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# Dachshund homologues play a conserved role in islet cell development

**Anna Kalousova**[1,*], **Anastasia Mavropoulos**[1,*], **Bruce A. Adams**[1], **Nada Nekrep**[1], **Zhongmei Li**[1], **Stephan Krauss**[2], **Didier Y. Stainier**[1,3], and **Michael S. German**[1,4,5]

[1]Diabetes Center, University of California at San Francisco, San Francisco, CA 94143, U.S.A

[2]Institute of Medical Microbiology and Centre for Molecular Biology and Neuroscience, University of Oslo, The National Hospital, 0027 Oslo, Norway

[3]Department of Biochemistry and Biophysics, [3]Cardiovascular Research Institute, University of California at San Francisco, San Francisco, CA 94143, U.S.A

[4]Department of Medicine, University of California at San Francisco, San Francisco, CA 94143, U.S.A

## Abstract

All metazoans use insulin to control energy metabolism, but they secrete it from different cells: neurons in the central nervous system in invertebrates and endocrine cells in the gut or pancreas in vertebrates. Despite their origins in different germ layers, all of these insulin-producing cells share common functional features and gene expression patterns. In this study, we tested the role in insulin-producing cells of the vertebrate homologues of Dachshund, a transcriptional regulator that marks the earliest committed progenitors of the neural insulin-producing cells in *Drosophila*. Both zebrafish and mice expressed a single dominant Dachshund homologue in the pancreatic endocrine lineage, and in both species loss of this homologue reduced the numbers of all islet cell types including the insulin-producing β-cells. In mice, *Dach1* gene deletion left pancreatic progenitor cells unaltered, but blocked the perinatal burst of proliferation of differentiated β-cells that normally generates most of the β-cell mass. In β-cells, Dach1 bound to the promoter of the cell cycle inhibitor *p27Kip1*, which constrains β-cell proliferation. Taken together, these data demonstrate a conserved role for Dachshund homologues in the production of insulin-producing cells.

## INTRODUCTION

The peptide hormone insulin regulates energy metabolism and growth in all metazoans (Leevers, 2001; Skorokhod et al., 1999). In mammals, the insulin-producing cells, the β-cells, reside in the pancreas, organized into the islets of Langerhans together with α-cells, δ-



[5]Corresponding author: UCSF Diabetes Center, University of California San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143-0534, Tel: (415) 476-9262, Fax: (415) 731-3612, mgerman@diabetes.ucsf.edu.
[*]contributed equally to this work



**CONTRIBUTIONS**

A.K. and M.S.G. initiated the project; A.K., A.M., B.A.A. and M.S.G. designed the experiments; A.K., A.M., B.A.A. and N.N. performed the research; A.K., A.M., B.A.A., N.N. and M.S.G. analyzed the data; and A.K., A.M., B.A.A. and M.S.G. wrote the paper.

cells, ε-cells and PP cells that produce the peptide hormones glucagon, somatostatin, ghrelin and pancreatic polypeptide, respectively. The β-cells act as part of an integrated information network regulating energy metabolism that includes the other islet cells, the endocrine cells of the gut, and neurons, especially specific neurons in the hypothalamus.

Our understanding of how the β-cells are generated during development, and the genes involved in that process, derives largely from studies in rodents (Murtaugh, 2007; Wilson et al., 2003), and more recently in zebrafish (Field et al., 2003). The generation of β-cells during mouse development can be divided into three phases. First, during the period starting with the budding of the initial pancreatic anlage from the gut endoderm at embryonic day 9.5 (E9.5) and ending with the "secondary transition" at E13, a small number of endocrine cells differentiate from the pancreatic progenitor cells. A minority of these "primary endocrine cells" express insulin, but these insulin-expressing cells contain low levels of insulin, often co-express glucagon, and lack mature β-cell markers Nkx6.1, MafA and Pdx1 (Kim and MacDonald, 2002; Wilson et al., 2002). During the second phase, starting at E13, much larger numbers of insulin-producing cells with mature β-cell characteristics differentiate via a pathway that involves the basic helix-loop-helix transcription factor Neurogenin3 acting upstream of the transcription factors NeuroD1, Nkx2.2, Nkx6.1, MafA, and Pax4, among others. This neogenesis of β-cells via the Neurogenin3$^{+}$ progenitor cells peaks around E14-15 in the mouse and has largely ceased by E18 (Jensen et al., 2000; Johansson et al., 2007; Schwitzgebel et al., 2000). The third phase, β-cell proliferation, starts shortly before the termination of β-cell neogenesis and lasts through the first few weeks of postnatal life, yielding a marked expansion of the β-cell population (Finegood et al., 1995; Sander et al., 2000).

The genes in the Neurogenin3-dependent pathway that drive β-cell neogenesis also function in neural development in the vertebrate central nervous system, especially in peptide- and monoamine-secreting neurons in the hypothalamus (Kurrasch et al., 2007) and hindbrain (Cordes, 2005). Interestingly, some cells in the vertebrate brain produce small amounts of insulin (Devaskar et al., 1994). While neuronal production of insulin is only a minor pathway in vertebrates, in invertebrates the principle insulin-producing cells (IPCs) are located in the nervous system (Rulifson et al., 2002). In *Drosophila*, the neural IPCs differentiate in a region analogous to the vertebrate hypothalamus, adjacent to the *Corpora cardiaca* cells, the fly equivalent of the vertebrate α-cells, with which they later functionally interact in regulating energy metabolism (Kim and Rulifson, 2004).

The parallels between the neural IPCs in *Drosophila* and the vertebrate β-cells suggest that the IPCs could be used to identify candidate genes involved in β-cell generation. A recent study of the development of the IPCs and *Corpora cardiaca* cells in *Drosophila* documented in detail the gene networks operating during the differentiation of each lineage (Wang et al., 2007). Whereas some of the genes identified -- such as *eyeless*, the homologue of vertebrate *Pax6* -- have been implicated previously in vertebrate islet development (Sander et al., 1997; St-Onge et al., 1997), most of the vertebrate homologues of the *Drosophila* IPC genes have not been interrogated in pancreas development.

In particular, a single gene, *dachshund*, not previously implicated in islet development, uniquely identified the earliest committed progenitor of the IPCs . The *dachshund* gene encodes a nuclear protein required for normal eye and leg development in *Drosophila* (Mardon et al., 1994). A highly interactive network of genes including *eyeless*, *eyes absent* and *sine oculis* work with *dachshund* in initiating eye formation in *Drosophila* (Gehring, 2004). The *dachshund* gene family is conserved in vertebrates; three homologues have been identified previously in zebrafish (Hammond et al., 2002) and two in mice and human (Caubit et al., 1999; Davis et al., 1999; Davis et al., 2001b; Kozmik et al., 1999). The

mammalian *Dach* genes have partially overlapping expression patterns in a variety of embryonic and adult tissues, including the eye, the hypothalamus, and the pituitary, where they also interact with mammalian members of the eyes absent (Eya) and sine oculis (Six) families, as well as other nuclear proteins (Hanson, 2001).

Dachshund family proteins lack obvious sequence similarity with other known transcription factors, but structural analysis has revealed that an N-terminal domain with sequence similarity to the ski/sno oncogenes has structural similarities to the winged-helix/forkhead DNA binding motif (Kim et al., 2002). Whether Dachshund proteins can bind DNA is unclear; but, like the Ski/Sno proteins, they can function as transcriptional repressors by linking to DNA-binding transcription factors and recruiting corepressors. For example, in regulating the proliferation of pituitary progenitor cells in mice, Dach2 binds to the Sine oculis homologue Six6, recruits the corepressors N-Cor and Sin3A/B and histone deacetylases, and suppresses the expression of the cell cycle inhibitor p27Kip1 (Li et al., 2002). In other contexts, however, such as in association with the phosphatase activity of Eya proteins (Li et al., 2003), Dachshund proteins can recruit coactivators and activate the expression of target genes. The function of Dachshund proteins, therefore, is highly dependent on their cellular and gene context.

To explore the possibility that Dachshund homologues play a role in the development of the vertebrate islet and β-cell, we determined the expression pattern and function of the genes in this family in the developing pancreas in zebrafish and mice. As in *Drosophila*, we found Dachshund family members expressed in the islet lineage and demonstrated their importance in expanding the endocrine cell population in both vertebrate species. These studies demonstrate a conserved role for Dachshund homologues in islet cell development, and validate the use of *Drosophila* to identify genes and pathways important for vertebrate islet development.

## Results

### Expression of dachb in the developing zebrafish pancreas

To determine if the vertebrate homologues of the Drosophila *dachshund* gene play a role in pancreas development, we started by examining their expression in the developing zebrafish pancreas. The zebrafish pancreas forms from two buds off the gut endoderm. The first anlage, generating only endocrine tissue, buds from the dorsal aspect of the developing gut by 24 hours post fertilization (hpf). The second anlage, located on the ventral aspect of the developing gut and anterior to the dorsal anlage, appears by 40 hpf and gives rise mainly to the pancreatic duct and exocrine cells. These two buds merge by 52 hpf to form the pancreas (Biemar et al., 2001; Field et al., 2003).

Three zebrafish *dachshund* homologues, *dacha*, *dachb* and *dachc*, have been described with distinct but overlapping expression patterns (Hammond et al., 2002); and we identified a candidate fourth homologue, *dachd*, in the zebrafish genome and demonstrated its expression by RT-PCR from RNA isolated from whole embryos at 24 and 48 hpf. We assessed the expression of all four *dachshund* homologues in the developing zebrafish pancreas by whole-mount *in situ* hybridization from mid-somitogenesis to 48 hpf.

We could detect expression of *dachb*, but not *dacha*, *dachc* or *dachd*, in the region of the developing pancreas (Fig. 1 and data not shown). *dachb* expression was first detected at the 14-somite stage in the mid-endoderm region from which the pancreas develops (Fig. 1A, E). *dachb* expression peaked at 24hpf, coincident with the formation of the dorsal pancreatic bud and persisted until 30 hpf (Fig1B–D;F–H). After 30 hpf, the level of expression dramatically decreased (data not shown).

The localization of *dachb* transcripts to the dorsal pancreatic bud was confirmed by double *in situ* hybridization with three pancreatic endocrine markers: insulin, somatostatin and glucagon (Fig1I–K). At 24 hpf, subsets of *dachb*-expressing cells co-expressed insulin or somatostatin. Similarly at 30hpf, a portion of the *dachb*-positive cells co-expressed glucagon.

## Knock-down of dachb reduces pancreatic endocrine cells

The function of Dachb in development of the zebrafish pancreas was investigated by injection of antisense morpholino oligonucleotides targeting the splicing of the *dachb* transcript into 1- to 2-cell stage embryos. We confirmed by RT-PCR that this morpholino oligonucleotide blocked efficiently the splicing of *dachb* mRNA (Supplemental Fig. 1). As negative controls, we used a morpholino oligonucleotide in which 4 nucleotides of the *dachb* targeting sequence were altered and an unrelated standard control morpholino oligonucleotide. Embryos were injected with 4 ng of morpholino oligonucleotide and allowed to develop until 30 hpf or 48 hpf. The expression of pancreatic markers was then analyzed quantitatively.

Injection of the *dachb* morpholino oligonucleotide decreased the number of hormone-expressing cells (Fig. 2C,F,I) as compared to control embryos (Fig. 2A,B,D,E,G,H). We observed a decrease of 36%, 35% and 64% in insulin-, somatostatin- and glucagon-expressing cells, respectively. In contrast, the expression of *ptf1a*, marking the exocrine tissue, was not affected (Supplemental Fig. 1C–E).

Taken together, these data demonstrate that one homologue of the Drosophila *dachshund* gene, *dachb*, is expressed in the developing pancreas and is involved in the formation of hormone-expressing islet cells in zebrafish.

## Expression of Dach1 in the mouse pancreas

The mouse genome contains 2 *dachshund* homologues, *Dach1* and *Dach2* (Davis et al., 2001b; Hammond et al., 1998). To assess the expression of the two *Dach* genes in the developing and adult mouse pancreas, we first performed RT-PCR on RNA from E12.5, E15.5, E17.5, adult total pancreases and adult islets using gene specific primers (Fig. 3A). *Dach1* mRNA was detected at all embryonic stages examined and also in RNA from adult mouse islets. *Dach2* mRNA was detected at lower levels throughout pancreas development and in mature tissue. Similar results were obtained by real-time RT-PCR (data not shown). Parallel RT-PCR analysis identified members of the *sine oculis* and *eyes absent* gene families (Supplementary Fig. 2).

The proendocrine bHLH transcription factor Neurogenin3 can induce endocrine differentiation in pancreatic duct cells *in vitro* (Gasa et al., 2004; Heremans et al., 2002). We found that adenovirus expressing Neurogenin3, but not NeuroD1, could induce *Dach1* expression in pancreatic duct cells (Fig. 3B), placing *Dach1* in a small group of genes induced by Neurogenin3, but not NeuroD1 (Gasa et al., 2008). *Dach2* expression, in contrast, was unaffected by Neurogenin3 or NeuroD1.

Next, we defined the pattern of Dach1 expression in the developing mouse pancreas by immunohistochemistry. The pancreas expressed detectable levels of Dach1 protein as early as E10.5 (data not shown); and at E12.5, Dach1 protein was detected throughout the pancreatic epithelium (Fig. 4A, C), where it co-localized with the pancreatic-duodenal homeodomain transcription factor Pdx1 in most cells (Fig. 4B). Double immunofluorescence staining also detected Dach1 in a subset of the glucagon-positive cells at E12.5 (Fig. 4D).

After the secondary transition at E13, when the undifferentiated pancreatic progenitor cells in the epithelium rapidly begin to differentiate into endocrine and exocrine cells, Dach1 expression was detected in a more restricted pattern. At E15.5, the majority of the insulin-producing and glucagon-producing endocrine cells expressed Dach1 protein (Fig. 4E, F), but Dach1 was absent from terminally differentiated exocrine cells. At this stage, the expression of Dach1 overlapped with other pancreatic endocrine transcription factors, including the homeodomain factors Pdx1 (Fig. 5A–C), which is expressed at high levels in mature β-cells, and Nkx6.1 (Fig. 5D–F), which is expressed at high levels in differentiating and mature β-cells, and to a lesser degree the bHLH factor Neurogenin3 (Fig. 5G–I), which marks islet cell progenitors (Schwitzgebel et al., 2000).

By E18.5 (data not shown) and in the adult pancreas (Fig. 4G–J), Dach1 expression was fully restricted to the islets of Langerhans, where it was detected in α-, β-, δ- and PP-cells.

## Pancreas development in mice lacking Dach1

To test the role of Dach1 in pancreatic development in the mouse, we examined mice with a targeted disruption of the *Dach1* gene. *Dach1*$^{-/-}$ mice survive to birth, but die shortly thereafter (Backman et al., 2003; Davis et al., 2001a). At E18.5, one day prior to birth, the pancreases of *Dach1*$^{-/-}$ embryos were normal in size and gross appearance (data not shown). Hematoxylin-eosin staining of the E18.5 pancreases revealed no obvious morphological discrepancy between wild-type and *Dach1*$^{-/-}$ mice (Fig. 6A, B). Immunofluorescent staining for acinar (Amylase), ductal (Mucin-1) and mesenchymal (Vimentin) markers at E18.5 also detected no abnormalities (Supplemental Fig 3, data not shown).

Immunohistochemical staining with antibodies against islet hormones insulin, glucagon, somatostatin and PP at E18.5 revealed that endocrine cells still clustered into islet structures, but the size of the aggregates was reduced in *Dach1*$^{-/-}$ embryos (Fig. 6D, F) compared to their wild-type littermates (Fig. 6C, E). Staining for Pax6, a marker for endocrine cells, confirmed the reduction in total endocrine cells in the *Dach1*$^{-/-}$ embryos (Supplemental Fig. S3). Cell counting normalized to pancreatic area at E18.5 demonstrated a 67% reduction in the number of insulin-producing β-cells in *Dach1*$^{-/-}$ pancreases relative to their wild-type littermates, and a 63% reduction in glucagon-producing α-cells (Fig. 6I). Although reduced in number, the remaining β-cells in the *Dach1*$^{-/-}$ embryos appeared to be fully differentiated, as judged by their expression of Glut2, a characteristic marker of mature β-cells (Supplemental Fig. S3). This defect in the pancreas was not apparent earlier at E15.5 (Supplemental Fig. S4).

## Role of Dach1 in islet cell proliferation

The reduction in islet cells observed in the *Dach1*$^{-/-}$ embryos could result from decreased generation of new islet cells from progenitor cells, or decreased proliferation or increased apoptosis of differentiated islet cells. Islet cells differentiate from pancreatic progenitor cells via islet progenitor cells that transiently express the bHLH transcription factor Neurogenin3. Staining for Neurogenin3 during the peak of islet cell genesis at E15.5, we did not detect a decrease in Neurogenin3-expressing islet cell progenitors in the *Dach1*$^{-/-}$ pancreases (data not shown). Similarly, the frequency of apoptotic β-cells as judged by co-staining of insulin with cleaved caspase-3 was very low at E18.5 in wild type embryos (0.38%) and was not increased in the *Dach1*$^{-/-}$ pancreases (0.2%) (data not shown).

Staining for Ki-67, a marker of actively proliferating cells, revealed approximately a three-fold decrease in the proliferation of insulin-positive cells in *Dach1*$^{-/-}$ embryos at E18.5 compared to their wild-type littermates (Fig. 6G,H, J). In contrast, the proliferation of Pdx1-

positive pancreatic progenitors at E12.5 was unaffected in the *Dach1*$^{-/-}$ embryos (Fig. 6J), a result that is consistent with the observation that the overall size of the pancreas was unchanged in the *Dach1*$^{-/-}$ embryos. Taken together, these results suggest that Dach1 plays a role specifically in the proliferation of in the terminally differentiated islet cells, but not in their differentiation or in the proliferation of the pancreatic progenitor cells.

### Dach1 binds to the p27Kip1 promoter

During the perinatal expansion of the islet cell population, the cyclin-dependent kinase inhibitor p27Kip1 plays a crucial role in limiting the proliferation of terminally differentiated β-cells (Georgia and Bhushan, 2006). We could readily detect a two-fold increase in p27Kip1 in the endocrine pancreas at E18.5 (Fig.7A, B and Supplemental Fig. 5). Dach2, in association with the homeodomain factor Six6, can regulate the proliferation of retinal and pituitary progenitors by directly repressing the promoter for the gene encoding p27Kip1 (Li et al., 2002). In the β-cell line βTC3, which expresses Dach1 (data not shown), chromatin immunoprecipitation demonstrated that Dach1 binds to the promoter of the *Cdkn1b* gene that encodes p27Kip1, but not to a site 2 KB downstream in the coding region (Fig.7C). Similarly, but to a lesser degree, the Dach1 antiserum also pulled down the promoter of the *Cdkn1c* gene that encodes p57Kip2.

## Discussion

Given the unique position of the *dachshund gene* in identifying the earliest committed progenitors of the insulin-producing cells in the *Drosophila* central nervous system (Wang et al., 2007), we explored whether *dachshund* homologues are similarly involved in the differentiation of vertebrate β-cells in the pancreatic islets of Langerhans. We found that all islet lineages expressed a *dachshund* homologue: *dachb* in zebrafish and *Dach1* in mice. Furthermore, we found that all islet lineages required these genes for generating a full complement of cells. Finally, for the β-cell lineage, we demonstrated in mice that the decrease in cell number resulted from a decrease in proliferation. These data demonstrate the conservation of a role for members of the Dachshund family of transcriptional regulators in the development of insulin-producing cells in both invertebrates and vertebrates.

The expression of Dach1 in the mouse pancreas followed a pattern common to a number of islet transcription factors including Sox4, Mnx1 (Hb9), Pdx1, Nkx2.2, and Nkx6.1: early expression in all pancreatic progenitor cells, followed by deactivation prior to the secondary transition and reactivation in the endocrine lineage (Murtaugh, 2007). Interestingly, these factors often have different functions during their different phases of expression. For example, despite their early broad expression, the loss of neither Nkx2.2 nor Nkx6.1 has any apparent effect on the growth and differentiation of the early pancreatic anlage; but during their second phase of expression their loss dramatically impairs the differentiation of the β-cells (Sander et al., 2000; Sussel et al., 1998). Similarly, in the *Dach1*$^{-/-}$ mouse embryos, the development of the early pancreatic buds proceeded normally, and it was only during the second expression phase that a phenotype became evident, and then only in the endocrine lineage.

This endocrine phenotype, a decrease in cell number late in fetal development, can be attributed to a decrease in proliferation as we observed in the example of the β-cells in the *Dach1*$^{-/-}$ embryos, since no decrease in Neurogenin3-expressing endocrine progenitors or increase in apoptosis was observed. In mice, the newly differentiated β-cells start to replicate late in embryonic development, in a wave of expansion that extends into the early postnatal period (Finegood et al., 1995; Georgia and Bhushan, 2004; Sander et al., 2000). This perinatal proliferation depends on the activity of cyclin D2 (Georgia and Bhushan, 2004; Kushner et al., 2005), which associates with cyclin-dependent kinase Cdk4 (Rane et al.,

1999) to move mouse β-cells from the $G_0$ to the $G_1$ phase of the cell cycle, and is restricted by the activity of the cyclin-dependent kinase inhibitor p27Kip1 (Georgia and Bhushan, 2006), which inhibits cyclin-Cdk complexes (for a review see (Cozar-Castellano et al., 2006)). We found that Dach1 was linked in β-cells to the promoter of p27Kip1, thus potentially keeping cell cycle inhibition in check and allowing β-cell proliferation.

The balance of Dach transcriptional repression and p27Kip1 inhibition of cell cycle progression also controls the expansion of key cell populations during the development of the pituitary and the retina in mice (Li et al., 2002). p27Kip1 controls the proliferation of retinal and pituitary precursors, and in its absence mice develop pituitary tumors and hyperplastic retina (Nakayama et al., 1996). In association with the Sine oculis homologue Six6, Dach2 binds to the p27Kip1 promoter in retinal and pituitary progenitor cells and regulates their proliferation by repressing p27Kip1 expression (Li et al., 2002). In an analogous manner in pancreatic endocrine cells, products of one or more of the *sine oculis* gene homologues that we detected in the pancreas may recruit Dach1 to the p27Kip1 promoter.

Unlike in the differentiated endocrine cells, loss of Dach1 from the pancreatic progenitor cells that normally express it prior to E13 had no impact on the proliferation of these cells in the *Dach1*$^{-/-}$ embryos. In place of p27Kip1, the related cyclin-dependent kinase inhibitor p57Kip2 plays a dominant role in regulating cell cycle progression of the early progenitors during pancreas formation in mice. In the absence of p57Kip2, increased numbers of these progenitors enter the cell cycle; while increasing p57Kip2 expression by removal of the transcriptional inhibitor Hes1 inhibits progenitor cell proliferation (Georgia et al., 2006). Interestingly, although we could detect the presence of Dach1 protein on the p57Kip2 promoter by chromatin immunoprecipitation in pancreatic cells, this binding did not translate into substantial effects on the proliferation of these cells.

The absence of a phenotype in the pancreatic progenitor cells in the *Dach1*$^{-/-}$ mouse pancreas underscores the context dependence of Dach function. The activity of Dach proteins on a particular promoter depends on the set of interacting proteins expressed in that cell and present in the nucleus and the subset of those proteins recruited to the promoter (Heanue et al., 1999; Ikeda et al., 2002; Li et al., 2003; Li et al., 2002). Neurogenin3 initiates the second phase of Dach1 expression in the pancreas by activating its expression in the endocrine lineage; and in parallel, it initiates the transcription of a unique set of genes involved in the differentiation, maintenance and function of the endocrine cells, including other transcription factors, signaling molecules, and regulators of the cell cycle (Gasa et al., 2004; Juhl et al., 2008; Petri et al., 2006; Treff et al., 2006; White et al., 2008). The proteins expressed by this set of genes then collaborate with Dach1 in regulating the proliferation of the differentiated endocrine cells and permit the remarkable proliferative plasticity that underlies the ability of β-cells to adjust to changing energy balance.

We propose that the Dachshund family of transcriptional regulators represents part of an evolutionarily conserved genetic network governing the formation of cells producing insulin and interrelated metabolic hormones. With the relocation of these cells during evolution from the ectoderm-derived central nervous system to the endoderm derived gut, much of this network moved intact. The example of the Dachshund family demonstrates that evolutionary conservation can be exploited by using invertebrate model organisms to identify genes and pathways relevant to the development of islet cells in fish, mice and humans. Understanding the determinants of β-cell production and expansion will provide new insights into the impairment of these processes in diabetes and new strategies for therapeutic β-cell replacement for people with diabetes.

## Materials and methods

### Animals

All animal studies were approved by the UCSF Institutional Animal Care and Use Committee. The Dach1 mutant mouse line and the mouse insulin I gene promoter (MIP)-GFP line have been described previously (Backman et al., 2003; Hara et al., 2003). Mice were housed on a 12-hr light-dark cycle in a controlled climate. Timed matings were carried out with embryonic day 0.5 being set as midday of the day of discovery of a vaginal plug. Zebrafish (Danio rerio) were raised and cared for according to standard protocols (Westerfield, 1995). Wild-type embryos from the AB and TL strains and ptf1::GFP embryos (Godinho et al., 2007) were used and staged according to Kimmel (Kimmel et al., 1995).

### Cell culture and infection of recombinant adenoviruses

Mouse mPAC L20 (Yoshida and Hanahan, 1994) and βTC3 (Efrat et al., 1988) were cultured in DMEM supplemented with 10% FCS and antibiotics. Viral infections were performed as previously described (Gasa et al., 2004).

### *In situ* hybridization

Using the N and C box conserved regions of the three known zebrafish *dachshund* homologues as well as translated full length reading frames, we searched the UCSC genome browser (www.genome.ucsc.edu) for other zebrafish *dachshund* homologs. We detected another candidate *dachshund* gene on zebrafish chromosome 9, which has been subsequently identified *in silico* and listed in the NCBI database as hypothetical protein LOC560080. Using primers unique to this sequence, we amplified from a mixture of RNA from 24 and 48 hpf embryos a single cDNA whose sequence matched this putative *dachshund* homologue.

Single and double hybridizations and detections were carried out as previously described (Hauptmann and Gerster, 1994) on whole-mount wild-type embryos. Anti-sense RNA probes were prepared by transcribing linearized cDNA clones with SP6, T7, or T3 polymerase using digoxigenin or fluorescein labeling mix (Roche, Basel, Switzerland). Experimental and control embryos were developed for the same amount of time in the final colorimetric reaction for each probe tested. The *dachb* (Hammond et al., 2002), *preproinsulin* (Milewski et al., 1998), *glucagon* (Argenton et al., 1999), and *somatostatin2* (Devos et al., 2002) probes were described previously.

Cell counting was performed directly after *in situ* hybridization under the microscope by focusing successively on each layer of stained cells in whole-mount embryos. The NBT/BCIP staining was monitored in order to avoid an overstaining which would have prevented the visualization of the individual cell boundaries. For mounting, the yolk was removed manually and the embryos where then mounted with the ventral side on top. Imaging was then performed using a Zeiss AxioImager Brightfield Microscope. Captured imaged were further processed with Adobe Illustrator CS2 for figure mounting.

### Morpholino oligonucleotide injection

The morpholino oligonucleotides (Gene Tools, Eugene, OR) used were a dachb antisense oligonucleotide that targets the exon2-intron2 splice boundary, CTCAATGAGGGTTTACCTGTGGGTG; its 4-mismatch version (mismatch bases are underlined),CTCAA**A**GAG**C**GATTA**G**CTGT**C**GGTG; and the standard control morpholino provided by Gene Tools. 4 ng of morpholino nucleotides diluted in water were injected into the yolk of each embryo. Rhodamine dextran was added at 0.5% to the samples to visualize injection efficiency.

To assess the effect of the dachb morpholino, we extracted RNA from 24hpf morpholino-injected embryos using Trizol according to manufacturer's instructions (Invitrogen, Carlsbad, CA). cDNA was prepared by in vitro transcription with 1μg of total RNA using SuperscriptII reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT) primers (Invitrogen). 2 μl of cDNA product was used for each PCR reaction under the following conditions: 1 cycle of 94°C for 2', 60°C for 1', 72°C for 1', followed by 35 cycles of 94°C for 1', 60°C for 1', 72°C for 1'. The primers used for the PCR amplification on the obtained cDNA were as follows: A: 5'-ACGACTGCACCAACGCAAGC-3', B: 5'-TGTACCGGCGTTAGAGTTCA-3', C: 5'-CTGCCTAAAACCAGAATATTACTGT-3', D: 5'-GAGGAAATTGAGGCTCATCT-3'.

## RT-PCR analysis

Mouse whole pancreata were dissolved in RLT-buffer (Qiagen, Valencia, CA) and total RNA was prepared according to the manufacturer's protocol RNAeasy (Qiagen). Total RNA was treated with DNase (Qiagen). cDNA was prepared by *in vitro* transcription using SuperscriptII reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT) primers (Invitrogen). MIP-GFP cell sorting, RNA preparation, reverse transcription and amplification were performed as described previously (Miyatsuka et al., 2009) from E17.5 pancreas and adult islets. cDNA product from 20 ng of original total RNA or 0.2 μl of the MIP-GFP amplified cDNA was used for each PCR reaction under the following conditions: 1 cycle of 94°C for 2', 60°C for 1', 72°C for 1', followed by 35 cycles of 94°C for 1', 60°C for 1', 72°C for 1'. Mouse β-actin was used as the internal control. Sequences of primers are available on request.

## Immunohistochemistry

The whole mouse embryos at E12.5 or E15.5 or isolated pancreases from E18.5 embryos or adults were fixed in 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 1 hour to overnight at 4°C and either paraffin embedded or frozen in OCT and then sectioned. Hematoxylin/eosin and immunofluorescence analysis were performed as described previously (Sander et al., 1997) . The primary antibodies used in these assays were the following: rabbit anti-Dach1 diluted 1:100 (Proteintech Group Inc., Chicago, IL), guinea-pig anti-insulin diluted 1:2000 (Linco, Billerica, MA), guinea-pig anti-glucagon diluted 1:2000 (Linco), mouse anti-glucagon 1:2000 (Sigma, St Louis, MO), rat anti-somatostatin diluted 1:500 (Chemicon, Billerica, MA), guinea-pig anti-pancreatic polypeptide diluted 1:100 (Linco), guinea-pig anti-PDX1 diluted 1:2000 (Sander et al., 2000), mouse monoclonal anti-Nkx6.1 diluted 1:50 (Developmental Studies Hybridoma Bank), guinea-pig anti-Ngn3 diluted 1:1000 (Schwitzgebel et al., 2000), mouse anti-Ki-67 diluted 1: 100 (BD Pharmingen, San Jose, CA), rabbit anti-p27Kip1 (C-19) diluted 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human caspase 3 diluted 1:400 (Cell signaling technology, Danvers, MA), rabbit anti-Pax6 diluted 1:500 (Chemicon, Billerica, MA), rabbit anti-Amylase diluted 1:750 (Sigma, St Louis, MO), hamster anti-Mucin diluted 1:200 (Thermo Scientific, Fremont, CA) and rabbit anti-Glut2 diluted 1:500 (Chemicon, Billerica, MA). The following secondary antibodies were used for immunofluorescence: Cy3-conjugated anti-rabbit 1:800, Cy3-conjugated anti-guinea pig 1:800, Cy3-conjugated anti-rat 1:800, FITC-conjugated anti-guinea pig 1:200, FITC-conjugated anti-rat 1:200, and FITC-conjugated anti-mouse 1:200 (Jackson Laboratories, Bar Harbor, Main). Fluorescence was visualized and photographed with a Zeiss Axiophoto2 plus microscope.

## Cell counting and protein quantifications in mouse

To obtain a representative average of the number of hormone-positive cells, entire pancreases were used for quantification. Immunofluorescence staining was performed on 6

μm sections and positive cells were counted on every fifth section throughout the pancreas at E18.5 from a minimum of four embryos per genotype. The average cell number was determined from all sections counted. After immunohistochemistry, pancreatic epithelial areas were outlined and measured with the OpenLab software. The values of cell counting were normalized to tissue area. Quantification of proliferating β-cells was performed on paraffin wax-embedded sections by counting of insulin and insulin/Ki-67 double-positive cells. The cells from every tenth section were counted throughout the pancreas at E18.5 from three embryos per genotype.

For the quantification of insulin cells at E15.5, five sections of pancreatic tissue from two *Dach1*$^{+/+}$ and from two *Dach1*$^{-/-}$ animals were stained for insulin. The insulin-positive cells were counted and the obtained numbers were normalized to the pancreatic tissue area. The tissue area was determined using ImageJ software (NIH).

For the quantification of p27Kip1, eight sections of pancreatic tissue from four *Dach1*+/+ and from four *Dach1*$^{-/-}$ animals (E18.5) were co-stained for p27Kip1 and insulin. The staining was visualized using a Zeiss confocal microscope (LSM 510Meta). The intensity of p27Kip1 staining in insulin-positive cells was quantified using ImageJ software. p27Kip1 was quantified in 50 insulin-positive cells per animal (a total number of 200 cells was quantified per condition). The intensity (pixels) of p27Kip1 staining in insulin-positive cells was normalized to the nuclear area.

### Chromatin immunoprecipitation assays

Mouse βTC3 cells were grown to 50–60% confluence; and cross-linking, chromatin preparation, immunoprecipitation and PCR were performed as previously described (Lynn et al., 2007) using 100 μg of chromatin and 2.5 μg normal rabbit IgG (sc-2027; Santa Cruz Biotechnology) or rabbit anti-Dach1 antibody (Proteintech Group Inc., Chicago, IL). The PCR primers for the *Cdkn1b* gene promoter (p27Kip1) located at −1.6 to −1.3 kb and coding sequence at +2.0 to +2.3 kb have been described previously (Li et al., 2002). The PCR primers for the *Cdkn1c* gene promoter (p57Kip2) were located at −1.5 kb (5’-ACACAGGGACAGAACAAAGC-3’) to −1.3 kb (5’-TCAAGTCAAACCCTGAAGCC-3’).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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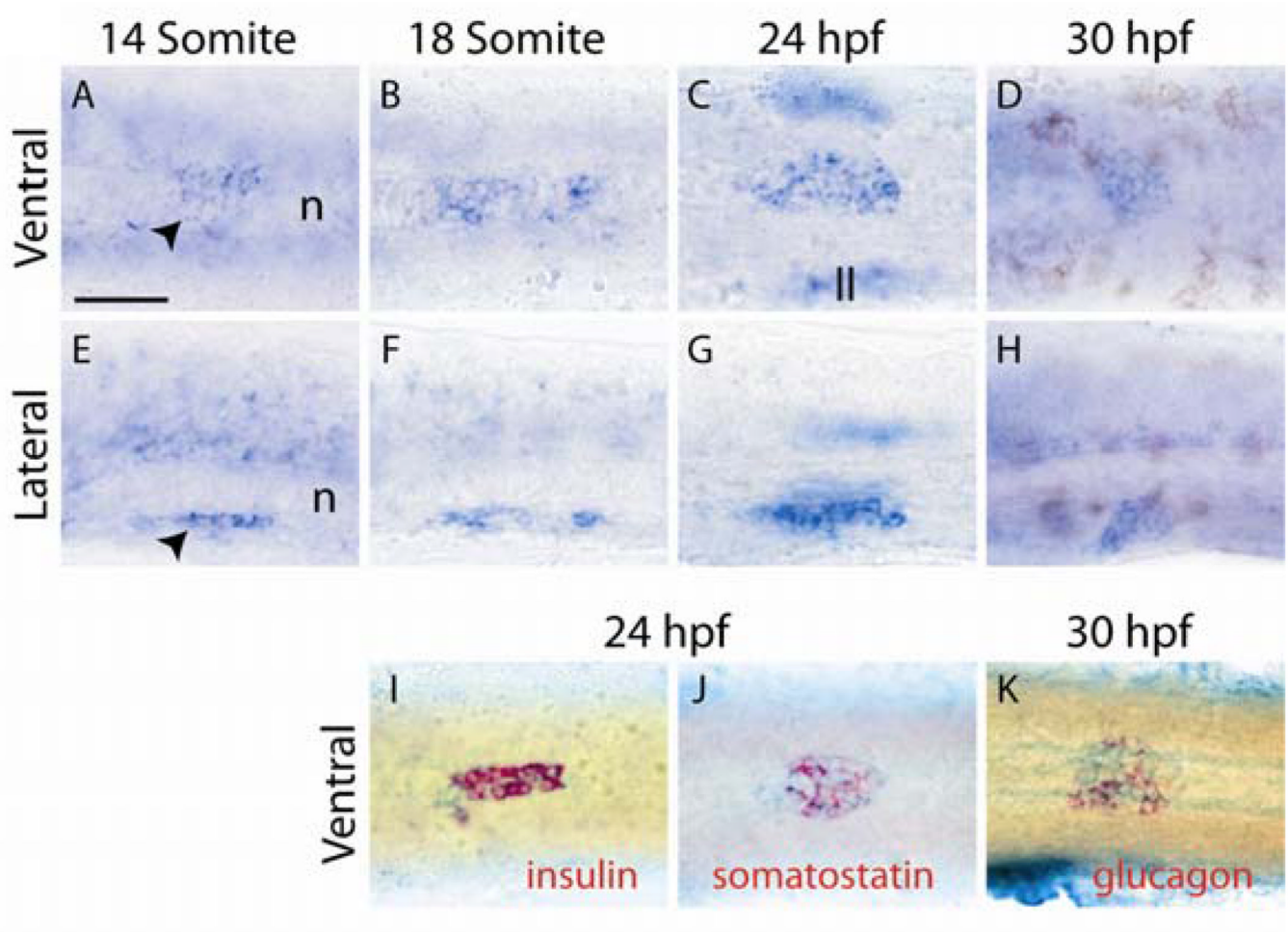


**Fig. 1.**

Expression of *dachb* in the zebrafish pancreas. Whole-mount *in situ* hybridization was performed for *dachb* (A–K, blue) and *insulin* (I, red), *somatostatin* (J, red), and *glucagon* (K, red) at the stages of zebrafish development shown. Ventral views (A–D;I–K), and lateral views (E–H) are shown with anterior to the left. On all the panels the yolk was manually removed from the embryos. The notochord (*n*) and the lateral line (*ll*) have been indicated in panels A, E and C. Black arrowheads: *dachb*-expressing cell in the pancreatic region. Scale bar, 50μm.

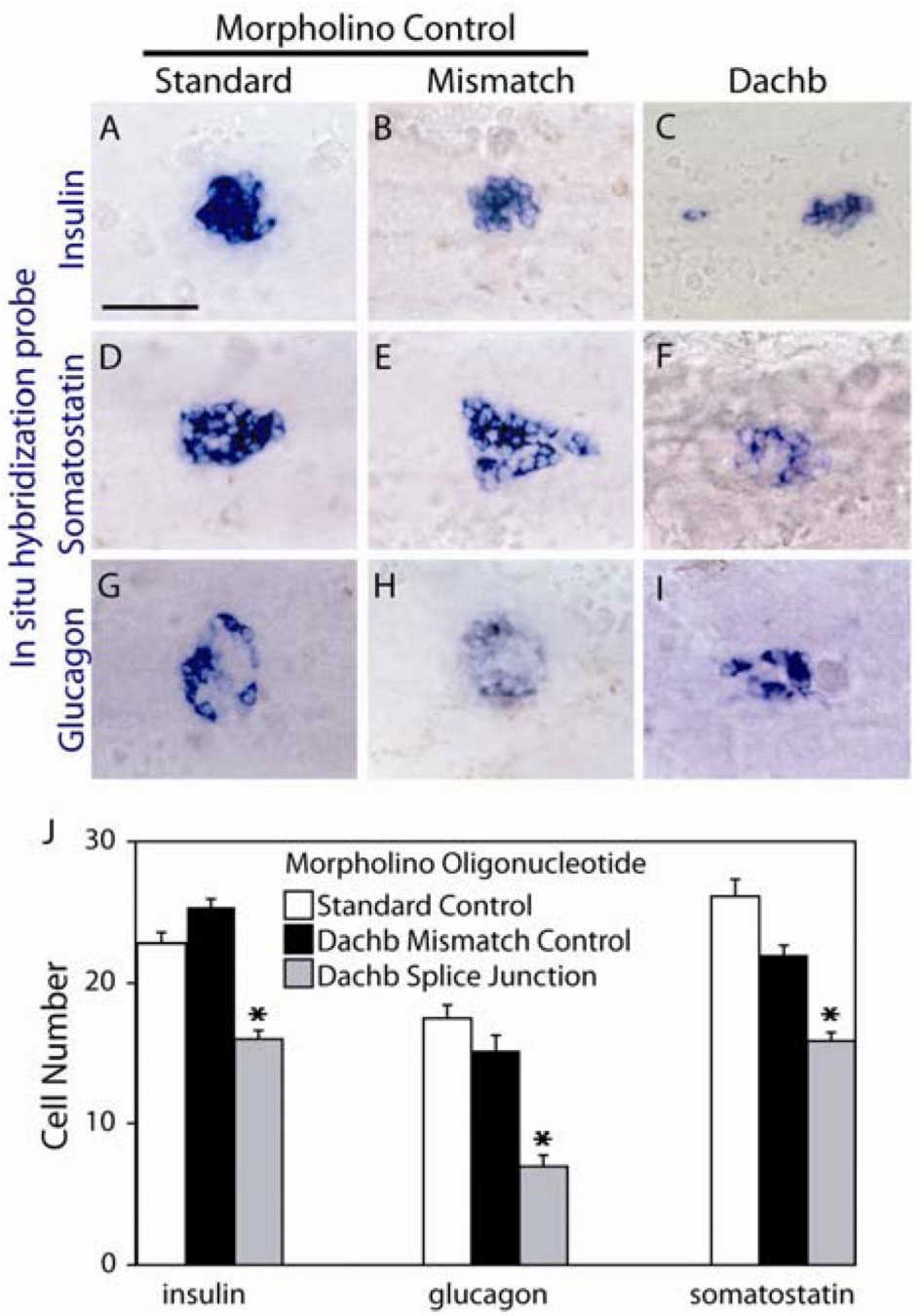


**Fig. 2.**

Inhibition of DachB expression by morpholino antisense oligonucleotides. Expression of insulin (A–C), somatostatin (D–F) and glucagon (G–I) was analyzed by whole-mount *in situ* hybridization at 30 hpf embryos injected with the morpholino oligonucleotides shown: a standard control (A, D, G), a mismatch control with 4 bases altered from the complimentary *dachb* sequence (B, E, H), and an oligonucleotide complimentary to a *dachb* splice junction (C, F, I). All panels present ventral views of yolk-free embryos with anterior to the left. In panel J, the numbers of cells expressing the hormones shown were assessed in morpholino-injected embryos at 30 hpf. Each data point represents the mean ± standard error of at least

40 embryos for each condition. ***p<0.001 compared with embryos injected with morpholino 4-mismatch control by Student's t test. Scale bar, 50μm.

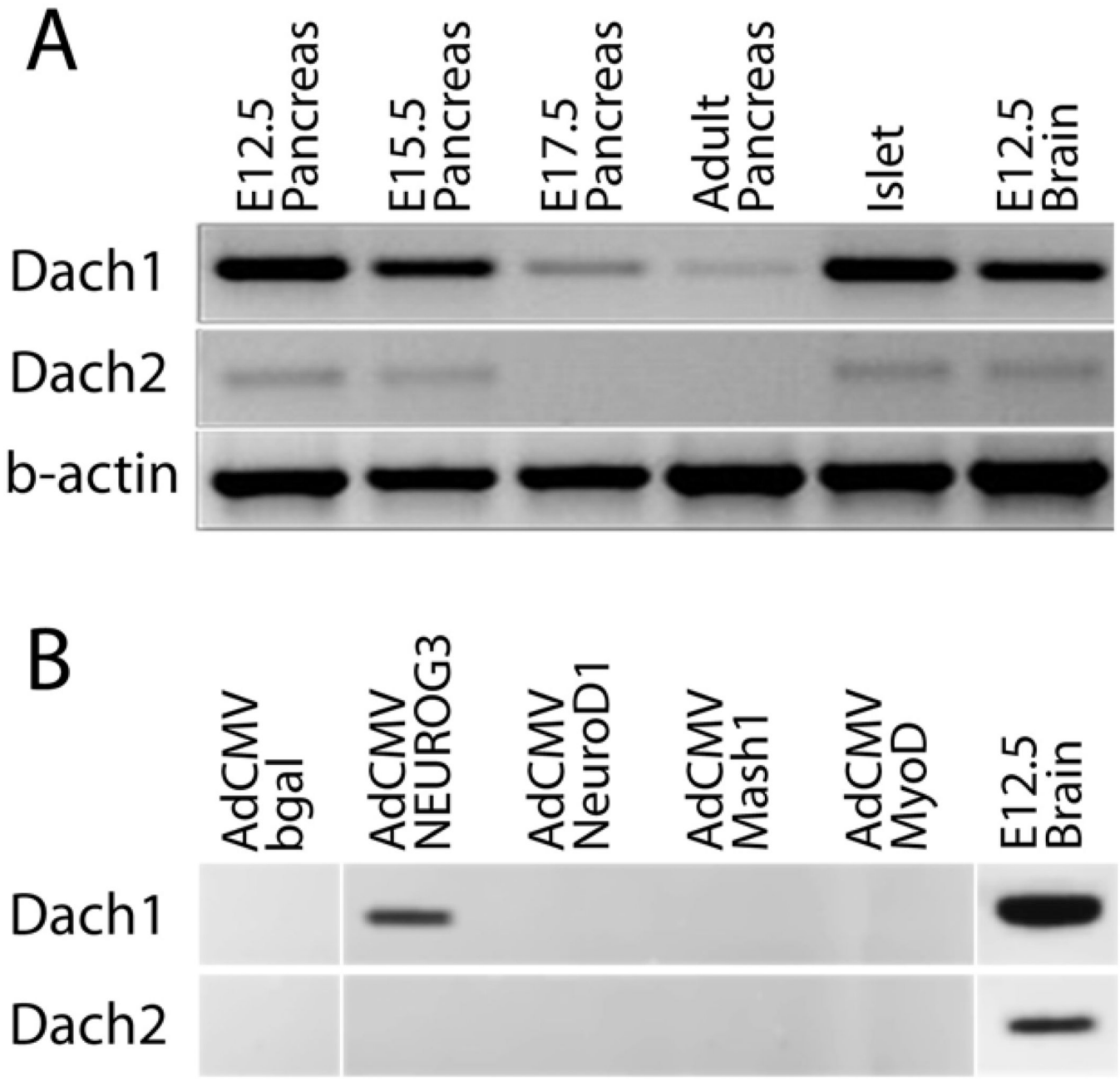


**Fig.3.**

Expression of *Dach* mRNA in the mouse. RT-PCR was performed with gene specific primers with RNA purified from isolated mouse tissues at the indicated ages in A, and from the mouse pancreatic ductal cell line mPAC L20 infected with adenovirus shown in B. Products were not amplified in the absence of RT (data not shown).

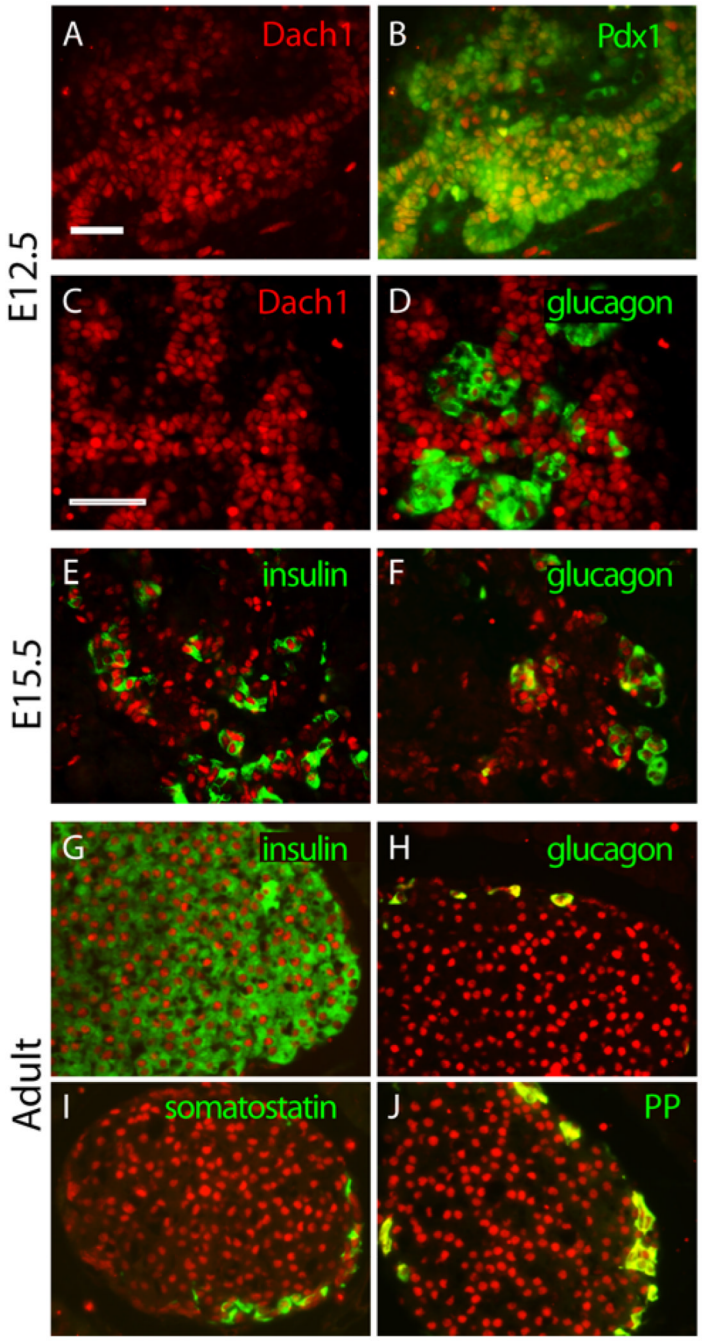


**Fig.4.**

Expression of Dach1 in the mouse pancreas. Immunofluorescence staining for Dach1 is shown in red at E12.5 (A–D), E15.5 (E, F), and adult (G – J). Double immunofluorescence staining for transcription factor Pdx1 or islet hormones (green) was performed for Pdx1 (B) glucagon (D, F and H), insulin (E and G), somatostatin (I), and pancreatic polypeptide (PP, in J). Scale bar, 100μm.

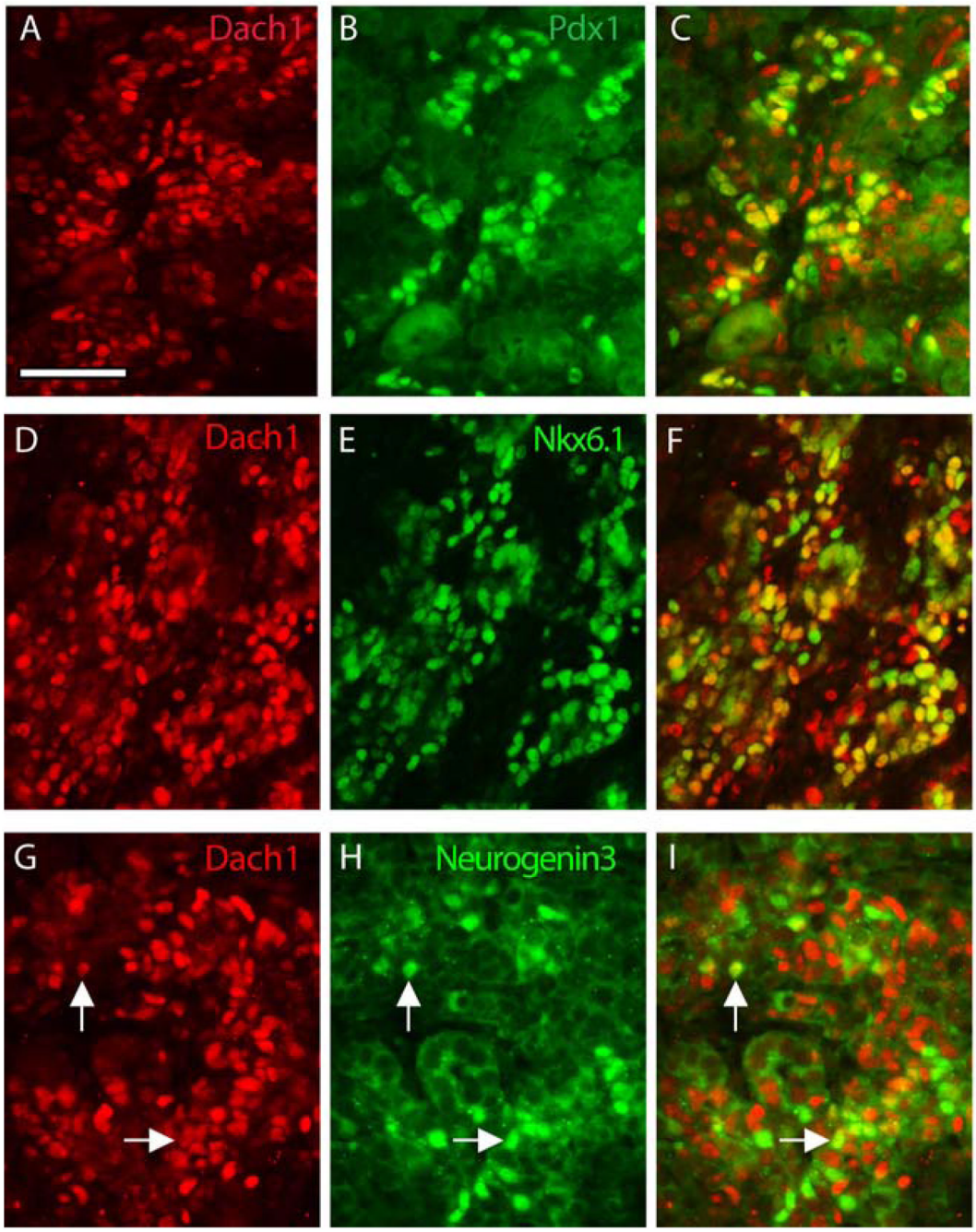


**Fig.5.**

Expression of Dach1with islet transcriptional regulators in the mouse pancreas at E15.5. Immunofluorescence staining for Dach1 is shown in red (A, C, D, F, G, I). Double immunofluorescence staining for islet transcription factors (green) was performed for Pdx1 (B, C), Nkx6.1 (E, F) and Neurogenin3 (H, I). Nuclei expressing both proteins appear yellow (C, F, I). Examples of co-staining nuclei in panels G–I are indicated with white arrows. Scale bar, 50μm.

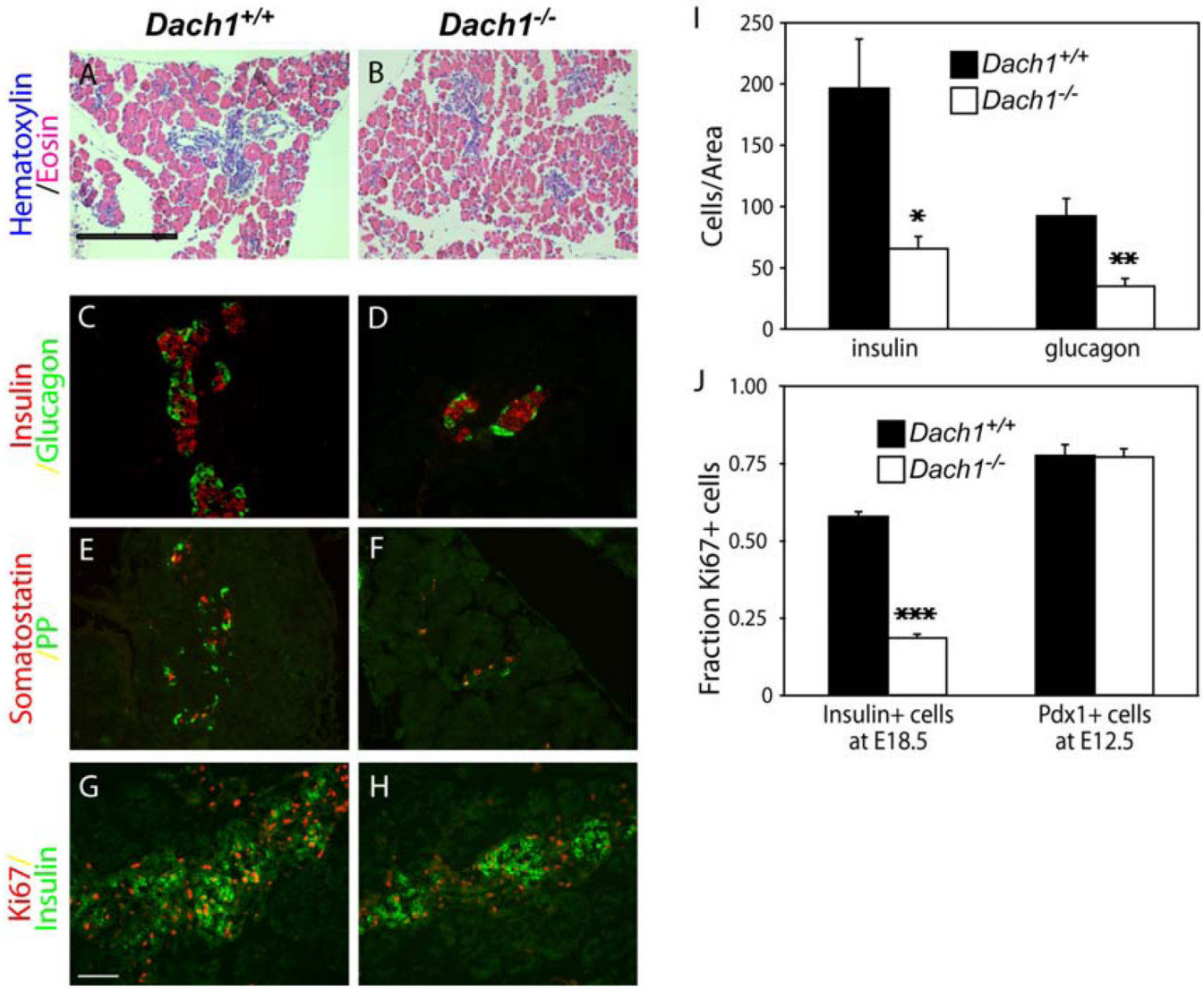


**Fig.6.**

Pancreas development in the absence of Dach1 at E18.5. Hematoxylin-eosin staining demonstrates the morphology of the pancreas in *Dach1*$^{+/+}$ (A) and *Dach1*$^{-/-}$ (B) mouse embryos. Scale bar, 500 μm. Immunofluorescent co-staining for insulin (C, D; red), glucagon (C, D; green), somatostatin (E, F; green) and pancreatic polypeptide (PP; E, F; red) was performed on the pancreas in *Dach1*$^{+/+}$ (C, E) and *Dach1*$^{-/-}$ (D, F) mouse embryos at E18.5. Scale bar, 100 μm. Immunofluorescent co-staining for insulin (green) and Ki67 (red) was performed on the pancreas in *Dach1*$^{+/+}$ (G) and *Dach1*$^{-/-}$ (H) mouse embryos at E18.5. Scale bar: 100 μm. I. Insulin- and glucagon-positive cells from *Dach1*$^{+/+}$ (black bars) and *Dach1*$^{-/-}$ (white bars) embryos were counted and expressed as the total number of cells per total pancreatic area. J. Percentage of β-cells replicating in *Dach1*$^{+/+}$ (black bars) and *Dach1*$^{-/-}$ (white bars) embryos at E18.5 was assessed by counting the number of cells co-staining for insulin and Ki67 and dividing by the total number of cells staining for insulin; and the percent of pancreatic progenitor cells replicating in *Dach1*$^{+/+}$ (black bars) and *Dach1*$^{-/-}$ (white bars) embryos at E12.5 was assessed by counting the number of cells co-staining for Pdx1 and Ki67 and dividing by the total number of cells staining for Pdx1. Each

data point represents the mean of 4 embryos ± standard error of the mean.*$p < 0.02$, **$p < 0.01$, and ***$p < 0.001$ compared with *Dach1*$^{+/+}$ embryos by Student's t test.

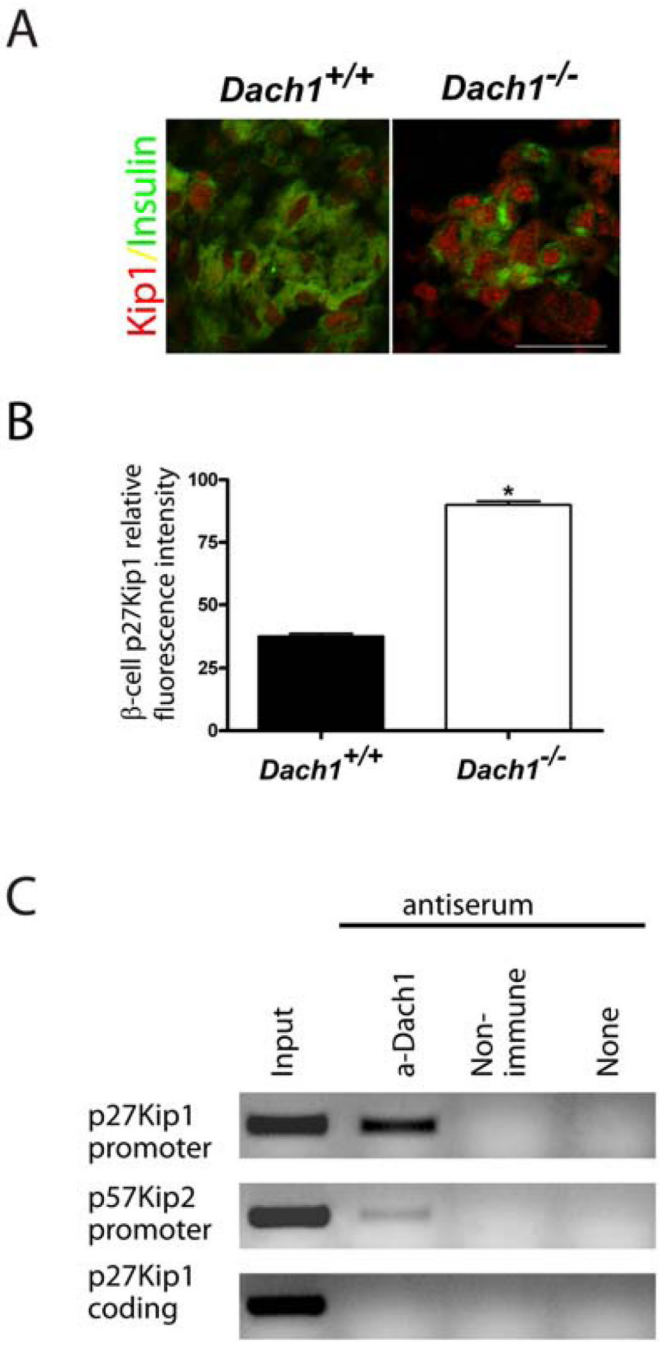


**Fig. 7.**

Cell cycle regulation by Dach1. A. Immunofluorescent co-staining was performed for cyclin-dependent kinase inhibitor p27Kip1 (red) and insulin (green) on sectioned pancreas from *Dach1*$^{+/+}$ and *Dach1*$^{-/-}$ mouse embryos at E18.5. Scale bar, 25μm. B. The intensity of p27Kip1 protein expression in insulin-positive cells in E18.5 pancreases from *Dach1*$^{+/+}$ (black bar) and *Dach1*$^{-/-}$ (white bar) embryos was quantified and expressed as the intensity normalized to cell area.*$p$<0.0001. C. Chromatin IP studies were performed by immunoprecipitating cross-linked chromatin with antiserum against Dach1, with control IgG or without antiserum. Fragments of the mouse genes shown were amplified by PCR from the

precipitates or the input DNA. Each data point represents the mean of quantification in 4 embryos ± standard error of the mean.