Published in final edited form as:
Mech Dev. 2009 ; 126(8-9): 687–699. doi:10.1016/j.mod.2009.05.005.

Jagged1 is a competitive inhibitor of Notch signaling in the embryonic pancreas

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Abstract

Pancreatic endocrine cells originate from precursors that express the transcription factor Neurogenin3 (Ngn3). *Ngn3* expression is repressed by active Notch signaling. Accordingly, mice with Notch signaling pathway mutations display increased *Ngn3* expression and endocrine cell lineage allocation. To determine how the Notch ligand Jagged1 (Jag1) functions during pancreas development, we deleted *Jag1* in foregut endoderm and examined postnatal and embryonic endocrine cells and precursors. Postnatal *Jag1* mutants display increased *Ngn3* expression, α-cell mass, and endocrine cell percentage, similar to the early embryonic phenotype of *Dll1* and *Rbpj* mutants. However, in sharp contrast to postnatal animals, *Jag1*-deficient embryos display increased expression of Notch transcriptional targets and decreased *Ngn3* expression, resulting in reduced endocrine lineage allocation. Jag1 acts as an inhibitor of Notch signaling during embryonic pancreas development but an activator of Notch signaling postnatally. Expression of the Notch modifier *Manic Fringe (Mfng)* is limited to endocrine precursors, providing a possible explanation for the inhibition of Notch signaling by Jag1 during mid-gestation embryonic pancreas development

The endodermally derived pancreas consists of both exocrine and endocrine compartments. The exocrine tissue is composed of acinar and duct cells and functions in the synthesis and secretion of digestive enzymes. The endocrine pancreas controls blood glucose homeostasis and is comprised of the islets of Langerhans, which in the adult murine pancreas contain insulin-secreting β-cells, glucagon-secreting α-cells, somatostatin-secreting δ-cells, and pancreatic polypeptide-secreting PP cells. Endocrine cell development requires expression of the basic helix-loop-helix (bHLH) transcription factor *Neurogenin3* (*Ngn*3). Deletion of *Ngn3* abolishes differentiation of all pancreatic endocrine cells (Gradwohl et al., 2000; Lee et al., 2002a), and

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Conflicting interests statement: The authors declare that they have no competing financial interests.

lineage-tracing experiments have shown that all mature endocrine cells are derived from Ngn3-positive precursors (Gu et al., 2002).

Ngn3 expression is regulated by the Notch signaling pathway. Notch signaling components are present in the developing pancreas as soon as the dorsal pancreatic bud appears on embryonic day 9.0 (E9.0) (Apelqvist et al., 1999). Notch, a plasma membrane receptor, is proteolytically cleaved upon binding of its ligands (Selkoe and Kopan, 2003), which in the murine pancreas are Delta-like1 (Dll1), Jagged1 (Jag1) and Jagged2 (Jag2) (Apelqvist et al., 1999; Jensen et al., 2000). Cleaved Notch translocates into the nucleus where it binds to and activates the transcription factor RBP-Jκ (Kramer, 2001; Mumm and Kopan, 2000). RBP-Jκ upregulates the Hes and Hey (also known as Hrt or Herp) families of repressors, which in the pancreas bind to the *Ngn3* promoter and inhibit its transcription (Bertrand et al., 2002; Iso et al., 2003; Kageyama and Ohtsuka, 1999). When the Notch signaling components *Rbpj*, *Dll1*, or *Hes1* are deleted, *Ngn3* expression in the pancreas increases, and pancreatic precursor cells differentiate prematurely into endocrine cells. This depletes the pool of progenitors and leads to decreased exocrine and endocrine mass and usually in an increase in the endocrine/exocrine ratio (Apelqvist et al., 1999; Fujikura et al., 2006; Jensen et al., 2000).

In addition to the core signaling components, additional proteins modulate the activity of the Notch pathway, including the *Fringe* family of glycosyltransferases. Fringe molecules glycosylate Notch, thereby modifying the receptor's response to its ligands (Haltiwanger and Stanley, 2002; Moloney et al., 2000; Panin et al., 2002). In *Drosophila*, Fringe limits the domain of Notch activation during wing and epidermis development (Klein and Arias, 1998; Walters et al., 2005). In general, glycosylation increases the response of Notch to Delta-like, but decreases Jagged-induced Notch signaling. In mammals, this decrease in Jagged-induced Notch cleavage occurs with no reduction in Jagged-Notch binding (Hicks et al., 2000; Yang et al., 2005). Of the three mammalian Fringe proteins (Lunatic, Radical, and Manic), only Manic Fringe (Mfng) expression can be detected up to E14.5, and then Lunatic Fringe (Lfng) is expressed only in differentiated acinar tissue (Svensson et al., 2009; Xu et al., 2006). *Mfng* expression partially overlaps with *Ngn3* expression in the developing murine pancreas (Svensson et al., 2009; Xu et al., 2006), and overexpression of Mfng induces *Ngn3* expression in chick endoderm, presumably by inhibiting Notch signaling (Xu et al., 2006).

Notch signaling can function at several stages during the differentiation of a single organ and can play multiple roles within a given tissue. For example, Notch signaling acts at several steps in hematopoiesis, myogenesis, and neurogenesis (Cagan and Ready, 1989; Fuerstenberg and Giniger, 1998; Hartenstein, 2006; Hirsinger et al., 2001). Additionally, during immune cell differentiation, various Notch components can possess non-redundant roles in the same tissue. For example, Notch1 is required to specify the T- vs. B-cell lineage, while Notch2 controls further differentiation into the various types of B-cells (Hartenstein, 2006). In the zebrafish, deltaA deficient mutants lack α-cells, jagged1b deficient embryos have an increase in α-cells, and jagged2 deficient embryos have an increased percentage of endocrine cells within the pancreas, indicating that different ligands have different roles in zebrafish pancreas development (Zecchin et al., 2007). In the murine pancreatic bud, Dll1 is expressed at E9.0, but Jag1 is not expressed until later (Apelqvist et al., 1999). Since various Notch ligands have different spatiotemporal expression patterns within the pancreatic anlage, they may also have diverse functions during mammalian pancreatic development.

The roles of the Notch ligands in mammalian pancreatic development have not been thoroughly studied due to the early embryonic lethality of *Dll1*$^{-/-}$ and *Jag1*$^{-/-}$ mice (Hrabe de Angelis et al., 1997; Xue et al., 1999). Here, we show that *Jag1* is the most abundant Notch ligand during mid-gestation pancreatic development. To delineate the role *of Jag1* during pancreatogenesis, we use the Cre-loxP system to delete the gene in the foregut endoderm, from which the pancreas

is derived. We demonstrate that although the postnatal phenotype is similar to the embryonic phenotype of other Notch signaling mutants, the embryonic phenotype is strikingly different. Surprisingly, in the fetal pancreas, the endocrine and pro-endocrine cell percentage is reduced in *Jag1* mutants while direct transcriptional targets of Notch are upregulated, mimicking Notch activation. We show further that Jag1 can act as a competitive inhibitor of Dll ligands *in vitro* when Notch is modified by Mfng glycosylation, providing a molecular model possibly explaining the upregulation of Notch targets observed in the *Jag1*-defecient fetal pancreas.

Results

Expression analysis Notch ligands in the developing pancreas

We analyzed the mRNA levels of Notch ligands to determine which of them is most abundant during pancreatogenesis. We developed primers for *Dll1*, *Jag1*, and *Jag2* that amplified their targets with equal efficiency, allowing for a quantitative comparison of mRNA levels for the three genes (Figure 1A). All three ligand mRNAs could be detected as early as E12.5 and throughout second wave endocrine cell development, the period during which most pancreatic endocrine cells differentiate. *Jag1* mRNA expression was consistently higher than that of the other two ligands (Figure 1B), however, we cannot exclude the possibility that the relative abundance of the three ligand protein levels is different due to different mRNA translatability or differences in protein stability.

Next we analyzed the localization of Jag1 protein in the developing epithelial pancreas. At E12.5, Jag1 protein was present in every cell positive for the epithelial pancreatic precursor marker Pdx1 (Figure 1, C–F). Co-expression of Jag1 and Pdx1 was also observed throughout the E14.5 pancreatic epithelium (data not shown). At E14.5, Jag1 protein was also present in differentiated endocrine cells, demonstrated with co-staining with glucagon, the most abundant endocrine marker at this timepoint (Figure 1, G–J). At E16.5, Jag1 protein was detectable in some but not all cells positive for markers of differentiated pancreatic cell types—glucagon, insulin, and somatostatin, and for the exocrine marker amylase (data not shown). By E18.5, Jag1 was limited to duct and endocrine cells (Figure 1, K–R). Peri- and postnatally, epithelial Jag1 is limited to the pancreatic ducts (Golson et al., 2009). Due to the widespread and abundant expression of *Jag1* throughout the early pancreatic epithelium, we evaluated the consequences of deleting *Jag1* in the developing pancreas.

Since early embryonic lethality of $Jag1^{-/-}$ mice has precluded analysis of the role of Jag1 in pancreatic development thus far (Mitsiadis et al., 1997), mice with a conditional allele of *Jag1* were bred to *Foxa3-Cre* mice or to $Pdx\text{-}Cre^{early}$ mice to produce $Jag1^{loxP/loxP}$;*Foxa3-Cre* mice or $Jag1^{loxP/loxP}$;$Pdx1\text{-}Cre^{early}$ mice and control littermates (Gu et al., 2002; Lee et al., 2005; Loomes et al., 2007). Because Foxa3-Cre is expressed in the foregut endoderm beginning at E8.5, *Jag1* was deleted in the epithelium of several derivative organs, including the pancreas, liver, and stomach (Lee et al., 2005), while Pdx1-Cre deletes throughout the epithelium of the pancreas only, beginning at E9.0 (Gu et al., 2002; Heiser et al., 2006). While $Jag1^{loxP/loxP}$;$Pdx1\text{-}Cre^{early}$ mice developed severe pancreatitis, precluding postnatal endocrine analysis, only ~25% of $Jag1^{loxP/loxP}$;*Foxa3-Cre* mice did, even when backcrossed onto a C57Bl6 background. To examine the role of endocrine cell development, we therefore used $Jag1^{loxP/loxP}$;*Foxa3-Cre* mice, excluding those with pancreatitis or fatty infiltration of the pancreas.

Quantitative RT-PCR analysis of *Jag1* mRNA in embryonic and postnatal pancreas with primers specific to exons 4 and 5 indicated a reduction of 50–65% in *Jag1* message beginning at E15.5 and continuing through postnatal development (Figure 2A), with a similar reduction at the protein level (Figure 2B). Thus, the extent of *Jag1* deletion remains constant throughout fetal development and postnatal life of the mutants, suggesting that *Jag1* deficient epithelial

cells are not at a competitive disadvantage in this model. Remaining Jag1 is probably in endothelial cells, where *Foxa3-Cre* is not expressed, indicating that about half of the *Jag1* mRNA observed in wildtype animals is from endothelial cells. Despite the activation of *Foxa3-Cre* at E8.5, significant deletion of *Jag1* occurs later in this model than in $Jag1^{loxP/loxP}$;*Pdx1-*Cre^{early} mice, in which *Jag1* mRNA was reduced to 54% of control levels by E14.5 (p=0.017; Supplementary Figure 1). Immunostaining indicates that by E18.5, no *Jag1* expression remains in epithelial cells (Figure 2, C–J).$Jag1^{loxP/loxP}$;Foxa3-Cre^{+} mice were born at the expected Mendelian frequency (16/56), and no significant differences compared to controls were observed in their appearance or body weight at birth. However, postnatal survival of $Jag1^{loxP/loxP}$;Foxa3-Cre^{+} mice was impaired, with only 11 of 16 mice surviving to P18 versus 33 of 40 controls.

α-cell mass is altered postnatally in *Jag1* mutants

Based on the phenotype of other Notch pathway mutants, endocrine cell lineage allocation in postnatal mutant animals was examined using several cell-type specific markers. Using immunostaining, we found an approximate 2.5-fold increase in α-cell mass in P21 *Jag1* mutants versus controls (131±36 vs. 48±13 μg), with no change in β-cell (194 ±62 vs 200±59 μg), δ-cell (23±14 vs 17±4 μg), or PP-cell mass (9±4 vs 7±1 μg) (Figure 3 A–G). The expected increase in total endocrine mass did not reach significance (Figure 3G).

Expression levels of endocrine genes were examined to confirm our histological findings. As expected, mRNA levels of several α-cell-specific genes were increased significantly at P12—pre-proglucaon by ~70%, the pro-α-cell transcription factor Arx by ~80%, and the α-cell specific transcription factor Brn4 by ~100% (Figure 3H) (Collombat et al., 2005; Collombat et al., 2007; Collombat et al., 2003; Heller et al., 2004). In concordance with an unchanged β-cell mass, no difference was detected in insulin mRNA at P12 (Figure 3H), in fed or fasted blood glucose or in glucose tolerance at P20–21 (data not shown).

Ngn3 is upregulated in postnatal *Jag1* mutants

We examined Ngn3-positive cells at P0 and observed just a few cells that were $Ngn3^{+}$ in controls as expected since *Ngn3* is dramatically downregulated around birth (Figure 4A) but many that were positive in mutants (Figure 4B). We also investigated *Ngn3* expression by utilizing a *Ngn3-eGFP* "knock-in" allele (Lee et al., 2002a). P7 pancreata from both $Jag1^{loxP/loxP}$;Foxa3-Cre^{+}; $Ngn3^{+/eGFP}$ and $Jag1^{+/loxP}$; Foxa3-Cre^{-}; $Ngn3^{+/eGFP}$ mice were examined for eGFP-expressing cells. Controls had very few $eGFP^{+}$ cells at P7 (Fig 4C). In contrast, mutant pancreata contained large numbers of eGFP-expressing cells, indicating persistent *Ngn3* gene activation (Figure 4D). The abnormal postnatal expression of *Ngn3* partially explains the *Jag1* mutant phenotype described above, i.e. the increased percentage of endocrine cells within the pancreas and the increased percentage of α-cells within the endocrine compartment.

Exocrine mass is reduced in postnatal *Jag1* mutants

Since other Notch mutants have reduced pancreas weight due to precocious endocrine differentiation and loss of pancreatic precursors, we examined the pancreas weight *of Jag1* mutants. At P21, total pancreas weight was reduced in *Jag1* mutants postnatally (Figure 5A). This reduction in weight can be attributed to loss of exocrine mass since approximately 95% of pancreas weight in wildtype animals is exocrine tissue. Note that this reduction in pancreas size indicates that endocrine *percentage* in *Jag1* mutants is increased compared to controls (0.89±0.25 vs 0.43±0.11% of total pancreas mass), since endocrine *mass* is maintained in *Jag1* mutants. Because *Jag1* was not deleted significantly until E15.5, we examined embryonic pancreatic area to determine when the onset of decreased pancreatic size occurred and detected no difference at E12.5, E16.5, or E18.5 (Figure 5B). We thus inferred that decreased exocrine

mass in the *Jag1* mutants did not result from a depletion of general pancreatic progenitors and therefore examined acinar cell death using TUNEL staining. At P0-P1, the number of TUNEL-positive acinar cells was increased in *Jag1* mutants (Figure 5, C–E), indicating that the decrease in exocrine tissue was due to exocrine cell death, not a change in lineage allocation. This increase in acinar cell death is reduced compared to that in $Jag1^{loxP/loxP}$;$Pdx1\text{-}Cre^{early}$ mice, and fibrosis was never observed at P0 or P14 in $Jag1^{loxP/loxP}$;*Foxa3-Cre* animals.

Embryonic phenotype mimics Notch activation

Since the size of the mutant embryonic pancreas was not decreased, we suspected that the *Jag1*-deficient embryonic pancreas may have unexpected differences compared to other Notch signaling mutants. We examined embryonic pancreata at E12.5 and E14.5, but detected no significant differences between controls and mutants. We then examined E16.5 embryonic pancreata. Unlike *Dll1* and *Hes1* null embryos and pancreas-specific *Rbpj* mutants, *Ngn3, glucagon*, and *insulin* mRNA were significantly downregulated in total pancreas at E16.5 while the Notch targets *Hes5* and *Hey1* were significantly upregulated, indicating an increase in Notch signaling despite ablation of the most abundant Notch ligand from the pancreatic epithelium (Figure 6A). No difference was detected in amylase mRNA levels (Figure 6A).

$Ngn3^{+}$, α- and β-cell numbers were quantified, showing that the number of endocrine precursors and glucagon-positive cells was reduced by approximately one-half at E16.5 (Figure 6B–G). Insulin-positive cell numbers were the same in controls and mutants, suggesting that the reduction in *insulin* mRNA resulted from reduced transcription rather than a reduced cell number, possibly indicating immaturity of the β-cells. We examined *Jag2* and *Dll1* levels to determine whether upregulation of these two ligands could account for the increase in Notch signaling at E16.5, but no significant increase was observed (Supplemental Figure 2). $Jag1^{loxP/loxP}$;$Pdx1\text{-}Cre^{early}$ mice also exhibited reduced levels of *Ngn3, glucagon, and insulin* mRNA at E16.5 but at E14.5 only *Ngn3* mRNA was reduced, indicating that the earlier deletion in the $Jag1^{loxP/loxP}$;$Pdx1\text{-}Cre^{early}$ mice does not change the timing of the decrease in endocrine cells (Supplemental Figure 3).

To address when *Jag1* mutant endocrine cell numbers begin to catch up to those of control mice, we examined *ngn3, ins, and glu* mRNA at E18.5. All three genes were expressed at the same level in control and *Jag1* mutant pancreas at this stage (Figure 6H). To confirm this finding, we also quantified the number of α- and β-cells and found no difference, indicating that *Jag1* mutant embryos begin to recover from the increased Notch signaling observed at E16.5 by E18.5 (Figure 6I). To determine the mechanism of recovery of the Ngn3- and glucagon-positive cells, we examined the percentage of these cells that had entered or passed through S-phase using BrdU. No difference was apparent at E16.5 or E18.5 in BrdU-positive α- or β-cells (Figure 6J), and there were very few BrdU-positive Ngn3-positive cells in either mutants or controls (data not shown), indicating that the increase in endocrine precursors and in α-cells after E16.5 is the result of *de novo* generation of these cell populations or a perdurance of pre-existing Ngn3-positive cells.

We hypothesized that the paradoxical phenotype observed in the fetal $Jag1^{loxP/loxP}$;$Foxa3\text{-}Cre^{+}$ pancreas might result from modifications of the Notch signaling pathway. In mammals, Fringe glycosyltransferases decrease the Jagged-Notch signal without affecting binding of Jagged and Notch. *Mfng* is expressed in some $Ngn3^{+}$ cells, and it induces Ngn3 expression in undifferentiated chick endoderm (Svensson et al., 2009; Xu et al., 2006), the same phenotype as a decrease in Notch signaling effected by a *Rbpj, Hes1*, or *Dll1* knockout (Apelqvist et al., 1999; Fujikura et al., 2006; Jensen et al., 2000). We postulated that Mfng may be acting within the endocrine precursor lineage as a modifier of Notch signaling by causing Jag1 to act as a competitive inhibitor of Dll1 signaling. We therefore examined *Mfng* expression in endocrine progenitors, sorted on the basis of *Ngn3-eGFP* expression. Cells positive for eGFP expression

(i.e. endocrine precursors and early endocrine descendents) expressed *Mfng* at levels that were between 10- and 20-fold greater than those of eGFP^{-} cells at all time points examined; *Mfng* expression was extinguished in mature islets (Figure 7A).

To further test our hypothesis that Jag1 is acting as a Notch inhibitor in the presence of Mfng expression, we examined Notch cleavage in cells containing either a control plasmid (3T3E) or a Mfng-expressing plasmid (3T3M). We plated these cells on Jag1 ligand alone, Dll1 ligand alone, or a combination of both ligands. Jag1 and Dll1 alone as well as in combination with each other induced Notch activation in control cells (Figure 7B, lanes 3, 5 &7). *Mfng* expression reduced Notch1 activation in cells plated on Jag1 ligand slightly, confirming previous results (lanes 3&4;(Hicks et al., 2000; Klein and Arias, 1998; Moloney et al., 2000; Yang et al., 2005)). Notch activation was unchanged in cells plated on Dll1 (Figure 7B, lanes 5&6), which was not very surprising since Mfng potentiates Dll1 signal through Notch1 less than Rfng or Lfng in 3T3 cells (Yang et al., 2005). 3T3M cells plated on a combination of Jag1 and Dll1 ligands showed low levels of cleaved Notch compared to cells plated on Dll1 alone, indicating that Jag1 can act as a competitive inhibitor of Dll1 in the presence of Mfng expression (Figure 7B, compare lanes 7&8 to lanes 5&6). These data support a model in which Jag1 and Mfng induce the expression of Ngn3 by suppressing Dll1-Notch signaling (Figure 7C).

Discussion

The robust expression of *Jag1* in the embryonic pancreas suggested that this gene plays a major role in pancreatogenesis. Of course, translational efficiency of the various Notch ligands differ, so although mRNA levels *of Jag1* are higher than those *of Dll1* and *Jag2*, protein levels may not exactly reflect mRNA levels. Deletion of *Jag1* in the foregut endoderm, which gives rise to the pancreatic epithelium, leads to a postnatal phenotype similar to early embryonic phenotypes of *Hes1* and *Rbpj* mutants (Fujikura et al., 2006; Jensen et al., 2000). This similarity may be surprising since a recent report detailing a compound knockout of *Notch1/2* describes a very mild pancreatic phenotype compared to those *of Hes1* and *Rbpj* mutants; these *Notch1/2* double knockout mice did not have any dysregulation of *Ngn3*. The mild phenotype observed in the *Notch1/2* compound mutant may, however, be due to expression of *Notch3/4* in the pancreatic epithelium. Although one group has reported that only *Notch1/2* are expressed in the pancreatic epithelium (Lammert et al., 2000), others have demonstrated epithelial expression of *Notch3/4* as well (Apelqvist et al., 1999).

Jag1$^{loxP/loxP}$*;Foxa3-Cre*$^{+}$ mice display important differences in comparison with previously reported Notch pathway mutants. *Jag1*-deficient pancreata have normal total endocrine mass and a full complement of β-cells while other Notch signaling mutants have reduced endocrine mass despite an increased percentage of endocrine cells (Apelqvist et al., 1999; Fujikura et al., 2006; Jensen et al., 2000). *Jag1* mutants also have a reduced Ngn3^{+} population at E16.5 but have persistent *Ngn3* expression after birth, while the pancreas-specific *Rbpj* mutant has an increased Ngn3^{+} population at E10.5 but reduced thereafter (Fujikura et al., 2006). Finally, *Jag1* mutants have decreased exocrine mass due to acinar cell death, not due to a change in cell lineage allocation with precocious differentiation of common pancreatic progenitors.

This increase in acinar cell death may indicate that Notch signaling promotes acinar cell survival; *Jag1*$^{loxP/loxP}$*;Pdx1-Cre*^{early} mice also exhibit acinar cell death, at even higher rates than *Jag1*$^{loxP/loxP}$*;Foxa3-Cre*$^{+}$ mice, with fatty replacement of the acinar tissue; the acinar cell death is probably due to ductal malformation (Golson et al., 2009). The acinar cell death in *Jag1*$^{loxP/loxP}$*;Foxa3-Cre*$^{+}$ mice may also result from a ductal malformation that could be less severe due to the later deletion *Jag1*$^{loxP/loxP}$*;Foxa3-Cre*$^{+}$ mice.

Like many other Notch signaling pathway mutants, *Jag1* mutants have an increased percentage of α-cells within the endocrine compartment. However, due to the timing of increased *Ngn3* expression, the increase in α-cells is unexpected since the competency of the fetal pancreas to produce α-cells because of *Ngn3* overexpression decreases dramatically with age (Johansson et al., 2007). Differences observed in these two models could be explained in at least two ways: 1) misexpression of Ngn3 under control of the Pdx1-Cre promoter initially causes it to be expressed in pancreatic progenitor but later in β- and δ-cells, and this misexpression leads to the change in percent of differentiating α-cells; or 2) embryonic Notch upregulation in the *Jag1* mutant pancreatic epithelium leads to competency alterations due to persistent general pancreatic precursors such, as in mice that overexpress Notch1 (Hald et al., 2003). Differentiated cells of all types, including acinar, islet, and duct cells are observed in neonates, however, and no markers of general pancreatic precursors are available that do not also mark differentiated cell types; therefore, determining whether more pancreatic progenitors are present in the late embryonic and peritnatal *Jag1* mutant pancreas has proven difficult.

Our most surprising finding is that during fetal life, *Jag1* mutants mimic an increase in Notch signaling rather than the expected decrease. E16.5 *Jag1* mutants have a reduced endocrine population, while direct transcriptional targets of Notch—Hes5 and Hey1—are upregulated, and *Ngn3* expression is decreased compared to control littermates. This striking phenotype may be explained by the spatially and temporally restricted presence of the Notch glycosyltransferase, Mfng, which is expressed highly in endocrine progenitors but is absent or very low in the rest of the embryonic pancreas or in differentiated pancreatic cells. Mfng has previously been reported to induce endocrine cell development when overexpressed in chick endoderm and to overlap partially with Ngn3 in the developing murine pancreas (Svensson et al., 2009; Xu et al., 2006).

A recent report suggests that Mfng is unnecessary for pancreas development (Svensson et al., 2009). Mfng null mice displayed no change in endocrine cell number or ratio between E9.5 and E14.5, and adults had normal pancreas morphology with no defects in blood glucose homeostasis. Endocrine cell counts after E14.5 were not reported, however, and endocrine cell number defects were not observed in *Jag1* mutants until after E14.5. Therefore, this recent report does not preclude the possibility that Mfng causes Jag1 to act as an inhibitor of Notch signaling in the embryonic pancreas.

Genetic evidence in *Drosophila* supports the hypothesis that Jag1 can act as an inhibitor of Notch signaling in the presence of Fringe (de Celis et al., 1998; Klein and Arias, 1998). Assays with Fringe glycosyltransferases in 3T3 cells reflect what is known of the function of Fringe in *Drosophila*; specifically, cells expressing both Serrate and Fringe are unable to respond to Serrate ligand. In mammals, glycosylation of Notch receptors by Mfng decreases Jagged-induced Notch cleavage without preventing binding between Jagged and Notch (Hicks et al., 2000; Yang et al., 2005). In addition to the reduced signaling efficacy from Jag1 in the presence of Mfng, we show that Jag1 can act as a competitive inhibitor of Dll1-induced Notch signaling when Mfng is expressed (Figure 7B).

We present a model in which Jag1 binds to but does not activate glycosylated Notch and interferes with Dll1 binding, leading to relatively low levels of Notch activation (Figure 7C). Since endocrine precursors express high levels of Mfng, and genetic ablation of *Jag1* leads to increased rather than decreased Notch signaling as assayed by *Hes5*, *Hey1*, and *Ngn3* expression at E16.5, the function of Jag1 during pancreatogenesis may be to limit the number of cells with activated Notch, just like *Serrate* in the *Drosophila* wing margin. Increased Notch signaling observed in *Jag1* deficient pancreata may be the result of more efficient binding between Dll1 and Notch receptors, leading to high levels of Notch activation and thus reduced *Ngn3* expression.

Many modifiers of the Notch signaling pathway exist and our experiments do not unequivocally determine that Mfng is the reason that *Jag1* mutants display increased Notch activation in the embryonic pancreas. However, we do show that Jag1 can act as an inhibitor of Dll1-induced Notch activation when Notch is glycosylated and that Mfng is highly expressed in endocrine progenitors. Our experiments also indicate that Jag1 normally inhibits Notch signaling embryonically but can stimulate it postnatally. In summary, we have shown that different Notch ligands have non-redundant roles in the developing pancreas and each serves to fine-tune the process of endocrine and exocrine cell differentiation from common precursors.

Experimental Procedures

Animals and genotype analysis

The derivation of *Jagged1*^{loxP}, *Ngn3-eGF*, and *Foxa3-Cre* mice has been reported previously (Gu et al., 2002; Lee et al., 2005; Lee et al., 2002a; Loomes et al., 2007; Soriano, 1999). All mice were maintained on a C57Bl/6J;129/SvEv background. Genotyping was performed by PCR analysis using genomic D*NA* isolated from the toe tips of newborn mice. Littermate *Jag1*$^{loxP/loxP}$ and *Jag1*$^{loxP/+}$ mice were used as controls in most experiments. All procedures involving mice were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Tissue culture

NIH 3T3 cells were transfected with human Mfng cDNA in a pCMV-XL4 plasmid or with pCMV-XL4 control plasmid (OriGene, Rockville, MD, USA) along with a neomycin-resistance containing vector (PL452) at a 5:1 (PL452) using Fugene6 (Roche, Nutley NJ, USA) and positive cells were selected using 750 μg/ml Geneticin. Mfng expression was confirmed by RT-PCR. HEK 293 cells were transiently transfected with Jag1-Fc or Dll1-Fc expression vectors (gifts from Drs. Tom Kadesch and Joseph Verdi) using Lipofectamine 2000 (Invitrogen), and conditioned media was collected. 10 cm plates were sequentially incubated for one hour at room temperature with 10 μg/mL anti-Fc antibody (Jackson) in sterile 1X PBS and then conditioned media containing Jag1-Fc, Dll1-Fc or a 1:1 combination of the two ligands. Mfng-expressing or control cells were plated and whole cell extracts (WCE) were collected 24 hours later and assessed for cleaved Notch1 by immunoblotting. For quantification, QuantiOne software (Bio-Rad; Hercules, CA, USA) was used. Blots were normalized to the ratio between cleaved Notch1 and α-tubulin in Jag1-Fc induced 3T3 cells.

Immunoblotting (IB) and immunostaining

The following antibodies were used: goat anti-Jag1 (C-20, 1:200; Immunoblot; Santa Cruz Biotechnologies, Santa Cruz, California, USA), rabbit anti-Jag1 (28H8, 1:200–500; immunostaining; Cell Signaling Technology, Danvers, MA, USA), goat anti-Pdx1 (A17, 1:200; Santa Cruz), mouse anti-Ngn3 (F25A1B3, 1:1000; Hybridoma Bank, Iowa City, Iowa) guinea pig anti-insulin (1:800; Linco Research, Inc., St. Charles, Missouri, USA), rabbit anti-glucagon (undiluted; Zymed Laboratories, Inc., South San Francisco, California, USA), guinea pig anti-glucagon (1:3000–5000; Linco), goat anti-glucagon (1:200; Santa Cruz) goat anti-somatostatin (1:5000; Santa Cruz), rabbit anti-pancreatic polypeptide (1:50; Zymed), rabbit anti-Chromogranin A (1:1500; DiaSorin, Stillwater, Minnesota, USA), goat anti-amylase (1:200; Santa Cruz), rat anti-CK19 (1:50, TromaIII, Hybridoma Bank), sheep anti-BrdU (1:1000; US Biologicals, Swammscott, MA, USA), rabbit anti-cleaved Notch1 (1:1000; Abcam), mouse anti-acetylated α-tubulin (1:5000; Sigma, St. Louis, MO, USA), Cy3-conjugated donkey anti-rabbit IgG (1:600; Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania, USA), Cy2-conjugated donkey anti-rabbit IgG (1:400; Jackson), Cy3-conjugated donkey-anti sheep (1:600; Jackson), Cy2-conjugated donkey anti-guinea pig IgG (1:600; Jackson) and Cy2-conjugated donkey anti-goat IgG (1:600; Jackson), biotinylated anti-

rabbit IgG (1:200; Vector Laboratories, Burlingame, CA, USA), biotinylated anti rat-IgG (1:200; Vector), HRP-conjugated anti-rabbit IgG (1:10,000; Jackson; IB), HRP-conjugated anti-mouse IgG (1:2,000; IB; Jackson) and HRP-conjugated anti-goat IgG (1:2,000; Jackson; IB).

Indirect immunofluorescence was performed as previously described on paraffin-embedded tissue (Sund et al., 2001) and examined using epifluorescent microscopy (Nikon Eclipse E600 microscope; Cool Snap CF camera). Immunohistochemistry was performed as previously described (Gupta et al., 2005). DBA staining was performed according to manufacturer's instructions (Vector).

For determination of postnatal endocrine cell mass, pancreata were laid flat during the paraffin-embedding process and cut for maximal footprint. Quantification of endocrine cell mass was performed by immunostaining against Chromogranin A and counterstaining with hematoxylin (Zymed). The entire pancreas of each animal was imaged with at 60X and the area of both exocrine and endocrine compartment was analyzed using Metamorph software (Molecular Devices, Downingtown, PA, USA). The percentage of area occupied by endocrine cells was then multiplied by pancreas mass to determine endocrine cell mass. For individual cell types, slides were labeled with antibodies against insulin and glucagon, against insulin and somatostatin, or against insulin and pancreatic polypeptide. Images of the first 10–20 islets encountered per mouse were taken with a 240X. IPLab software was used to quantify the area positive for each hormone per slide in square microns (BD Biosciences, Rockville, Maryland, USA). Total microns for each hormone per mouse were added together and the insulin-positive area per mouse was averaged over the three experiments. The percentage of each cell type was calculated based on square microns. Masses of the individual cell types were determined by multiplying the percentage of each cell type by the total endocrine mass. For assessment of TUNEL-positive acinar cells, TUNEL-positive cells were counted at 240X and entire pancreas sections were imaged at 60X. Area of acinar tissue was quantified using IPLab.

For assessing whether Ngn3-expressing cells were present in postnatal mice, we both stained pancreata for Ngn3 expression and collected pancreata from P7 *Jagged1*$^{loxP/loxP}$;*Foxa3-Cre*$^{+}$ and *Jagged1*$^{loxP/+}$; *Foxa3-Cre*$^{-}$ pups that were determined by PCR to contain a Ngn3-eGFP allele were dissected and fixed for 2 hours in 4% PFA, rinsed in PBS, and then embedded in agarose before sectioning with a vibratome at 100 μm. These sections were examined using confocal microscopy (Leica, Longueuil, Canada).

For quantification of embryonic endocrine precursors, sections were stained with Ngn3 and counterstained with DAPI. Ngn3-positive nuclei were hand-counted at 240X. Entire sections were imaged at 120X and DAPI-stained nuclei were counted using the "auto-segment" function of IPLab. For quantification of embryonic glucagon- and insulin-positive cells, images of every glucagon- or insulin-positive cell were taken at 120X or 200X and counted by hand. Then entire sections were imaged at 40X or 24X and the auto-segmentation function of IPLab was used to quantify area in square microns.

WCE from tissue for immunoblot were collected as described (Lee et al., 2002b) and from cell culture with 250 μl RIPA buffer which was then incubated on ice for 15 minutes before sonication and centrifugation. 75–80 μg WCE were loaded on an 8% SDS-PAGE gel or 4–12% Bis-Tris gel (Invitrogen, Carlsbad, California, USA) and transferred to a membrane before being probed with primary and secondary antibodies and then developed with the Amersham ECL-Plus kit (Amersham, Piscataway, NJ, USA).

Collection of *Ngn3-eGFP*-positive and -negative sorted cells

Collection of *Ngn3*-e*GFP*-positive cells, RNA extraction, and method of normalization for qRT-PCR has been reported previously (White et al., 2008).

Quantitative reverse transcriptase PCR (qRT-PCR)

RNA was isolated and reverse-transcribed as previously described (Gupta et al., 2005). PCR reaction mixes were assembled and reactions were performed on an Mx3000^{TM} or Mx4000 ^{TM} Multiplex Quantitative PCR System (Stratagene). For comparison of Notch ligand RNA levels, primers were designed within one exon within the last 700 bp of mRNA. The primers were tested on a dilution series of ES cell DNA. Unless otherwise indicated, TBP was used as the housekeeping gene for all qRT-PCR experiments. Primer sequences are listed in Table 1.

Statistics

Data were analyzed using Student's t-test with equal distribution and either one or two-tailed analysis, as appropriate

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Acknowledgments

The authors thank Dr. Rana Gupta, Athanasios Arsenlis, Beth Helmbrecht, Sophia Hammani and James Fulmer for technical contributions to this work and Drs. Tom Kadesch and Joe Verdi for Notch pathway vectors. Our studies were facilitated by the University of Pennsylvania Diabetes Center (P30DK19525), the Morphology Core of the Penn Center for Molecular Studies in Digestive and Liver Disease (P30DK50306) and the Flow Cytometry and Cell Sorting Resource Laboratory. This work was supported by the NIDDK (DK55342 to KHK, DK61226 to CLM, and DK02796 to KML).

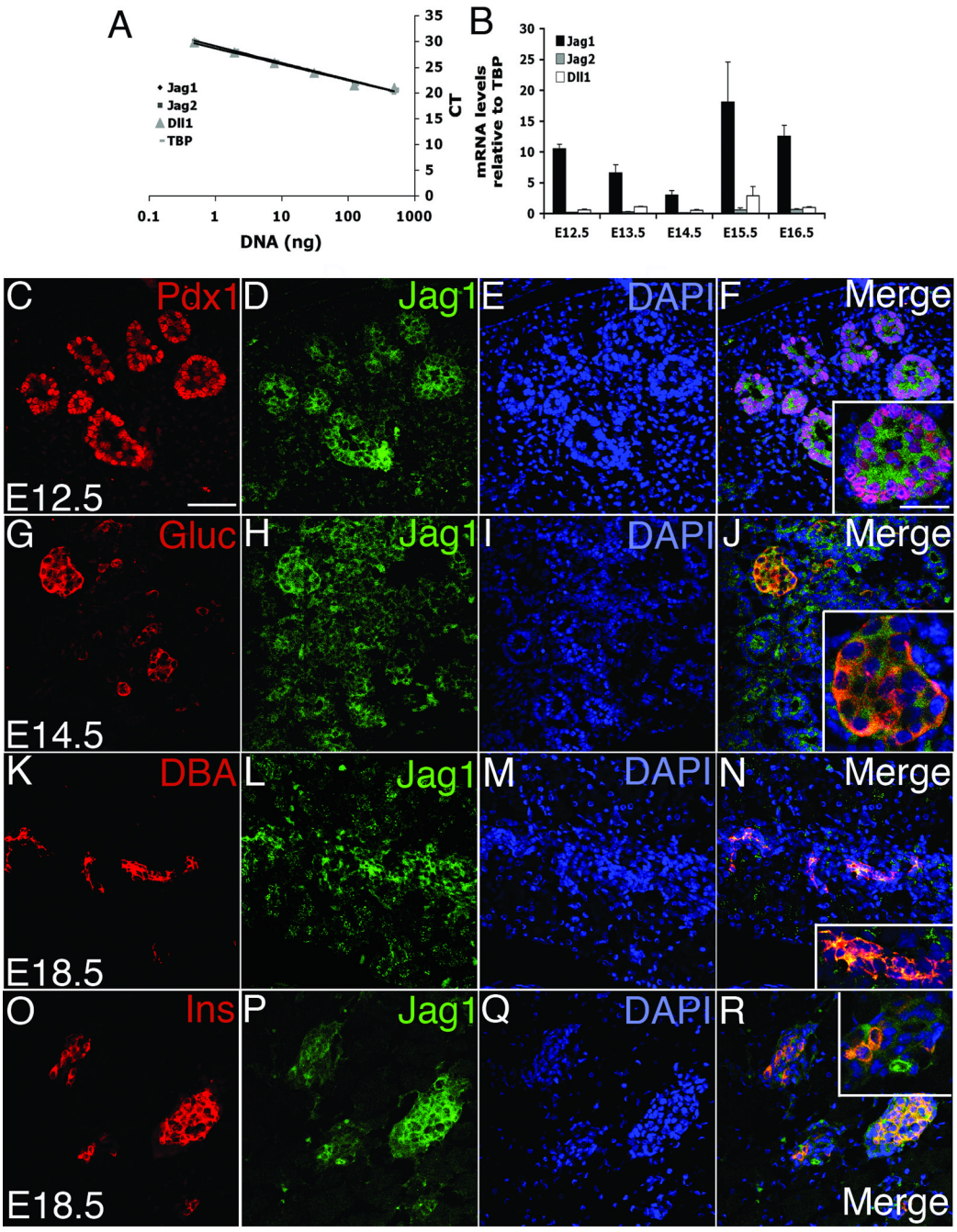

Figure 1.

Jagged1 is initially expressed throughout the pancreatic epithelium but gradually becomes limited to ducts. (A) Primers for Jag1, Jag2, Dll1 and TBP amplify with equal efficiency. Not all of icons can be seen because they lie on top of each other. (B) qRT-PCR on total pancreas RNA for Jag1, Jag2 and Dll1 (normalized to TBP) indicate that Jag1 is expressed at much higher levels than the other Notch ligands throughout pancreas development (n=4). (C–R) Indirect immunofluorescence, shown both as single channel images of Pdx1, glucagon, DBA lectin, or insulin (column 1) plus Jag1 (column 2), the nuclear marker DAPI (column three) and merged images (column 4). (C–F) At E12.5, immunostaining indicates that Jag1 (D) is expressed in all Pdx1-expressing cells (C), which mark the undifferentiated pancreatic

epithelium. (G–J) Jag1 (H) is expressed in differentiated α-cells (glucagon expression, G) at E14.5, the predominant endocrine cell at this timepoint. (K–N) At E18.5, *Jag1* (L) is expressed in ducts labeled with DBA lectin (K). (O–R) Jag1 (P) is also expressed in differentiated islets at E18.5 (insulin, O). For all images, bar = 25 μm except insets, where bar = 50 μm.

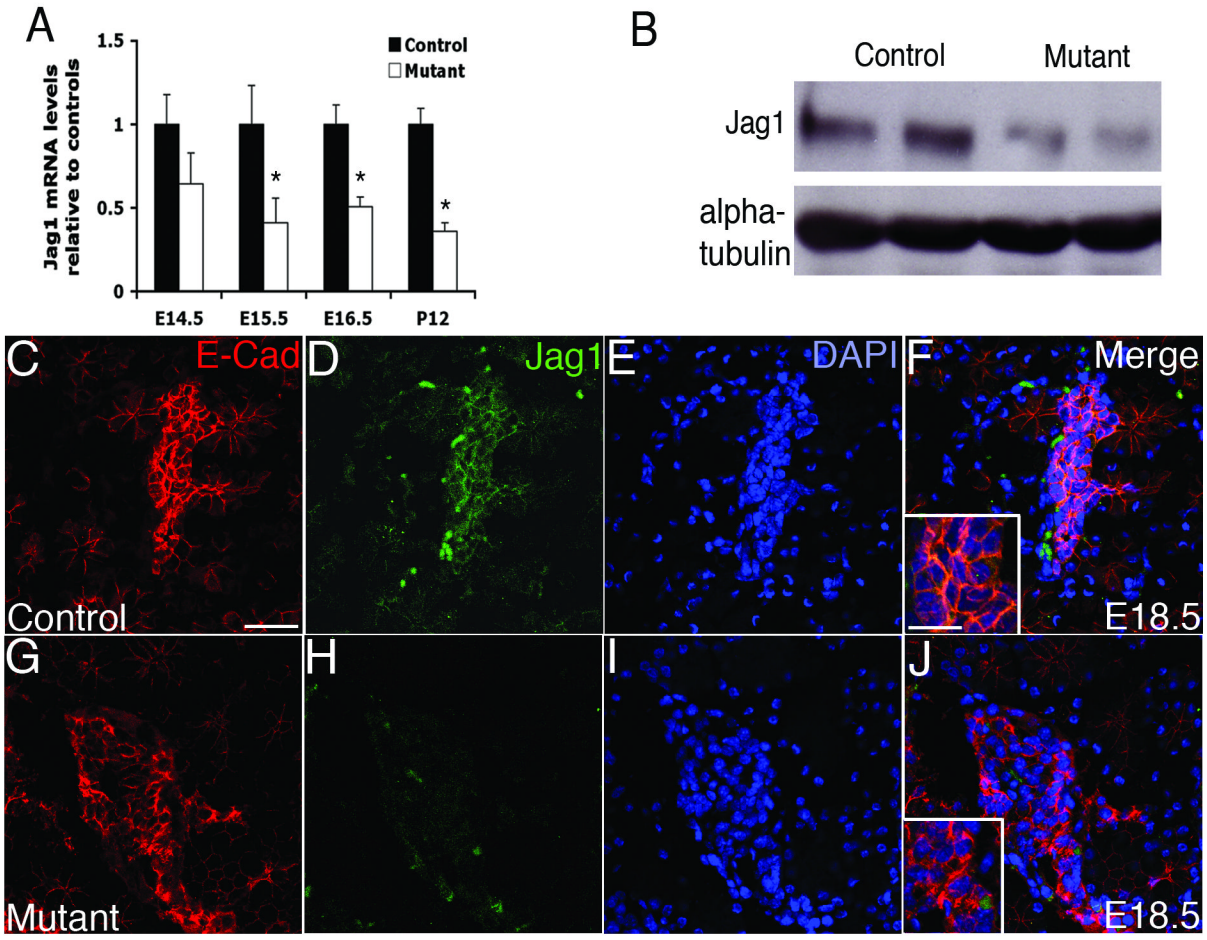

Figure 2.

Conditional ablation of *Jag1* in the pancreatic epithelium. (A) qRT-PCR analysis of *Jag1* mRNA levels (relative to TBP) in total pancreas (n=4–7). (B) Western blot on P8 pancreata confirms that Jag1 levels are decreased in *Jag1*$^{loxP/loxP}$;*Foxa3-Cre*$^{+}$ mutants. α-tubulin is used as a loading control. (C–J) Immunofluorescence confirms deletion of Jag1 in pancreatic epithelium by E18.5. (C–F) At E18.5, Jag1 co-labels with the epithelial, transmembrane E-cadherin in control pancreas islet clusters. (G–J) In contrast, E18.5 *Jag1*$^{loxP/loxP}$;*Foxa3-Cre*$^{+}$ animals no longer display Jag1 in E-cadherin-expressing islet clusters.

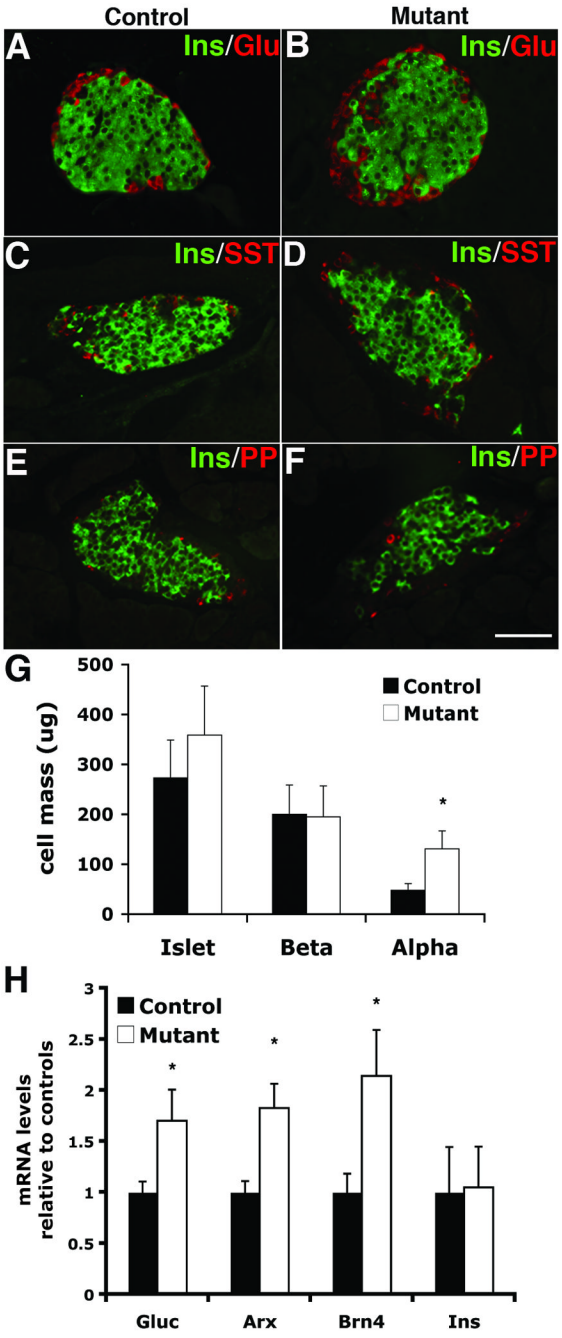

Figure 3.

The α-cell compartment is expanded and the exocrine compartment reduced in the postnatal pancreas of *Jag1*$^{loxP/loxP}$;*Foxa3-Cre*$^{+}$ mice. Indirect immunofluorescence detection at P21 of insulin and glucagon (A,B), insulin and somatostatin (C,D) or insulin and PPY (E,F) in control (A,C,E) and mutant (B,D,F) mice (bar = 50 μm). (G) Mutants display increased α-cell mass with no change β-cell mass. (n=4). (H) mRNA levels for the α-cell hormone glucagon and the α-cell transcription factors Arx and Brn4 are increased in the pancreas of P12 *Jag1*$^{loxP/loxP}$;*Foxa3-Cre*$^{+}$ mutants while the expression of *insulin* mRNA is not changed (n=4). (*p<0.05).

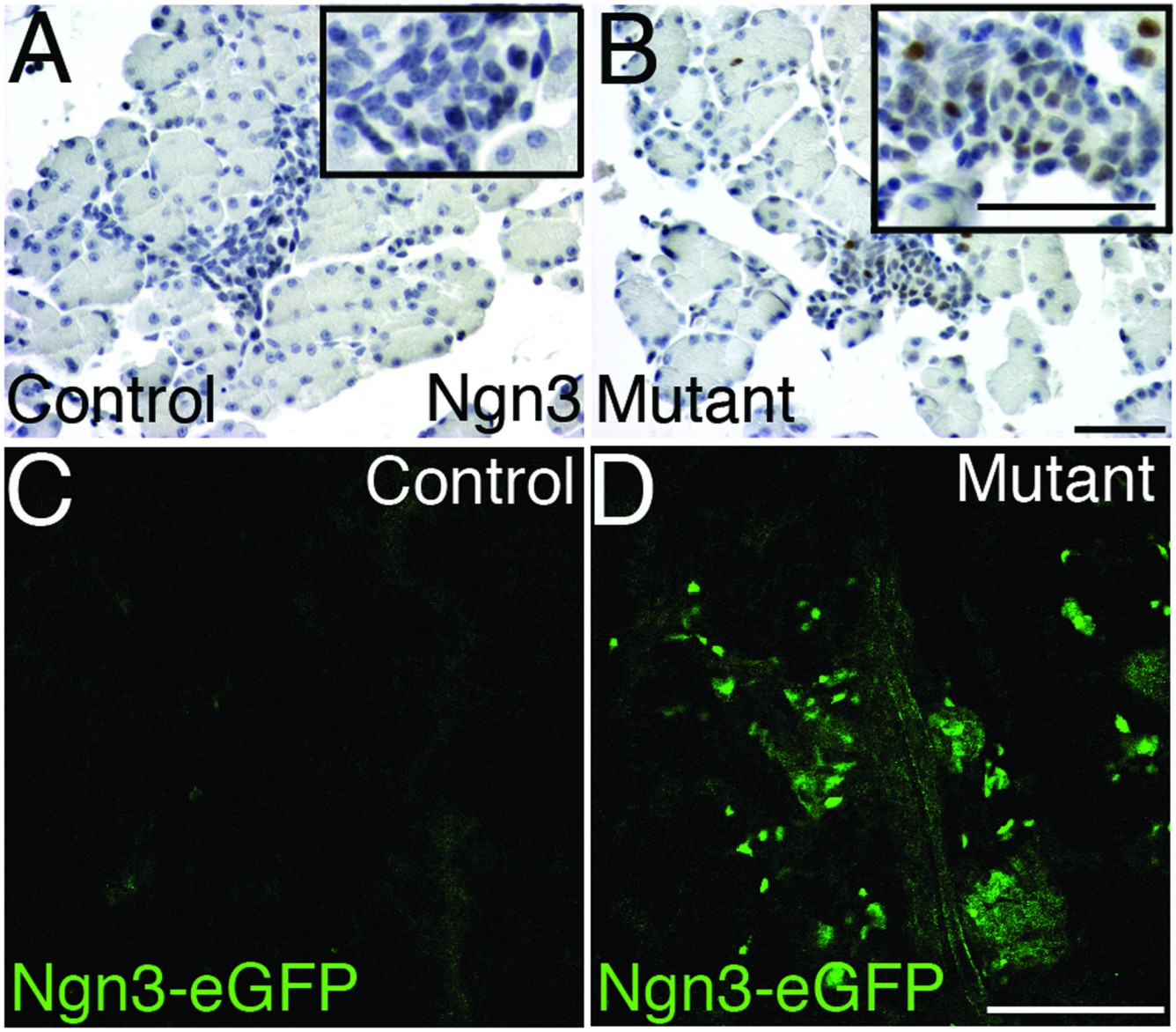

Figure 4.

Ngn3-eGFP is upregulated and *Hes3* mRNA downregulated in *Jag1* mutants. (A–B) At P0, brown Ngn3 staining is detected in only a few cells in control (A) but in many mutant cells (B). (C–D) Ngn3-eGFP expression is not detected in P7 control (C) but many $eGFP^{+}$ cells can be detected in *Jag1* deficient pancreas (D) (bars = 50 μm).

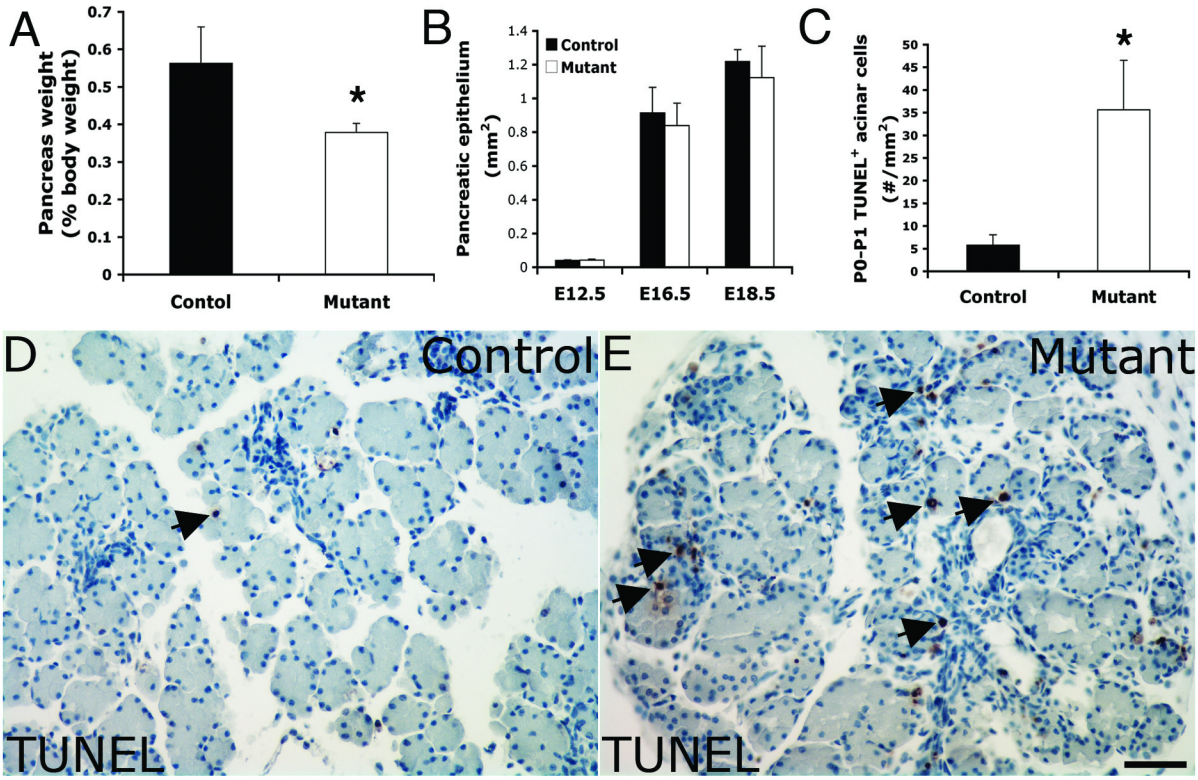

Figure 5.

Acinar tissue is reduced due to postnatal cell death. (A) Pancreas weight is decreased at P21. (B) Total pancreatic epithelium is unchanged at E12.5, E16.5, or E18.5. (C–E) Acinar cell death, marked by TUNEL staining, is increased by approximately 6-fold in P0-P1 *Jag1* mutants. (C) Quantification of TUNEL$^+$ acinar cells as number of cells per acinar area (n=6–10). (D–E) Representative images of TUNEL staining for control (D) and mutant (E) pancreas. Arrows designate TUNEL-positive cells. (bars = 50 μm).

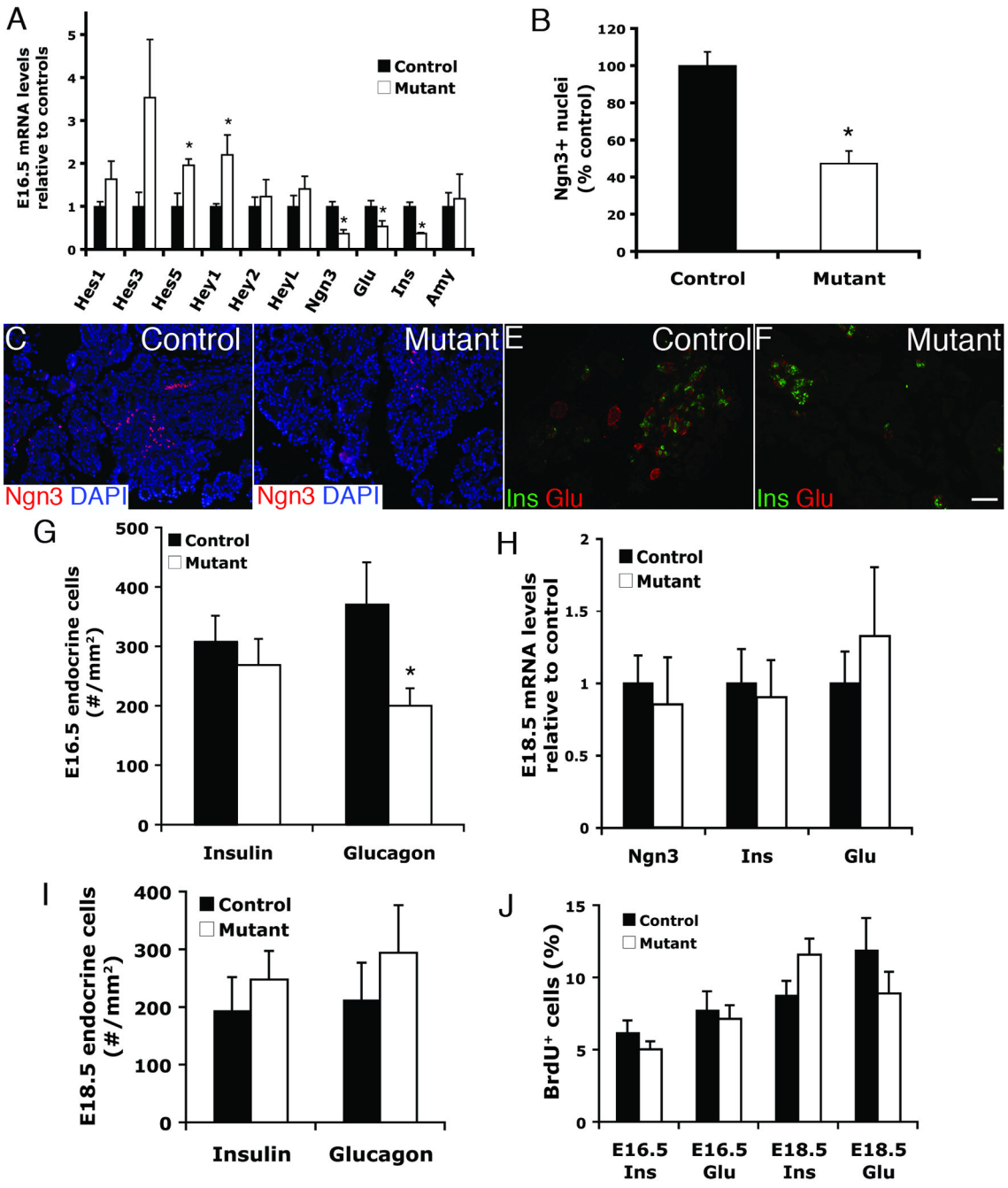

Figure 6.

Paradoxical reduction of the endocrine lineage in E16.5 *Jag1* mutant embryos. (A) qRT-PCR on E16.5 total pancreas mRNA. mRNA of transcriptional targets of Notch is increased and that for endocrine markers is reduced in E16.5 Jag1 mutants. (n=4; for Hes3, p=0.065). (B) Quantification of percentage of nuclei positive for $Ngn3^{+}$ (representative images shown in C&D) indicates a smaller percentage of endocrine precursors within Jag1 mutants at E16.5 (n=4–5). (C,D) Representative images of indirect immunofluorescence detection of Ngn3 with DAPI counterstaining in control (C) and mutant (D) pancreas. The red signal in the upper righthand corner of the image is due to autofluorescent blood cells. (E–F) Indirect immunofluorscence of insulin and glucagon in control (E) and mutant (F) pancreas. (G)

Quantification of insulin$^+$ and glucagon$^+$ cells as cells per square millimeter. Glucagon$^+$ cells are reduced by approximately one-half in *Jag1* mutants (n=10–14). (H) qRT-PCR for *ngn3, ins, and glu* mRNA shows that *Jag1* mutants begin to recover endocrine mass by E18.5 (n=4) (I) Quantification of E18.5 α-and β-cells confirms that endocrine mass starts to recover by E18.5 (n=6). (J) Recovery of endocrine mass does not occur by proliferation of α- or β-cells (n=5–10). (*$p<0.05$; bar = 50 μm).

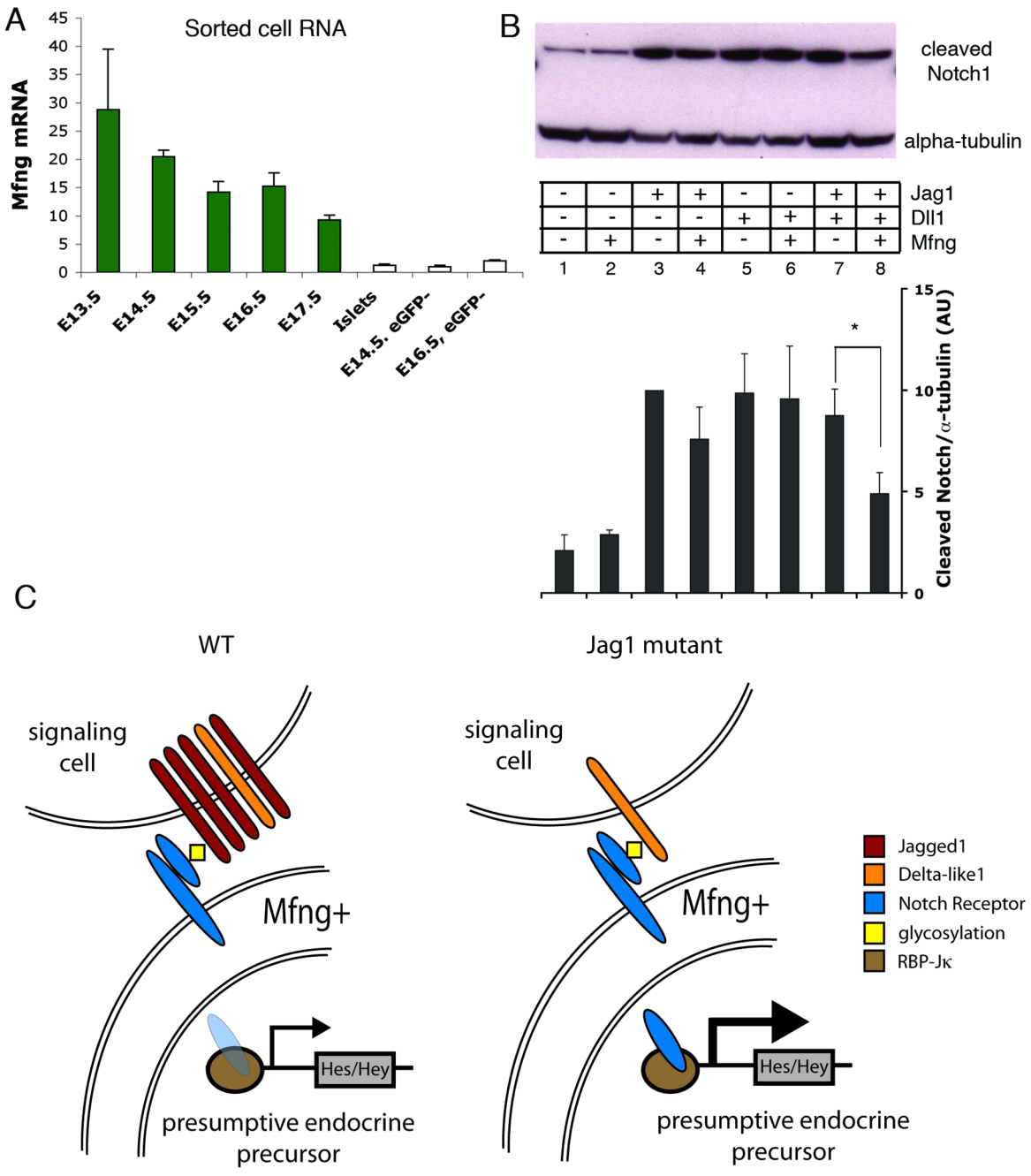

Figure 7.

Manic Fringe is expressed exclusively in endocrine precursors of the fetal pancreas and causes Jag1 to act as a competitive inhibitor of Dll1. (A) In the pancreas, *Mfng* expression is limited to cells that express *Ngn3-eGFP*, i.e. endocrine precursors and their immediate descendants. Mfng expression was normalized to the mean of four housekeeping genes (TBP, GAPDH, HPRT, UBC) (B) Jag1 acts as a competitive inhibitor of Dll1-Notch signaling in NIH 3T3 cells expressing Manic Fringe. Representative Western blots and quantification (n=3 for lanes 1–2 and n=5) for Lanes 3–8). Control cells (lanes 1, 3, 5 & 7) or cells expressing Mfng (lanes 2, 4, 6 & 8) were plated on Jag1 ligand (lanes 3–4), Dll1 ligand (lanes 5–6), or a combination of both (lanes 7–8) and Notch activation was measured by immunoblotting for cleaved Notch1.

All ligands activated Notch cleavage compared to uninduced cells (lanes 1&2), and the combination of Dll1, Jag1, and Mfng yielded low levels of Notch activation (*$p<0.05$). (C) Model: Manic Fringe promotes endocrine differentiation by causing Jag1 to act as a competitive inhibitor of Dll1 during pancreatogenesis. When *Mfng* expression is high, resulting in high levels of Notch glycosylation, Jag1 binds to but does not activate Notch, thus acting as a competitive inhibitor of Dll1. In this situation, Notch signaling decreases, *Hes/Hey* gene expression decreases, and Ngn3 is expressed. However, in Jag1 mutants, Dll1 has increased access to the Notch receptor, and glycosylation potentiates the cleavage of Notch when bound by Dll1, leading to relatively high Notch activation in cells that express *Mfng*. Active Notch signaling represses *Ngn3* expression, resulting in fewer endocrine precursors and differentiated endocrine cells.