTCTACTGA
Mst1qPCR	GCAGAAGGGAGCAAACTGTC	AAGCTTCTGAATGGGAGCAA
Mst2 qPCR	AGGCCCTATGTCCAACAGTG	ATGGCATCCAGGATAGGTTG
Mob1 qPCR	AGCCAGGGCTACACAGAGAA	GTCCCAACTGCACAGGTCTC
Lats1 qPCR	AAAGACGTTCTGCTCCGAAA	TTCAGGAAAGATGCCCATTC
Lats2 qPCR	GTGTCCACAAGATGGGCTTT	AACATCGTCCCAGAGGTCAC
Sav1 qPCR	GGCTCTGAGACACAGCACAG	GGAAGGAATGACAGCCTGTTT
Yap qPCR	AAAGGATGGCGTCTTCTGTG	ACCTTGTGGGGGAGGTTAAG
Taz qPCR	ATGTTGACCTCGGGACTTTG	CCACACGGACAAGGAGTGTG
Tead1 qPCR	ACAAGGACCAGGAAGCAGGTGT	TGTGCTGCAGGGCCTTGTCC
Tead2 qPCR	TAGATCCCCAACACGAGGAC	AGCAGGAAGGTTACAAGGCA
Tead3 qPCR	CTGGGCTGAAGCCTAGACAG	TGGGGGCACAGTACTTCATT
Tead4 qPCR	GCATCTGTGAGGATGCAGAA	CTGTGTGAGGCACTGTCCAT
Ccna2 qPCR	GGAGTCGCTCGGAGTCCATGC	TCTTGACAGTTGGCAGCACCAATGT
Ccnb1 qPCR	TTAGGCTAACGGAAGTTGTC	ATGGAACAAAGGACTAGCTG
Ccnc qPCR	GTTTGCAGAACTTTCTGTGG	GCTAGAGTTCTGACTTCCAT
Ccnd1 qPCR	GCGTACCCTGACACCAATCT	CTCCTCTTCGCACTTCTGCT
Ccnd2 qPCR	CAAACACAGACAGTGGACTA	TCCGAAGTTGGGGTATAGAT
Ccne1 qPCR	TCCTGACACCCCCACCCAGC	CAGACCCCTGCTGCTCCGGA
Cdk4 qPCR	GTGAGGTGGCCTTGTTAAG	ATCCTTAATGGTCTCAACCG
Cdk6 qPCR	GACTTGACCACTTACTTGGA	AAACTATAGATGCGGGCAAG
Cdkn1a qPCR	AATTGGAGTCAGGCGCAGAT	AAAGTTCCACCGTTCTCGGG
Cdkn2a^{p16} qPCR	CGAACTCGAGGAGAGCCATC	TACGTGAACGTTGCCCATCA
Cdkn2a^{p19} qPCR	TCACTGTGAGGATTCAGCGCGC	CTGCTACGTGAACGTTGCCCA
Cdkn2b qPCR	TCCAAAACTTGAACCCTACC	GATCCAAGAATTTCCCTTGC
Ezh2 qPCR	AGCGTGTAAAATCAGAGTACA	GTGACTGAACACTCCCTAGT
Ins1 qPCR	AGCGTGGCTTCTTCTACACA	AACGCCAAGGTCTGAAGGT
Ins2 qPCR	AAGCAGCACCTTTGTGGTTC	TCTACAATGCCACGCTTCTG
Gcg qPCR	TGAATTTGAGAGGCATGCTG	TGGTGCTCATCTCGTCAGAG
PP qPCR	TACTGCTGCCTCTCCCTGTT	GTTCTCCTCCTCGGCTCTCT
Sst qPCR	CCCAGACTCCGTCAGTTTCT	CATTGCTGGGTTCGAGTTG
Ghrelin qPCR	AGCTAAACTGCAGCCACGAG	GGCGCCTCTTTGACCTCT
Pdx1 qPCR	GAGGACCCGTACTGCCTACA	GGGTCCCGCTACTACGTTTC
Mafa qPCR	ATCATCACTCTGCCCACCAT	ATGACCTCCTCCTTGCGGAA
Mafb qPCR	TTCGACCTTCTCAAGTTCGACG	TCGAGATGGGTCTTCGGTTCA
Ngn3 qPCR	AAGAGCGAGTTGGCACTCAG	TCTGAGTCAGTGCCCAGATG
Neurod1 qPCR	CTCTGGAGCCCTTCTTTGAA	AAGATTGATCCGTGGCTTTG
Glut2 qPCR	ATTCGCCTGGATGAGTTACG	CGCAATGTACTGGAAGCAGA
Gck qPCR	AGGAGGCCAGTGTAAAGATGT	CTCCCAGGTCTAAGGAGAGAAA
Sur1 qPCR	CTTCATCATGGACGAAGCAA	GAAGCTTCTCCGGTTTGTCA
Kir6.2 qPCR	GGCCGCTATTCTGTGGACTA	GGACAAGGAATCTGGAGAGATG
Nkx2.2 qPCR	CAGCGACAACCCCTACACTC	GCTTTGGAGAAGAGCACTCG
Nkx6.1 qPCR	GCAGGACCAAGTGGAGAAAG	GTCAGAGTTCGGGTCCAGAG
Top1 qPCR	GGTCCAAGAAAAACAAAAACCA	GGAATGGACTCTGCACACAC
Ppia qPCR	CTGTTTGCAGACAAAGTTCCA	AGGATGAAGTTCTCATCCTCA
β-Actin qPCR	ACCCAGGCATTGCTGACAGGA	GGAGGGGCCGGACTCATCGT
Cdkn2a Chip	AGAGCACATAGCCAAAAAGTCA	TCCTAGGGCTGCCATTTGAG
Pdx1 Chip	CCCTGTTAGCCCCATGTTGT	CCAGGCAGGACCATTAACGA
Mafa Chip	TTGGACAAGGCCAGTTGCTT	GCATGAGGAAACCCCTACAA
Nkx6.1 Chip	GTTCTAGGAGCGACGCCTT	CCTCCCTGCCTCTCTTTAGC
Ucn3 Chip	AGCCCAGGCTACATACTGAG	GGGACATGTAATCGCAAGCC
P1	ATCCGGGGGTACCGCGTCGAG	
P2		AGAAGCCACAGTGCCCTGGAAGTGT
P3	GCAGTGCTCTCAGGAGTGCTGAGTGCGAC	
P4		CACTGCAGCGCGCATAGCCTATGCTCCTTC

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Lee, J., Moulik, M., Fang, Z., Saha, P., Zou, F., Xu, Y., Nelson, D.L., Ma, K., Moore, D.D., and Yechoor, V.K. (2013). Bmal1 and beta-cell clock are required for adaptation to circadian disruption, and their loss of function leads to oxidative stress-induced beta-cell failure in mice. Mol. Cell Biol *33*, 2327-2338.

Scharfmann, R., Pechberty, S., Hazhouz, Y., von, B.M., Bricout-Neveu, E., Grenier-Godard, M., Guez, F., Rachdi, L., Lohmann, M., Czernichow, P., et al. (2014). Development of a conditionally immortalized human pancreatic beta cell line. J. Clin. Invest *124*, 2087-2098.

Figure 1

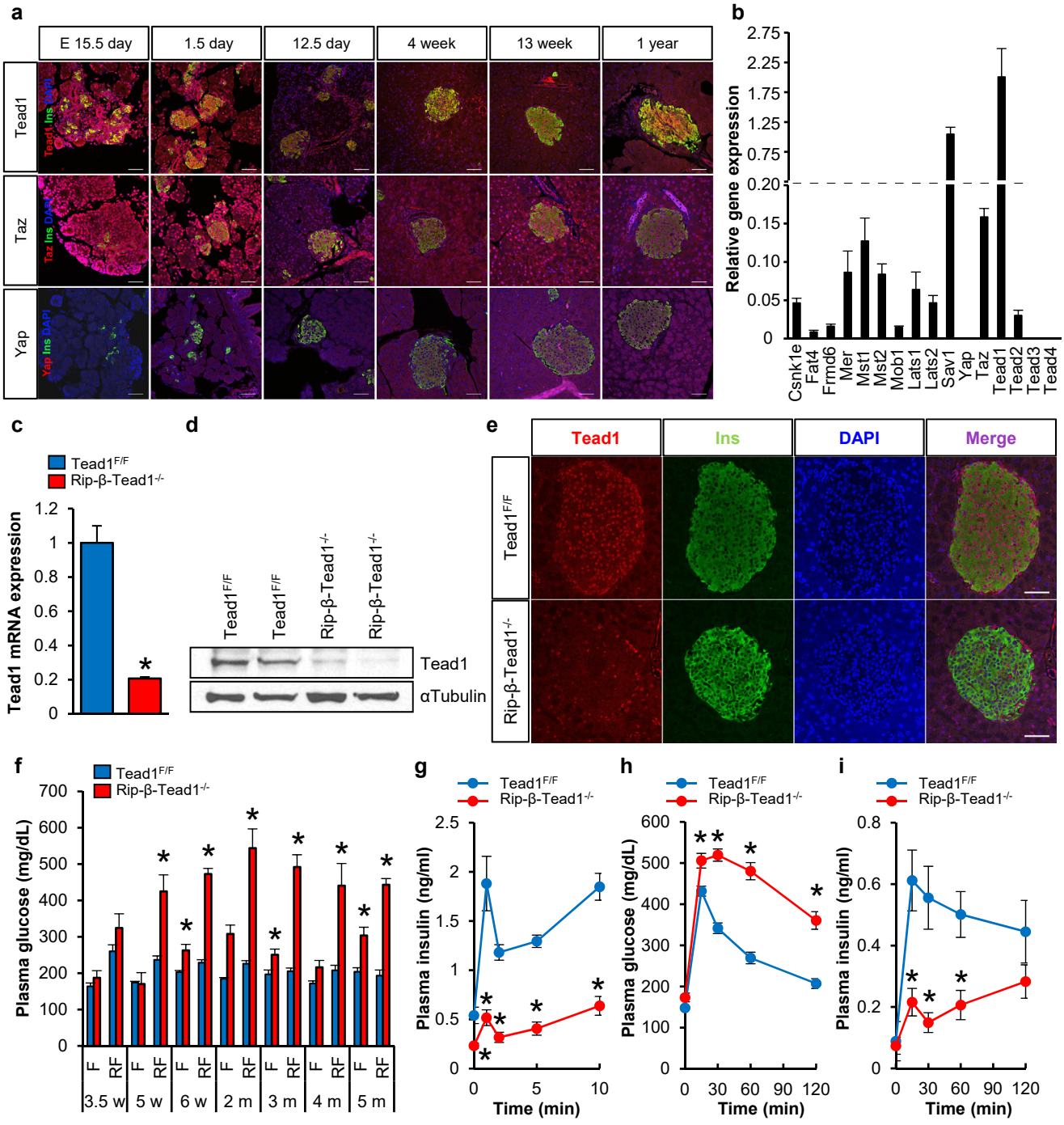

Figure 1: β-cell Tead1 deletion leads to diabetes. (a) Representative immunofluorescence staining of Tead1, Taz and Yap in mouse islets at different ages. Scale bar - 50μm. (b) Relative gene expression assessed by RT-qPCR of total RNA from isolated islets of 4 month old wild type mice. n=4 (c) RT-qPCR and (d) western blotting for Tead1 in isolated islets from Tead1$^{F/F}$ and Rip-β-Tead1$^{-/-}$mice. n=4-6. (e) Representative images of immunofluorescence staining of Tead1$^{F/F}$ and Rip-β-Tead1$^{-/-}$ pancreas. Scale bar - 50μm. (f) Plasma glucose level with overnight fasting and after 1hour refeeding in Tead1$^{F/F}$ and Rip-β-Tead1$^{-/-}$mice. n=4-6. W-weeks; M-months; F-fasting; RF-refeeding. (g) Plasma glucose and (h) insulin during glucose tolerance testing (GTT) in overnight fasted 8 weeks old male mice. n=5-6. (i) Acute insulin secretion after glucose stimulation in 10-week-old mice. n=5. All values are mean ± SEM, * - p≤ 0.05.

Figure 2

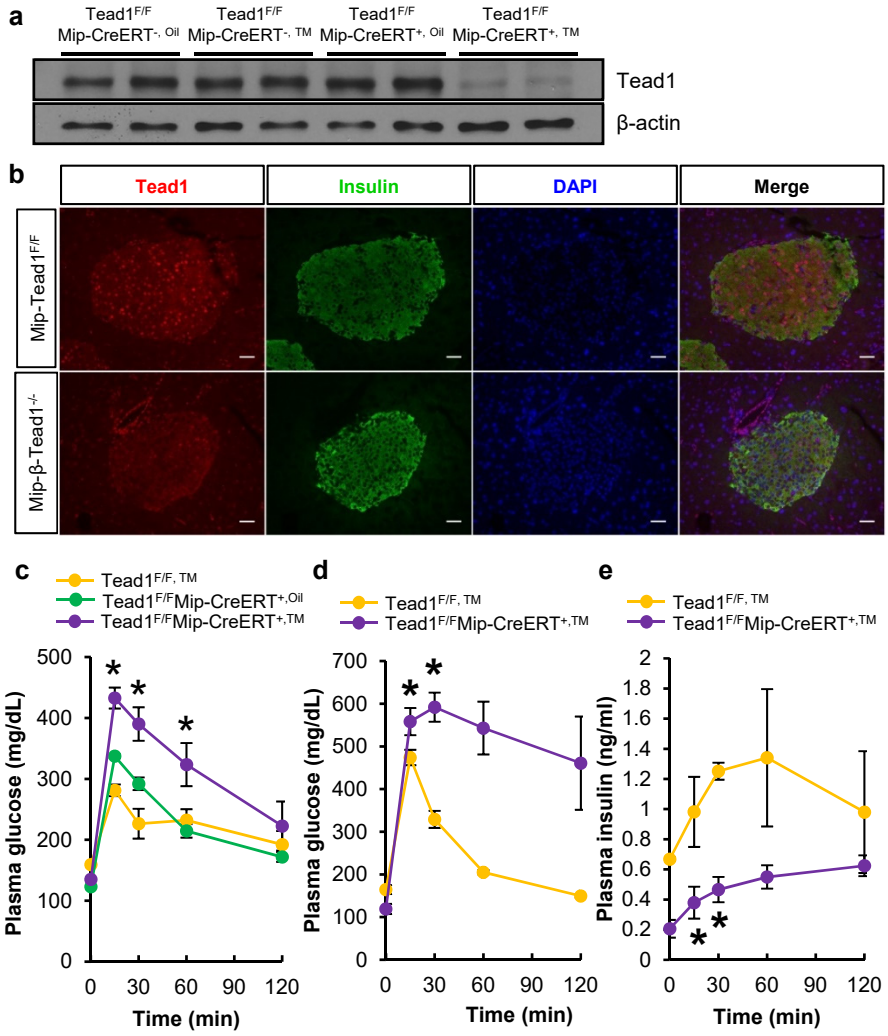

Figure 2: Induced deletion of Tead1 in adult β-cells in Mip-β-Tead1$^{-/-}$ leads to glucose intolerance. (a) Western blotting of isolated islets from Tead1$^{F/F}$ and Tead1$^{F/F}$Mip-CreERT^{+} mice treated with oil or TM. (b) Nuclear Tead1 staining is absent from most insulin expressing β-cell in TM-treated Mip-β-Tead1$^{-/-}$ islets. Scale bar – 50μm. (c) Plasma glucose level during the GTT in Tead1$^{F/F}$ and Tead1$^{F/F}$Mip-CreERT^{+} mice treated with oil or TM, 5 month old male mice (d) plasma glucose and (e) insulin levels during the GTT in Tead1$^{F/F}$ and TM-treated Tead1$^{F/F}$Mip-CreERT^{+} (Mip-β-Tead1$^{-/-}$), 11 month old female mice. GTT was performed 2 months after the last dose TM treatment. n=4. TM, Tamoxifen. All values are mean ± SEM, * - p≤ 0.05.

Figure 3

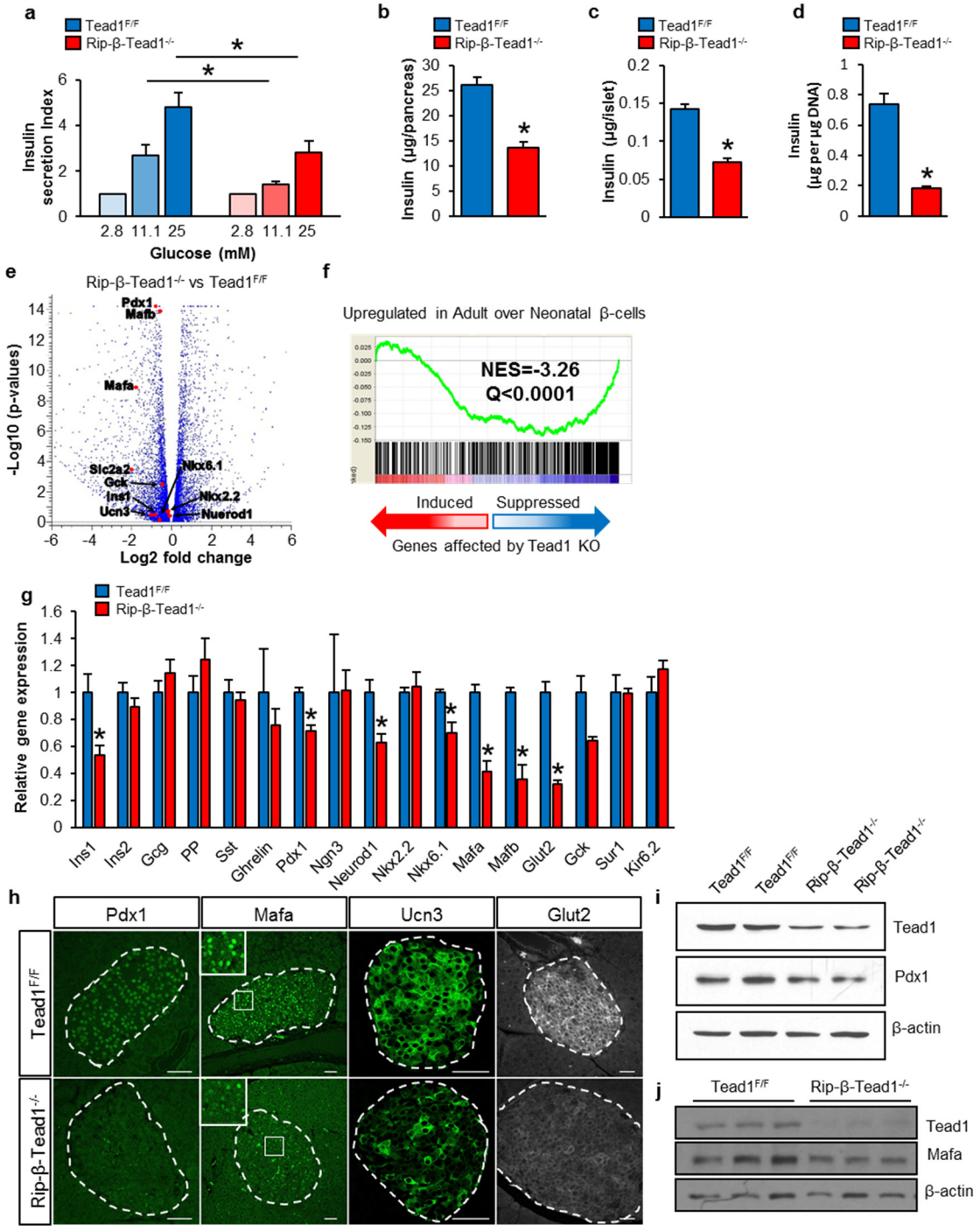

Figure 3: Tead1 is required for mature β-cell function. (a) GSIS from isolated islets (ten islets each from 5-6 individual mice of each genotype) on exposure to increasing glucose concentrations. Insulin secretion index is expressed as a fold change over basal secretion in 2.8mM glucose. Insulin content in (b) total pancreas, n=4; in isolated islets expressed as insulin content (c) per equally sized islet or (d) per µg DNA, n=5. (e) Volcano plot from RNA-seq data from differentially expressed genes from isolated islets of 1 year old Rip-β-Tead1$^{-/-}$ compared to Tead1$^{F/F}$. Some of the genes critical in mature β-cell function are highlighted. (f) GSEA analysis of the differentially expressed genes in isolated islets of Rip-β-Tead1$^{-/-}$ compared to Tead1$^{F/F}$ as compared to the published gene set (GSE47174) that was upregulated in adult β-cells to neonatal β-cells. Details in the accompanying text. NES – normalized enrichment score. FDR Q value is also shown. (g) Expression, by RT-qPCR, of genes critical to mature β-cell function in Tead1$^{F/F}$ and Rip-β-Tead1$^{-/-}$ islets. n=4. (h) Representative immunofluorescent images of Pdx1, MafA, Ucn3 and Glut2 staining in Tead1$^{F/F}$ and Rip-β-Tead1$^{-/-}$ islets. Scale bar - 50 µm. (i) Western blot of Pdx1 protein and MafA from isolated islets from Rip-β-Tead1$^{-/-}$ and Tead1$^{F/F}$ mice. All values are mean ± SEM, * - p≤ 0.05.

Figure 4

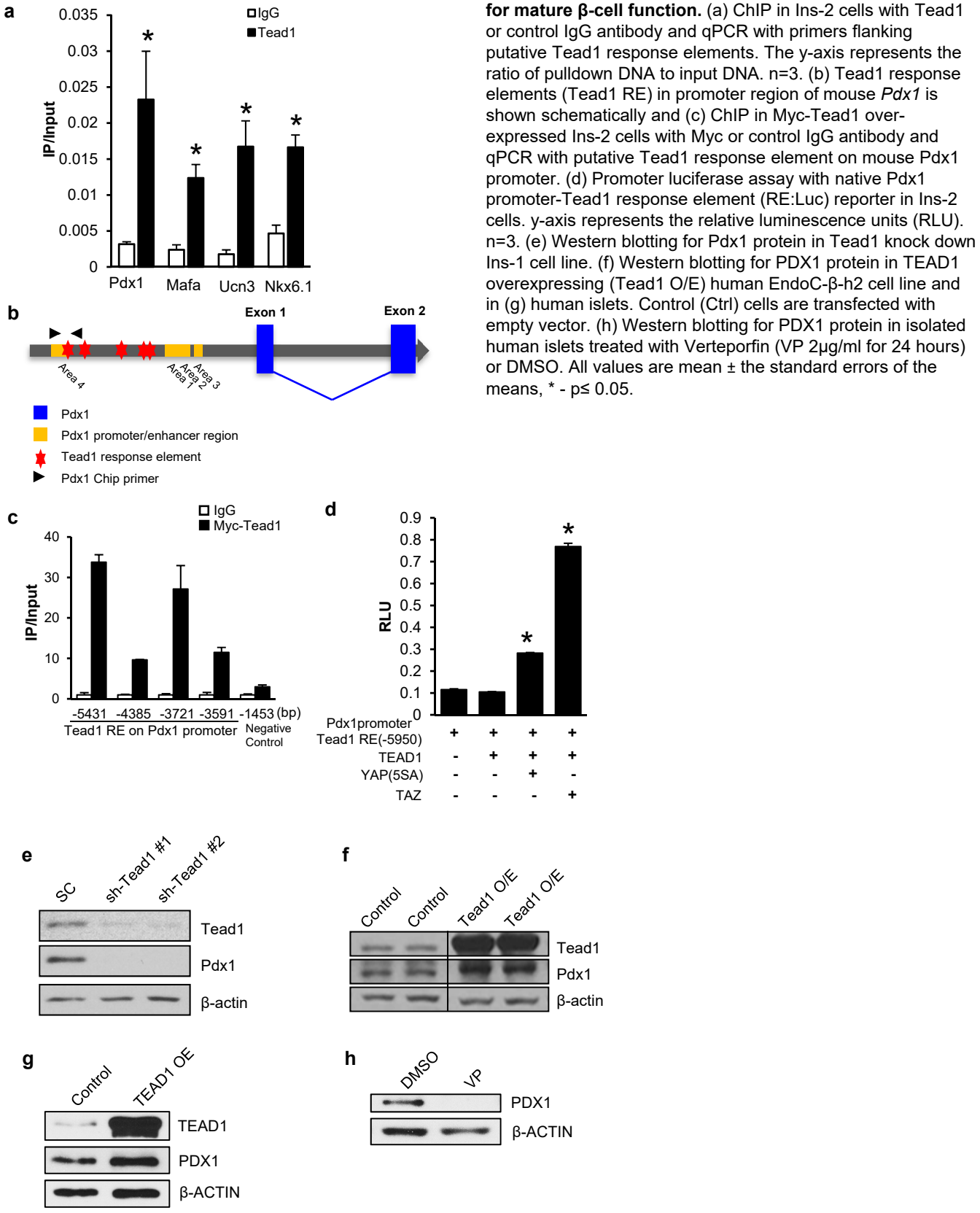

Figure 4: Tead1 is direct transcriptional regulator of genes for mature β-cell function. (a) ChIP in Ins-2 cells with Tead1 or control IgG antibody and qPCR with primers flanking putative Tead1 response elements. The y-axis represents the ratio of pulldown DNA to input DNA. n=3. (b) Tead1 response elements (Tead1 RE) in promoter region of mouse *Pdx1* is shown schematically and (c) ChIP in Myc-Tead1 over-expressed Ins-2 cells with Myc or control IgG antibody and qPCR with putative Tead1 response element on mouse Pdx1 promoter. (d) Promoter luciferase assay with native Pdx1 promoter-Tead1 response element (RE:Luc) reporter in Ins-2 cells. y-axis represents the relative luminescence units (RLU). n=3. (e) Western blotting for Pdx1 protein in Tead1 knock down Ins-1 cell line. (f) Western blotting for PDX1 protein in TEAD1 overexpressing (Tead1 O/E) human EndoC-β-h2 cell line and in (g) human islets. Control (Ctrl) cells are transfected with empty vector. (h) Western blotting for PDX1 protein in isolated human islets treated with Verteporfin (VP 2μg/ml for 24 hours) or DMSO. All values are mean ± the standard errors of the means, * - p≤ 0.05.

Figure 5

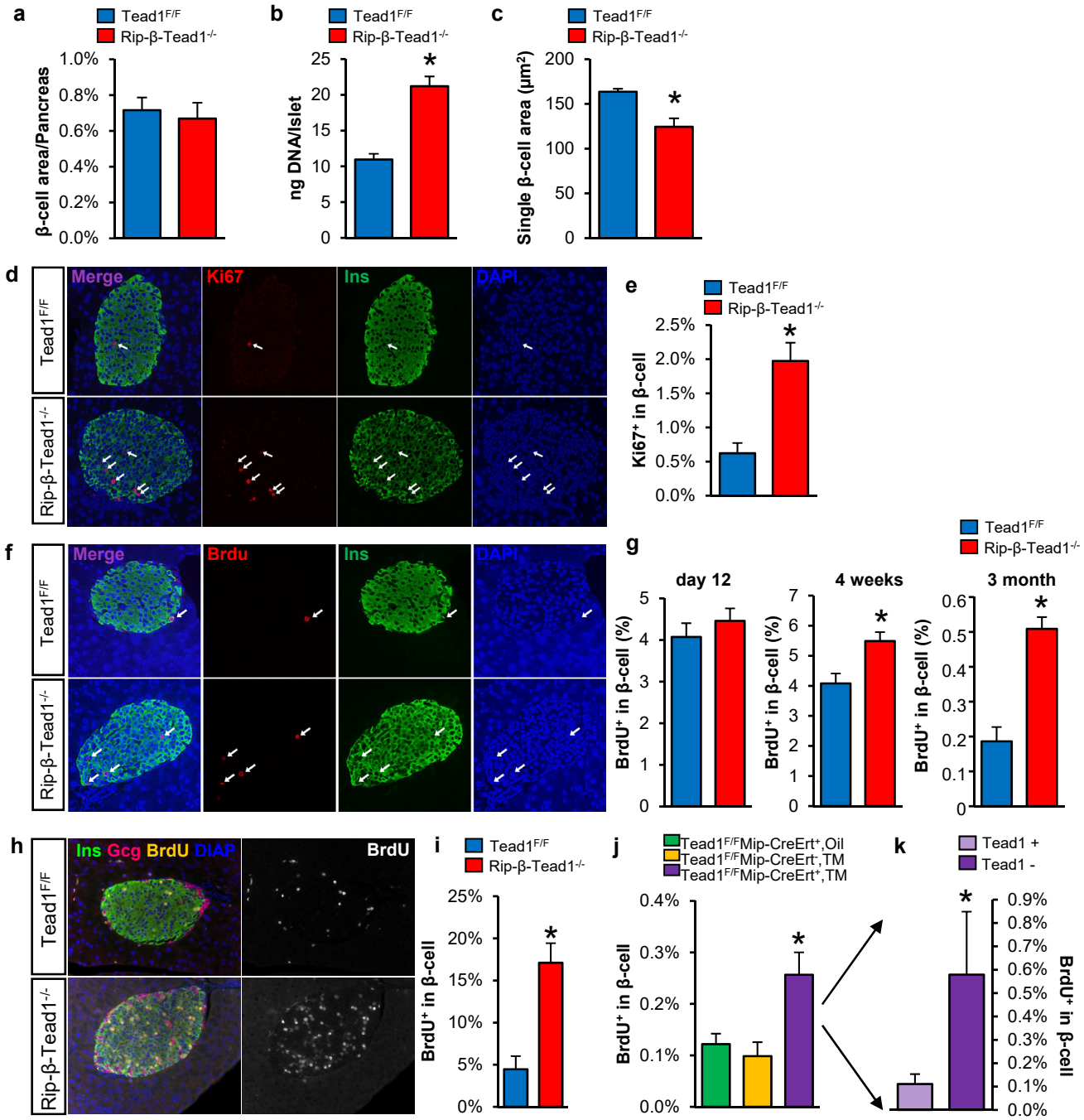

Figure 5: Tead1 deletion enhances β-cell proliferation. (a) Total pancreatic β-cell area from Rip-β-Tead1$^{-/-}$ mice and Tead1$^{F/F}$ mice at 3 months age. n=3. (b) Genomic DNA content in similar sized islets. n=5. (c) Single β-cell area in Rip-β-Tead1$^{-/-}$ mice and Tead1$^{F/F}$ mouse pancreas at 3 months age. n=3. (d-g) Quantification of β-cell proliferation in Tead1$^{F/F}$ and Rip-β-Tead1$^{-/-}$ mice at 3 months. n=3. BrdU was injected (f, g) 2 hrs prior to sacrifice, or (h, i) administered continuously by water for 6 days prior to sacrifice. Representative confocal microscopy images (d, f, h) of Rip-β-Tead1$^{-/-}$ mice and Tead1$^{F/F}$ mouse pancreas at 3 months age and quantification of (e) Ki67 expressing and (g and i) BrdU positive β-cells. n=4. (j) Quantification of Brdu-positive β-cells in in 11 month old TM-treated Mip-β-Tead1$^{-/-}$ mice and controls. n=4. (k) Brdu-positive cells in Tead-negative and Tead1-positive β-cell subpopulations in 11 month old TM-treated Mip-β-Tead1$^{-/-}$ mice. n=4. All values are mean ±the standard errors of the means, * - $p \leq 0.05$. N.S.-not significant.

Figure 6

a

GO analysis – genes up in Rip-β-Tead1$^{-/-}$

Category	Term	Count in selected Genes	Count in Total Population	P-value
GO	regulation of DNA replication initiation	3	4	9.85E-06
GO	DNA replication checkpoint	2	2	0.000184
GO	negative regulation of DNA replication initiation	2	2	0.000184
GO	proteinaceous extracellular matrix	11	217	0.000199
GO	extracellular matrix	11	224	0.000262
GO	regulation of DNA replication	3	11	0.000379
GO	G1/S transition of mitotic cell cycle	4	27	0.000459

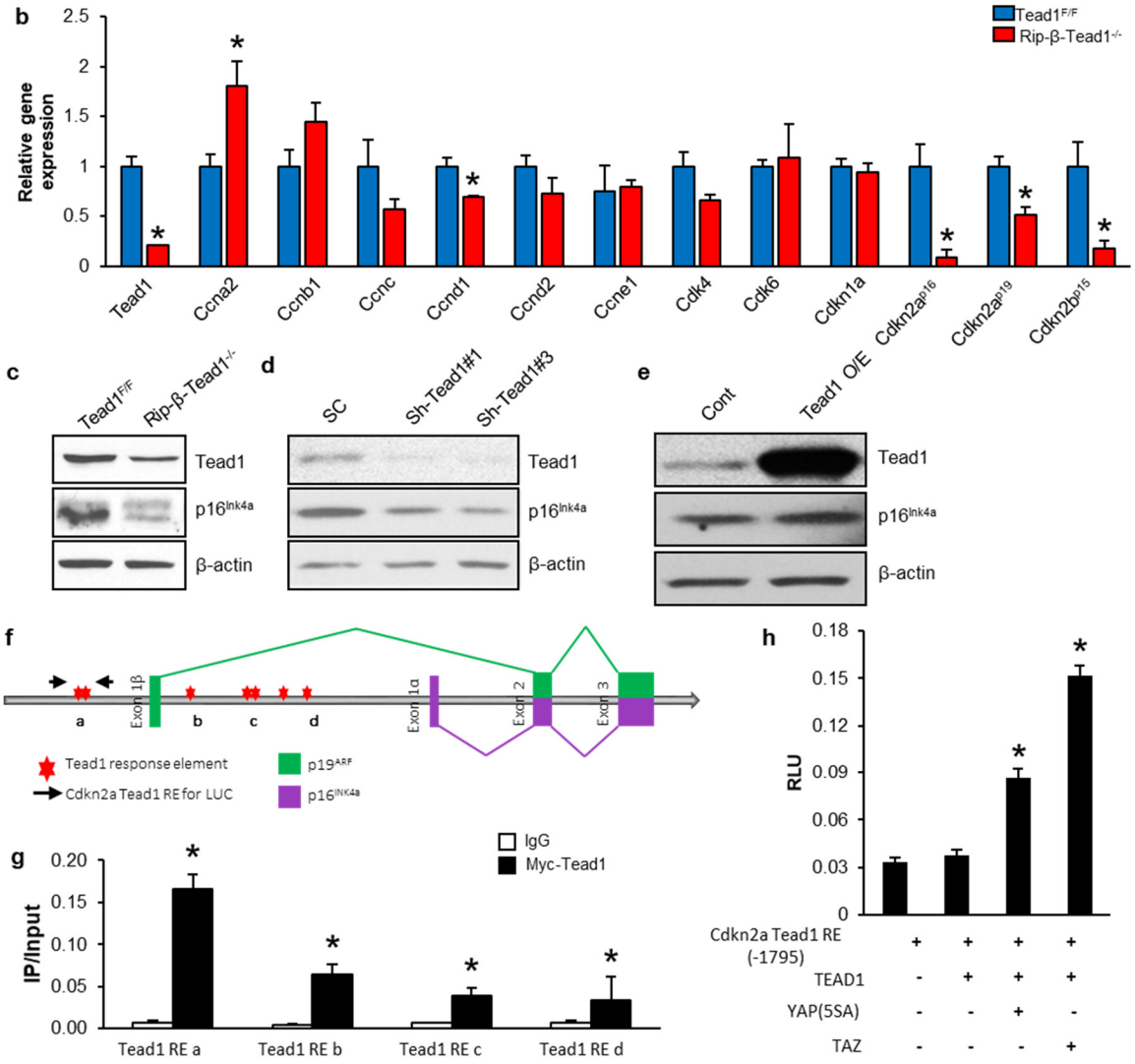

Figure 6: Tead1 regulates p16^{INK4a}. (a) Microarray analysis of isolated Rip-β-Tead1$^{-/-}$ and control Tead1$^{F/F}$ islets from 12 weeks old male mice with significantly enriched pathways by Gene Ontology pathway analysis. All proliferation related pathways are highlighted in red. (b) Relative expression of cell cycle related genes in Rip-β-Tead1$^{-/-}$ and Tead1$^{F/F}$ islets. n=4. Western blot of p16^{INK4a} protein in (c) isolated islets from Rip-β-Tead1$^{-/-}$ and Tead1$^{F/F}$ mice, (d) in Tead1 knockdown Ins-2 cells and (e) Tead1 overexpressing Ins-2 cells. (f) Tead1 response elements (Tead1 RE) in promoter region of mouse *Cdkn2a*. The *Cdkn2a* locus encoding p16^{INK4a} and p19^{ARF} is shown schematically. (g) ChIP of Myc-Tead1 over-expressing Ins-2 cells with Myc or control IgG antibody and qPCR with primers flanking putative Tead1 response elements. The y-axis represents the ratio of pulldown DNA to input DNA. n=3. (h) Promoter luciferase assay with native Cdkn2a promoter-Tead1 response element (RE:Luc) reporter in Ins-2 cells. y-axis represents the relative luminescence units (RLU). n=3. All values are mean ±the standard errors of the means, * - p≤ 0.05.