The ATPase activity of Asna1/TRC40 is required for pancreatic progenitor cell survival

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ABSTRACT

Asna1, also known as TRC40, is implicated in delivery of tail anchored (TA) proteins into the ER, in vesicle-mediated transport, and in chaperoning unfolded proteins during oxidative stress/ATP depletion. We recently showed that Asna1 inactivation in β-cells resulted in impaired retrograde transport, ER stress and diabetes in mice. Here we show that Asna1 inactivation in pancreatic progenitor cells leads to redistribution of the Golgi TA SNAREs Syntaxin-5 and Syntaxin-6, Golgi fragmentation, and accumulation of cytosolic p62+ puncta. Asna1−/− multipotent progenitor cells (MPCs) selectively activate integrated stress response signalling and undergo apoptosis, thereby disrupting endocrine and acinar cell differentiation, resulting in pancreatic agenesis. Rescue experiments implicate the Asna1 ATPase activity and CXXC di-cysteine motif in ensuring Golgi integrity, Syntaxin-5 localization and MPC survival. Ex vivo inhibition of retrograde transport reproduces the perturbed Golgi morphology and Syntaxin-5 and Syntaxin-6 expression, whereas modulation of p53 activity using PFT-α and Nutlin-3, prevents or reproduces apoptosis in Asna1 deficient and wildtype MPCs, respectively. These findings support a role for the Asna1 ATPase activity in ensuring survival of pancreatic MPCs, possibly by counteracting p53 mediated apoptosis.
INTRODUCTION

Asna1, also known as TRC40, and its yeast homolog GET3 has been implicated in a variety of cellular processes including proteasome assembly and degradation of ubiquitinated proteins, growth under oxidative or metal stress, membrane trafficking within the secretory pathway and insulin secretion (Akahane et al., 2013; Auld et al., 2006; Costanzo et al., 2010; Jonikas et al., 2009; Norlin et al., 2016; Schuldiner et al., 2005; Shen et al., 2003). Many of these phenotypes have been attributed to the ability of Asna1/GET3 to mediate the ATP dependent delivery of tail anchored (TA) proteins for post-translational insertion into the ER via the CAML/WRB receptor complex, a pathway that has been delineated in cell free systems (Favaloro et al., 2008; Johnson et al., 2013; Schuldiner et al., 2008; Stefanovic and Hegde, 2007). The physiological range of TA-protein clients that critically rely on Asna1/GET3 for their membrane insertion may, however, be limited (Norlin et al., 2016; Rivera-Monroy et al., 2016). In addition to its ATPase dependent functions, studies in yeast have suggested an additional role of Asna1/GET3 as a holdase chaperone under conditions of oxidative stress and ATP depletion (Powis et al., 2013; Voth et al., 2014). The shift from an ATPase dependent function to the holdase function is associated with a structural reorganization of Asna1/GET3 from a dimer to a tetramer and involves the oxidation of two di-cysteine motifs (Voth et al., 2014). In Get3 yeast mutants, the holdase function can be selectively rescued by Asna1/Get3 constructs that carry mutations in the ATPase domain or hydrophobic groove, i.e. domains that mediates TA-protein insertion (Voth et al., 2014), suggesting that the part of Asna1 that ensures the holdase function is distinct from that required for the ATPase dependent and TA-targeting activities. In Asna1 deficient C.elegans, re-expression of Asna1 mutated in the CXXC di-cysteine motif rescues the severe growth phenotype displayed by worms lacking
Asna1 but not the sensitivity to cisplatin, an oxidative stress inducing drug (Hemmingsson et al., 2010), suggesting that Asna1 performs multiple functions also in higher eukaryotes. The relative contribution of these functions in mammalian cells in vivo remains, however, unknown.

Through conditional inactivation of Asna1 in insulin producing β-cells of mice we recently demonstrated a role for Asna1 in ensuring retrograde transport and thereby ER homeostasis and insulin biosynthesis in β-cells (Norlin et al., 2016). Notably, the proposed Asna1 target TA-proteins Syntaxin-5 (Stx5) and Syntaxin-6 (Stx6) were redistributed from their Golgi compartments both in Asna1 mutant β-cells and after pharmacological inhibition of retrograde transport using Retro-2 (Norlin et al., 2016; Stechmann et al., 2010). Together these findings suggested a key role for Asna1 in ensuring retrograde transport and Golgi localization of Stx5 and Stx6 in adult β-cells. To gain further insight in the role(s) for Asna1 in mammalian cells in vivo, we have here conditionally inactivated Asna1 in pancreatic progenitor cells.

Pancreatic development is initiated as two evaginations from the primitive gut epithelia. Over time, the specified pancreatic epithelia grow into the surrounding mesenchyme and form a tubular epithelium that undergo extensive branching morphogenesis. Mouse pancreatic progenitor cells undergo two major rounds of differentiation (Shih et al., 2013). During the early phase between E9-12 (i.e. 1st transition), multipotent progenitor cells (MPCs), capable of generating acinar, endocrine and ductal cell lineages, proliferate and generate a small number of endocrine cells primarily expressing glucagon. During the 2nd transition between E12-14, pancreatic progenitor cells undergo extensive growth and branching
morphogenesis and the initial Ptf1a⁺/Sox9⁺ MPC population segregates into two populations; a branch tip population containing Ptf1a₅/Sox9Low proacinar cells, and a bipotential Ptf1a⁺/Sox9High branch trunk population containing Ngn3⁺ proendocrine cells and Ngn3⁻ duct progenitor cells (Schaffer et al., 2010; Solar et al., 2009). After E14.5, Ptf1a₅ proacinar tip cells differentiate and initiate expression of mature acinar cell markers, e.g. amylase. In the branch trunks, duct progenitor cells form the pancreatic ducts that connect the acinar cells to the intestine whereas the Ngn3⁺ proendocrine cells migrate into the surrounding mesenchyme and initiate expression of endocrine hormones as they differentiate into α-, β-, δ-, γ-, and ε-cells that eventually form the endocrine islets. Thus, the different cell types in the developing pancreas serves a model for evaluating the requirement for Asna1 in several cellular processes including proliferation, differentiation, morphogenesis, and hormone secretion.

We here show that inactivation of Asna1 in pancreatic progenitor cells results in severe pancreatic agenesis. Loss of Asna1 in pancreatic progenitor cells leads to rapid redistribution of the TA proteins Stx5 and Stx6, followed by perturbed Golgi morphology, apoptotic cell death, and impaired acinar and endocrine cell differentiation from E13 onwards. In contrast, Asna1 deficient Sox9⁺ duct/MPC–like cells, as well as differentiated α-cells and β-cells specified at earlier stages of pancreatic development, survive. Apoptotic cell death in embryonic Asna1 deficient pancreatic epithelium correlates with an increase in integrated stress response (ISR) signaling whereas the adaptive UPR and oxidative stress responsive pathways appear unaltered. Pharmacological inhibition of retrograde transport reproduces the redistribution of Stx5 and Stx6 and the fragmentation of the Golgi, but is insufficient
to provoke substantial apoptosis and impaired acinar and endocrine cell differentiation. Re-introduction of an ATPase deficient mutant of Asna1 failed to restore Golgi integrity and differentiation of pancreatic progenitor cells lacking Asna1, suggesting that the ATPase dependent and TA-targeting activities of Asna1 are required for pancreatic progenitor cell survival.

RESULTS

Deletion of Asna1 in pancreatic progenitor cells leads to severe pancreatic hypoplasia due to apoptosis

Asna1 was broadly expressed in the developing pancreatic epithelium from ~E10.5 and by E13.5 the expression became prominent in the pro-acinar branch tip cells (Fig. S1A). To elucidate a potential functional role of Asna1 in mouse pancreatic progenitor cells in vivo, we crossed Asna1flx/flx mice (Norlin et al., 2016) with Ipf1-nlsCre mice (Svensson et al., 2009) yielding Asna1flx/flx;Ipf1-nlsCre+ mice (denoted Asna1Panc−/−) (Fig. S1B). Ipf1-nlsCre ensures pancreas and duodenal specific Cre-mediated recombination in Rosa26loxP-stop-loxPLacZ reporter mice (Soriano, 1999) as early as E10.5 (Fig. S1C) and in agreement with this, qRT PCR analysis revealed 68% reduction of Asna1 expression in pancreatic epithelium of Asna1Panc−/− embryos at E11.5 (Fig. S1D). Asna1PancWT and Asna1Panc+/− embryos did not show any apparent phenotype at any stage examined and were thus used as controls, collectively denoted Asna1Panc:Ctrl. The resulting Asna1Panc−/− mice were born alive but died soon after birth due to severe pancreatic and duodenal agenesis (Fig. 1A).

To identify the cause of the pancreatic agenesis in Asna1Panc−/− embryos, we next characterized the developing dorsal pancreatic epithelia (DPE) in more detail. X-gal staining of Asna1Panc−/−;Rosa26loxP-stop-loxPLacZ embryos revealed that severe dorsal and
ventral pancreatic agenesis and reduced branching complexity was evident already by E15.5 (Fig. 1B). Analyses of earlier developmental stages showed that the size of Asna1Panc-/ DPE was normal at E12.5, but reduced by 28% at E13.5 (Fig. 1C). Moreover, the rapid expansion of the pancreatic epithelia area of control mice between E13.5-E15.5 was severely impaired in the Asna1Panc-/ DPE (Fig. 1C). Notably, the growth defect of Asna1Panc-/ DPE coincided with the prominent appearance of apoptotic cells at E13.5, preferentially in branch tips along the epithelial/mesenchymal border of the Asna1Panc-/ DPE (cleaved Caspase3+ cells in Fig. 1D, E and tunel assay+ cells in Fig. S2A). In contrast, proliferation, as judged by the amount of phospho-H3 (pH3) positive cells in the Asna1Panc-/ DPE, was unaffected at E13.5. (Fig. 1F). At E15.5 apoptosis was still drastically increased in Asna1Panc-/ DPE as compared with Asna1Panc:Ctrl DPE and in addition proliferation was reduced in Asna1Panc-/ DPE at this stage (Fig. 1E,F). Apoptosis was also evident in E13.5 Asna1Panc-/ ventral pancreatic and E14.5 Asna1Panc-/ duodenal epithelia (Fig. S2B). These data show that Asna1Panc-/ mice develop pancreatic and duodenal epithelial hypoplasia as a consequence of apoptosis.

**Loss of Asna1 impairs pancreatic acinar, endocrine, and ductal cell lineages**

To identify the pancreatic cell types that undergo apoptosis in Asna1Panc-/ DPE, we next analyzed the expression of cell specification and differentiation markers in E12.5-E16.5 Asna1Panc-/ DPE. Cells in the branch trunk regions of E12.5 Asna1Panc-/ DPE showed normal Sox9 expression characteristic of endocrine and ductal progenitor cells (Fig. 2A). Moreover, like that of control littermates, branch tips of Asna1Panc-/ DPE contained a subset of cells that showed Ptf1aHigh expression characteristic of pro-acinar cells, and Ptf1aLow and Carboxypeptidase A+ (CPA+) cells
that may represent Ptf1a\textsubscript{Low}/Sox9\textsuperscript{+}/CPA\textsuperscript{+} MPCs (Pan et al., 2013; Zhou et al., 2007) (Fig. 2A). Ngn3\textsuperscript{+} endocrine progenitor cells and differentiated glucagon\textsuperscript{+} α-cells were dispersed within and around the branch trunk region (Fig. 2A) and quantification revealed no significant difference in the numbers of these cells at E12.5, i.e. prior to the onset of apoptosis, when comparing \textit{Asna1\textsubscript{Panc/-}} and control littermates (Fig. 2B,C). Thus, the initial lineage specification of MPCs, endocrine and ductal progenitor cells, as well as differentiation of α-cells is unaffected in \textit{Asna1\textsubscript{Panc/-}} mice up to E12.5.

Following the onset of apoptosis at E13.5, branching morphogenesis was clearly perturbed by E14.5 onwards (Fig. 2D-F). The expression of Sox9 and Ipf1/Pdx1 appeared unaltered in the bipotential branch trunk progenitors arguing against a role for these transcription factors in mediating impaired morphogenesis and progenitor cell apoptosis in E14.5 \textit{Asna1\textsubscript{Panc/-}} DPE (Fig. 2D). Consistent with the localization of apoptotic cells in branch tips of the E13.5 DPE (Fig. 1D, Fig. S2A), Ptf1a\textsuperscript{+} tip cells appeared reduced in \textit{Asna1\textsubscript{Panc/-}} DPE at E14.5 (Fig. 2D). Moreover, although some Ptf1a\textsubscript{Low} cells remained at E15.5 (Fig. 2E insets), Ptf1a\textsubscript{High} pro-acinar cells were essentially absent (Fig. 2E) and pro-endocrine Ngn3\textsuperscript{+} cells severely reduced at this stage (Fig. 2E,G). Together, these results provide evidence that \textit{Asna1} is required for survival of MPCs and consequently their progression into Ptf1a\textsubscript{High} pro-acinar and Ngn3\textsuperscript{+} pro-endocrine cells. Coherent with this scenario, definitive amylase\textsuperscript{+} acinar cells were essentially absent, and differentiated glucagon\textsuperscript{+} α-cells and insulin\textsuperscript{+} β-cells where significantly reduced in E15.5-E16.5 \textit{Asna1\textsubscript{Panc/-}} DPE (Fig. 2F,H, Fig. S3). Notably, the number of α-cells and β-cells still increased in \textit{Asna1\textsubscript{Panc/-}} DPE between E13.5-E15.5 (Fig. 2H), suggesting that they derive from Ngn3\textsuperscript{+} pro-endocrine cells.
already specified at E13.5. The remaining E16.5 \textit{Asna1}^{Panc-/-} epithelium consisted of proliferating Sox9\textsubscript{Low}/DBA-lectin\textsubscript{Low} cells (Fig. 2F), which survive and differentiate in the absence of \textit{Asna1}. Taken together these data suggest that MPCs and/or their immediate offspring undergo apoptosis from E13.5 onwards in \textit{Asna1}^{Panc-/-} embryos, which impairs the generation of pro-endocrine and pro-acinar cells and consequently the subsequent differentiation of endocrine and acinar cell types.

\textit{Asna1}^{Panc-/-} pancreatic agenesis is associated with p53 activity and integrated stress response signalling.

Mouse genetic models presenting with severe pancreatic agenesis and acinar hypoplasia appear to be preferentially associated with premature differentiation, impaired proliferation or altered specification (Apelqvist et al., 1999; De Vas et al., 2015; Fukuda et al., 2008; Jensen et al., 2000; Krapp et al., 1996; Murtaugh et al., 2005; Nakhai et al., 2008; Papadopoulou and Edlund, 2005; Seymour et al., 2007) and not primarily with apoptosis. However, pancreas specific ablation of DNA methyltransferase 1 (Dnmt1) (Georgia et al., 2013), was shown to provoke pancreatic progenitor cell apoptosis, which involved the de-repression of \textit{p53}, an oncogene that induces cell cycle block or apoptosis in response to cellular stress such as genotoxic and ER stress (Brooks and Gu, 2010; Vogelstein et al., 2000). To investigate a potential role of p53 mediated apoptosis in \textit{Asna1}^{Panc-/-} DPE we modulated p53 activity in explants of \textit{wildtype} (WT) and \textit{Asna1}^{Panc-/-} pancreas. Exposure of E10.5 \textit{Asna1}^{Panc-Ctrl} explants to the p53 inhibitor Pifithrin-\alpha (PFT-\alpha) (Komarov et al., 1999), resulted in reduced Ngn3\textsuperscript{+}, insulin\textsuperscript{+} and amylase\textsuperscript{+} cell area implying a requirement of p53 in \textit{β}-cell and acinar cell differentiation (Fig. 3A, B). Similarly, \textit{β}-cell differentiation was reduced in \textit{Asna1}^{Panc-/-} explants exposed to PFT-\alpha (Fig. 3A, C). In
addition, glucagon⁺ area was also reduced suggesting that p53 may play a role in α-cell survival and/or differentiation in Asna1Panc⁻/⁻ embryos. In contrast however, amylase⁺ cell differentiation was, albeit partially, restored in Asna1Panc⁻/⁻ explants exposed to PFT-α (Fig. 3A, C), suggesting that excess p53 activation may provoke the apoptosis of Asna1Panc⁻/⁻ MPCs. To test this idea we exposed WT E12.5 explants to the p53 agonist Nutlin-3 (Vassilev et al., 2004) (Fig. 3D), which resulted in increased epithelial apoptosis after 24h (Fig. 3E) and a dramatic reduction of epithelial area and Amylase⁺ cells after 3 days (i.e. E15.5) (Fig. 3D,F), whereas insulin⁺ cells appeared unaffected (Fig. 3F). Total p53 protein levels in E13.5 dorsal pancreas buds were however not increased (Fig. S4), suggesting that p53 activity, rather than expression levels, were increased in Asna1Panc⁻/⁻ DPE. Taken together, these results leave open the possibility that apoptosis in Asna1Panc⁻/⁻ DPE, at least in part, is mediated by p53.

Since inactivation of Asna1 in mouse, C. elegans, and yeast has been associated with both ER-stress and increased sensitivity to oxidative stress, we next investigated stress signaling responses in Asna1Panc⁻/⁻ embryonic pancreatic epithelium. Several cellular stress responses converge on the phosphorylation of eIF2α that, together with its downstream effector genes, collectively are referred to as the integrated stress response (ISR) pathway. qRT-PCR analysis of Asna1Panc⁻/⁻ dorsal pancreatic buds, i.e. pancreatic epithelia and surrounding mesenchyme, revealed that the expression of genes associated with the ISR, such as ATF4 and its target genes Chop10, ATF3 and Trib3, was increased at E13.5 (Fig. 3G). Additionally, immunohistochemical analyses revealed strong nuclear expression of ATF4 in tip cells of Asna1Panc⁻/⁻ DPE at E13.5, but not at E12.5 (Fig. 3H), thus correlating with the appearance of apoptotic
progenitor cells (Fig. 1D). Taken together, these results suggest that ISR signaling and apoptosis occur in a spatially restricted subset of $Asna1^{Panc-/-}$ DPE cells.

The expression of oxidative stress genes, e.g. $Nrf2$ and its target genes $Nqo1$ and $HO-1$, appeared unaltered at E13.5 (Fig. 3I). Although the expression of the UPR regulated gene $Herpud1$, which encodes a component of the ER-associated degradation (ERAD) machinery, was upregulated in $Asna1^{Panc-/-}$ DPE, the expression of genes associated with the adaptive UPR, encoding chaperones ($DnaJc3, Hsp90b1$ [i.e. $Grp94$] and $BiP$) and oxidative folding enzymes ($Pdia4$) rather showed a tendency to be reduced in E13.5 pancreatic buds (Fig. 3I), arguing against ER-stress as the underlying cause for apoptosis observed in $Asna1^{Panc-/-}$ DPE. Together, these data suggest that in $Asna1^{Panc-/-}$ DPE tip cells, the ISR activates a program, possibly involving $p53$, which leads to MPC apoptosis and subsequent pancreatic agenesis.

**Redistribution of Stx5 and Stx6 precedes Golgi fragmentation and apoptosis in $Asna1^{Panc-/-}$ pancreatic and duodenal epithelia.**

We next tried to establish a connection between the previously proposed functions of $Asna1$, ISR signaling, and pancreatic progenitor cell apoptosis. Since $Asna1$ is implicated in the targeting of TA proteins to the ER-membrane, we first analyzed the structural integrity of endomembrane. Since apoptosis itself is associated with the breakdown of cellular structures, we analyzed the structural integrity of endomembrane compartments in $Asna1^{Panc-/-}$ DPE at E12.5, i.e. one day prior to the onset of apoptosis. Immunohistochemical analyses using markers for the ER (KDEL), endoplasmic reticulum Golgi-intermediate compartment (ERGIC53), endosome (EEA1) and lysosome (Lamp1) revealed that these compartments appeared
structurally indistinguishable from that of controls (Fig. S5A). However, analyses using markers for the cis-Golgi (Gm130) and trans-Golgi network (TGN) (TGN46) showed that these markers were uncharacteristically dispersed in the cytoplasm of cells throughout the \( Asna1^{Panc-/} \) DPE (Fig. 4A). Transmission electron microscopy (TEM) revealed the presence of small distended membrane stacks in \( Asna1^{Panc-/} \) DPE as opposed to the thin multilayered membrane stacks characteristic of the cis- and medial Golgi compartment observed in control littermates (Fig. 4B). No apparent difference was, however, observed in ER or mitochondrial morphology at E12.5 (Fig. S5B). Together, these results show that cis-Golgi and TGN morphology is perturbed in cells throughout the \( Asna1^{Panc-/} \) DPE prior to apoptosis.

Loss of Asna1/GET3 function in both yeast and mouse is associated with altered subcellular localization of the TA-SNAREs Stx5/Sed5 and Stx6, which undergo a striking re-localization from the cis-Golgi and TGN respectively (Jonikas et al., 2009; Norlin et al., 2016; Schuldiner et al., 2008). In \( Asna1^{Panc-/} \) DPE at E11.5, both Golgi integrity as well as Stx5 and Stx6 expression was normal (Fig. 4C). However, at E12.5, Stx5 and Stx6 immunoreactivity was severely reduced and virtually absent in the fragmented cis-Golgi and TGN compartments of \( Asna1^{Panc-/} \) DPE (Fig. 4D). Total Stx5 protein levels were however unaltered in E12.5 pancreas buds (Fig. S5C), suggesting that, alike Asna1 deficient β-cells (Norlin et al., 2016), Stx5 protein become redistributed rather than downregulated in \( Asna1^{Panc-/} \) DPE. An intermediate phenotype was observed in embryos carrying a germline \( Asna1^{null} \) allele in combination with a conditionally inactivated \( Asna1^{flox} \) allele, denoted \( Asna1^{null/Panc-} \), in which Stx5 and Stx6 immunoreactivity was perturbed already at E11.5, whereas Golgi compartments appeared intact (Fig. 4E). Nonetheless, this earlier depletion of
Asna1 in \( Asna1^{null/Panc-} \) embryos was insufficient to provoke an earlier onset of increased apoptosis at E12.5 (Fig. S5D). Taken together, these results provide evidence that redistribution of Stx5 and Stx6 precedes Golgi fragmentation, which in turn is followed by overt apoptosis at E13.5. Moreover, similar changes in Golgi morphology and syntaxin distribution preceded apoptosis also in the ventral pancreas and the duodenum epithelia (Fig. S5E,F), suggesting a general \( Asna1 \) dependent function(s) in endodermal progenitor cells.

We next investigated whether Stx5 redistribution and Golgi fragmentation was retained in surviving cell types at E16.5. The Sox9\(^{low} / DBA-lectin^{low} \) duct-like cells, \( \beta \)-cells, and most \( \alpha \)-cells all showed redistribution of Stx5 and fragmented Golgi compartments (Fig. 4F), suggesting that Stx5 redistribution and Golgi fragmentation does not affect cell survival of these cell. Notably, since the \( Ipf1-nlsCre \) transgene is inactive in \( \alpha \)-cells, these results further suggest that most \( \alpha \)-cells are derived from \( Asna1 \) deficient Ngn3\(^+ \) pro-endocrine cells. However, a minor fraction of the \( \alpha \)-cells showed intact Golgi structure and Stx5 localization (data not shown) suggesting that they derive from the early pool of \( \alpha \)-cells that are generated prior to E11.5, and may thus have escaped \( Ipf1-nlsCre \) mediated inactivation of \( Asna1 \). Finally, treatment of \( Asna1^{Panc-/-} \) explants with the p53 inhibitor PFT-\( \alpha \) did not restore Stx5 distribution in epithelial cells, including the rescued amylase\(^+ \) cells (Fig. 4G), suggesting that p53 inhibition did not prevent apoptosis by restoring Stx5 localization or by interfering with CRE-mediated inactivation of \( Asna1 \). However, the integrity of the Golgi compartment in the rescued amylase\(^+ \) cells appeared partially restored (Fig. 4G). These results demonstrate that \( Asna1 \) inactivation, Stx5 redistribution, and Golgi...
fragmentation is tolerated by most cell types in the developing pancreas, thus highlighting the selective sensitivity of MPCs.

**p62** protein aggregates accumulate in *Asna1*<sup>Panc-/−</sup> DPE.

Apart from a role in TA-protein targeting, endomembrane transport and ER homeostasis, Asna1/GET3 is also implicated as a redox regulated holdase chaperone during oxidative stress and ATP depletion (Powis et al., 2013; Voth et al., 2014). Thus, one consequence of *Asna1* inactivation might be the aggregation of misfolded TA, as has been suggested for Stx5 (Rivera-Monroy et al., 2016), and/or non-TA Asna1 target proteins, which may be potentially cytotoxic. In keeping with this idea, the E13.5 pancreatic epithelium showed increased numbers of distinct puncta positive for p62 (Fig. 5A), an ubiquitin-like protein conjugate that targets proteins for autophagic degradation. These results suggest that protein inclusions destined for proteasomal and/or autophagic degradation accumulates in *Asna1*<sup>Panc-/−</sup> DPE at the time of apoptotic onset. An alternative explanation could be that autophagic degradation *per se* is impaired in *Asna1*<sup>Panc-/−</sup> DPE as autophagy is dependent on vesicular transport. To separate these possibilities, we first investigated whether autophagic degradation was decreased by analyzing the accumulation of LC3<sup>+</sup> autophagosomes in GFP-LC3 reporter mice on an *Asna1*<sup>Panc-/−</sup> background. Although occasional cells exhibited increased GFP-LC3 levels, a general accumulation in LC3-GFP levels was not observed in *Asna1*<sup>Panc-/−</sup> DPE (Fig. 5B). Next, we specifically inactivated autophagy in the developing pancreas by generating *Atg5*<sup>flou/flox</sup>, *Ipfl-nlsCre* mice (denoted *Atg5*<sup>Panc-/−</sup>). At E14.5, *Atg5*<sup>Panc-/−</sup> DPE exhibited an increase in cytosolic p62 protein in the pancreatic trunk epithelia but not in pro-acinar tip cells (Fig. 5C insets). Notably, no corresponding increase in p62<sup>+</sup> puncta or apoptosis was
observed in any part of the Atg5Panc−/− DPE (Fig. 5C). Taken together, these results strongly argue against decreased autophagic degradation as the primary cause for the accumulation of p62^+ puncta in Asna1Panc−/− cells. Instead, it appears likely that these p62^+ puncta consists of aggregating Asna1 client TA- or non TA-proteins, which in turn may contribute to apoptotic cell death.

**Progenitor cell survival and exocrine cell differentiation depend on the ATPase activity of Asna1.**

Rescue experiments in yeast have demonstrated that both the ATPase activity and the TA binding ability are required for the TA-targeting function of GET3/Asna1 as defined by the Golgi localization of Sed5/Stx5, but not for growth under oxidative stress (Voth et al., 2014). The GET3/Asna1 holdase activity is associated with a tetramer configuration and involves the formation of di-sulfide bonds within two di-cysteine motifs, CXC(246-248) and CXXC(285-288) (Voth et al., 2014). Accordingly, the CXXC motif is required for growth under oxidative stress in yeast (Metz et al., 2006). Moreover, in C. elegans, the CXXC motif of Asna1 is required for resistance to the oxidative stress inducing drug cisplatin, but not for rescuing the growth defect of Asna1 mutant worms (Hemmingsson et al., 2010). *In vitro*, mutations in the CXXC(285-288) motif affects GET3/Asna1 homodimerisation, TA-protein binding and ATPase activity (Mateja et al., 2009; Metz et al., 2006), suggesting a possible dual role for the CXXC(285-288) motif in both configurations of Asna1. The ability of the Asna1 CXXC(285-288) mutant to restore Stx5 localization *in vivo* has, however, not been directly tested. The more N-terminal CXC(246-248) motif is not required for dimer formation *in vitro* or for growth under oxidative stress conditions (Metz et al., 2006). To explore which of Asna1’s activities,
i.e. the ATPase dependent chaperone (TA-targeting) or holdase function, that is required for pancreatic progenitor survival and differentiation, we next used a lentivirus delivery system to reintroduce Asna1 versions carrying mutations in different functional domains into Asna1<sup>Panc-/-</sup> DPE. E11.5 Asna1<sup>Panc-/-</sup> pancreatic explants infected with empty lentivirus control vector and cultivated 5 days did not show any evidence of exocrine differentiation, the Golgi compartments were fragmented, and Stx5 expression was faint (Fig. 6A), thus reproducing the phenotypes of E16.5 Asna1<sup>Panc-/-</sup> DPE.

Infection of Asna1<sup>Panc-/-</sup> pancreatic explants with Asna1(WT) construct partly restored acinar cell differentiation as well as Stx5 expression in the Golgi (Fig. 6A,B). Similar results were obtained when re-expressing the Asna1(C246S-X-C248S) mutant (Fig. 6A,B). In contrast, an Asna1(D74N) construct, that carry a mutation in the ATPase domain, failed to restore Golgi morphology, Stx5 expression, and acinar cell differentiation (Fig. 6A,B). Similar to the Asna1(D74N) construct, re-expression of Asna1(C285S-XX-C288S) failed to rescue the Asna1 deficiency in E11.5 Asna1<sup>Panc-/-</sup> pancreatic explants (Fig. 6A,B). These results suggest that, in agreement with its proposed role in the dimerization of Asna1 (Mateja et al., 2009; Metz et al., 2006), the CXXC(285-288) motif also affects the ATPase dependent activities of the Asna1 dimer, and thus cannot be used to assess the contribution of a putative ATPase independent holdase function of Asna1 in Asna1<sup>Panc-/-</sup> mice. Thus, these data show that the ATPase dependent chaperone functions of Asna1 is required for ensuring both Stx5 expression/localization as well as survival of pancreatic progenitor cells from E13.5 onwards.
Inhibition of retrograde transport in pancreatic progenitor cells mimics Stx5 redistribution and Golgi fragmentation but does not provoke apoptosis.

The loss of ATPase/CXXC dependent activities in \( \text{Asna1}^{\text{Panc}-/-} \) mice is likely to affect an array of cellular processes including vesicle-mediated transport of various protein cargo. In yeast, the GET complex, including GET3/Asna1, genetically interacts with retrograde transport pathways (Jonikas et al., 2009; Schuldiner et al., 2005) and ß-cell specific inactivation of \( \text{Asna1} \) result in impaired endosome (EE)-to-TGN as well as COPI independent Golgi-to-ER transport (Norlin et al., 2016). Thus, we next investigated whether inhibition of retrograde transport with the small molecule inhibitor Retro-2 (Norlin et al., 2016; Stechmann et al., 2010) could reproduce \( \text{Asna1}^{\text{Panc}-/-} \) phenotypes. Exposure of E11.5 wild type (wt) dorsal pancreatic bud explants to Retro-2 for 48h resulted in reduced Stx5 and Stx6 expression in the cis-Golgi and TGN compartments (Fig. 7A). In analogy with \( \text{Asna1}^{\text{Panc}-/-} \) DPE, the cis-Golgi of Retro-2 treated WT DPE appeared partly fragmented and the TGN more diffuse (Fig. 7A insets). However, Retro-2 exposure did not induce prominent ATF4 expression in DPE explants (Fig. 7B) and extended exposure of E10.5 WT DPE explants to Retro-2 for 5 days (i.e equivalent to E15.5) did not significantly affect epithelial size or the differentiation of endocrine or acinar cell lineages (Fig. 7C and D), although it did provoke apoptosis in both epithelial and mesenchymal cells (Fig. 7E). Taken together, these data show that inhibition of retrograde transport by Retro-2 is sufficient to reproduce the redistribution of Stx5 and Stx6 and the fragmentation of the Golgi compartments but not the loss of acinar and endocrine cell types observed in \( \text{Asna1}^{\text{Panc}-/-} \) DPE.
DISCUSSION

Here we show that inactivation of Asna1 in pancreatic progenitor cells leads to selective apoptosis of multipotent progenitor cells, thereby depleting the pancreatic progenitor cell pool and perturbing subsequent growth and differentiation of the developing pancreatic epithelium, which ultimately results in severe pancreatic agenesis. Embryonic pancreatic epithelial cells lacking Asna1 exhibited perturbed localization of Stx5 and Stx6 in the cis-Golgi and TGN compartments respectively, aberrant Golgi morphology, activation of the ISR, and accumulation of cytosolic p62+ protein aggregates. Finally, we demonstrate that the ATPase activity of Asna1 is required to restore Golgi morphology and Stx5 expression as well as progenitor cell survival.

The most striking effect of Asna1 deficiency in pancreatic progenitor cells is the severe pancreatic agenesis that appears to be caused by selective apoptosis of multipotent progenitor cells at E13.5. However, the reduced pancreatic epithelial cell proliferation observed at E15.5, which likely is secondary to the apoptosis of MPC at E13.5, might also contribute to the pancreatic hypoplasia of Asna1Panc−/− mice. Whereas Asna1 seems to be functionally depleted throughout the E12.5 Asna1Panc−/− DPE, as indicated by perturbed Golgi morphology and Stx5 expression, the first signs of apoptosis and increased ISR signaling, i.e. ATF4 expression, is observed one day later and is primarily evident in the branch tips of the growing pancreatic epithelium that contain MPCs for both acinar and endocrine cell linages, thus providing an explanation for the perturbed endocrine and acinar cell differentiation in Asna1Panc−/− pancreatic epithelium beyond E13.5 (Zhou et al., 2007). Moreover, the few remaining endocrine cells observed in Asna1Panc−/− embryos do not appear to escape
recombination since Stx5 and Stx6 are mis-localized also in these cells, providing evidence that loss of Asna1 per se does not affect survival of already specified lineage specific progenitor cells nor their subsequent differentiation. Thus, the severe pancreatic hypoplasia observed in Asna1Panc-/- embryos appears to be the consequence of massive cell death within the domain of multipotent progenitor cells or their immediate offspring from E13.5 onwards.

The Asna1/GET3 pathway has been implicated in multiple functions in yeast and mammalian cells, including that of i) an ATPase dependent chaperone function required for Sed5/Stx5 localization to the Golgi (Schuldiner et al., 2008; Voth et al., 2014), ii) an ATP independent holdase chaperone function that supports growth under conditions of oxidative/metal stress (Powis et al., 2013; Voth et al., 2014), and iii) a proteostatic quality control network ensuring cellular proteostasis (Akahane et al., 2013; Auld et al., 2006). Accordingly, Asna1Panc-/- pancreatic progenitor cells display reduced Stx5 expression in the Golgi, which may be attributed to the loss of ATPase dependent chaperone activity of Asna1, and increased prevalence of cytosolic p62^+ puncta, which may be interpreted as an accumulation of protein aggregates in the absence of the holdase activity of Asna1 and/or due to the proposed role for Asna1 in ensuring cellular proteostasis. Notably, our results clearly demonstrate that the ATPase activity of Asna1 is required for restoring Golgi morphology and Stx5 and Stx6 expression as well as acinar cell differentiation, whereas a putative holdase function, which should be retained in the Asna1(D74N) mutant (Voth et al., 2014), is unable to rescue these phenotypes. We further find that the CXXC(285-288) motif, which is predicted to affect the holdase function (Hemmingsson et al., 2010; Metz et al., 2006), also interferes with the ATPase dependent Stx5 localization, presumably
by affecting dimerization of Asna1/GET3 (Metz et al., 2006), thus precluding a direct test of the putative role for the holdase function of Asna1.

The redistribution of Stx5/Sed5 is a common hallmark phenotype observed in yeast GET3 mutants, Asna1 deficient pancreatic progenitor and β-cells, as well as in cardiomyocytes and hepatocytes in which the Asna1/TRC40 receptor WRB was disrupted, and thus indicates the functional loss of the ATPase dependent GET3/Asna1/TRC40 pathway activity (Norlin et al., 2016; Powis et al., 2013; Rivera-Monroy et al., 2016; Schuldiner et al., 2008; Voth et al., 2014). The notion that membrane integration of Stx5, as well as that of other TA-proteins, is directly mediated by the ATPase dependent function of Asna1/GET3 relies heavily on studies in cell free systems whereas in vivo analysis show that many TA-proteins are not obligatory dependent on Asna1/GET3 for their membrane insertion (Norlin et al., 2016; Rivera-Monroy et al., 2016; Schuldiner et al., 2008). However, the ability of Retro-2 to phenocopy the redistribution of Stx5 and Stx6, raises the possibility that these Golgi syntaxins are appropriately inserted into the ER membrane in Asna1 deficient cells but subsequently redistributed from the Golgi due to impaired vesicle transport. These two explanations are not mutually exclusive and additional experiments are required to fully resolve the role of Asna1 and retrograde transport for the localization of Stx5 and Stx6.

Regardless of the exact mechanism, several phenotypes of Asna1 deficient pancreatic progenitors and β-cells can be attributed to perturbed Retro-2 sensitive retrograde transport. First, as mentioned above, the redistribution of Stx5 and Stx6 can be reproduced by exposure of both islets (Norlin et al., 2016) and pancreatic explants to
Retro-2. Second, impaired COPI independent Golgi-to-ER retrograde transport and increased UPR signaling in Asna1 deficient β-cells is recapitulated by treatment with Retro-2 (Norlin et al., 2016). Finally, Golgi fragmentation, which is observed in progenitor cells but not in mature β-cells, is selectively reproduced by Retro-2 in progenitor cells but not in β-cells, further emphasizing the overlap between Retro-2 sensitive- and Asna1 dependent processes, albeit in an apparent cell context specific manner. In contrast, the induction of ISR signaling and the apoptosis observed in Asna1<sup>Panc</sup>−/− MPCs cannot easily be attributed exclusively to impaired Retro-2 sensitive retrograde transport. Alike mature β-cells of Asna1<sup>ß</sup>−/− mice (Norlin et al., 2016), Sox9<sup>+</sup> duct-like cells, Ngn3<sup>+</sup> pro-endocrine cells and differentiated α-cells and β-cells present in the Asna1<sup>Panc</sup>−/− DPE, remain refractory to apoptosis despite apparent Stx5 and 6 redistribution as well as Golgi fragmentation. In addition, inhibition of retrograde transport by Retro-2 mimics the redistribution of Stx5 and 6 and the Golgi fragmentation in WT MPCs, but does not trigger extensive apoptosis or impair acinar cell differentiation. Thus, it appears that the requirement for Asna1 ATPase function for progenitor cell survival is mediated by pathways independent of Stx5 and Retro-2 sensitive retrograde transport, but which may mediate membrane insertion of other TA-protein(s) that still remain to be defined. We cannot, however, exclude the possibility that poor solubility of Retro-2 precludes sufficient concentration to achieve complete inhibition of Asna1 dependent retrograde transport in pancreatic progenitor cells.

Several possibilities may account for the cell specific differences in Asna1<sup>+/−</sup> phenotypes. For instance, the absence of Golgi fragmentation as observed in β-cells of Asna1<sup>ß</sup>−/− mice may be explained by the activation of UPR signaling that may help to
maintain Golgi integrity (Norlin et al., 2016). Another possibility is that, dependent on the proliferative status of cells (i.e. pancreatic progenitor cells as compared to differentiated β-cells), the redistribution of Stx5 may prevent the re-assembly of the Golgi compartments after cell division (Rabouille et al., 1998). The proliferative status may also influence the apoptotic response. Cell types with limited proliferative potential, such as specified pro-endocrine cells and differentiated endocrine cells, appear refractory to apoptosis. In contrast, Asna1Panc−/− progenitor cell apoptosis, which may be partly p53 dependent, is observed among the proliferating cells in the MPC domain (tip cells) in both the dorsal and ventral pancreatic as well as the proliferating duodenal epithlia in Asna1Panc−/− mice. The link between p53, an oncogene that can induce cell cycle arrest and/or apoptosis in response to a variety of cellular stresses, and pancreatic progenitor cell apoptosis in Asna1Panc−/− mice, suggests that Asna1 inactivation somehow interferes with cell cycle progression. Interestingly, cells deficient for one of the Asna1 ER-receptor subunits, CAML, exhibits mitotic defects including chromosome mis-segregation (Liu et al., 2009). The causal link between Asna1 deficiency and p53 activation remains however unknown and will require further analyses. To this end, it is worth noting that inactivation of RINT1, a component of the DSL1/NRZ and the RZZ complexes (Tagaya et al., 2014), interferes with retrograde transport and chromosome segregation resulting in Golgi fragmentation, genomic instability, and apoptosis (Grigaravicius et al., 2016), indicating a possible connection between the vesicle transport machinery and cell division control.

In summary, we show that Asna1 in pancreatic progenitor has dual functions; (1) ensuring retrograde transport pathway(s), thereby maintaining Golgi integrity, and (2)
ensuring progenitor cell survival, possibly by preventing p53 activation. Both these functions rely, directly or indirectly, on the ATPase activity of Asna1, presumably by promoting insertion of TA-protein. We cannot, however, rule out putative ATPase dependent functions of Asna1 that are unrelated to the actual membrane insertion of TA-proteins, such as influencing protein degradation, or direct effects of Asna1 in regulating retrograde transport. Elucidation of the molecular mechanism(s) underlying the redistribution of Stx5 and Stx6 as well the progenitor cell apoptosis observed in Asna1Panc−/− pancreatic and duodenal epithelium will require further analyses.

METHODS

Data reporting
For animal experiments, no sample-size estimate was calculated before the study was executed. The experiments were not randomized unless otherwise stated. Investigators were not blinded to allocation during experiments and outcome assessment. For in vivo data, each n value corresponds to a single mouse or mouse embryo. For ex vivo cultures each n value corresponds to independent explants. If technical replicates were performed, then their mean was considered as n = 1.

Mouse strains and generation of Asna1Panc mice.
Ipf1-nlsCre transgenic mice (Svensson et al., 2009) were bred with Asna1+/flox mice (Norlin et al., 2016), yielding Asna1+/flox, Ipf1-nlsCre+ mice (denoted Asna1Panc+/−), which thus exhibit pancreas and duodenum specific Cre mediated recombination from at least E10.5. Unless otherwise stated, male Asna1Panc+/− males were crossed with Asna1flox/flox females to yield Asna1Panc−/− mice. To achieve earlier inactivation of Asna1, we took advantage of the leakiness of the Ipf1-nlsCre transgene (Svensson et
al., 2009) in the germ cell lineage of female Asna1Panc+/− mice, which thus transmit wildtype Asna1 (Asna1+) or recombined Asna1null alleles. Hence, crossing male Asna1flox/flox mice with female Asna1+/−;Ipf1-nlsCre+ mice yields Asna1null/flox;Ipf1-nlsCre+ mice (denoted Asna1null/Panc−). Cre mediated recombination was visualized in situ by breeding a Rosa26loxP-stop-loxP-LacZ transgene (Soriano, 1999) onto an Ipf1-nlsCre+ or Asna1Panc+/− or Asna1Panc− background. For evaluation of autophagic degradation, Asna1Panc+/− mice were bred onto a background of GFP-LC3 transgenic mice (Mizushima et al., 2004) (provided by RIKEN BRC [RBRC00806]); and pancreas progenitor specific deletion of Atg5 was performed by breeding Atg5flox mice (Kuma et al., 2004) (provided by RIKEN BRC [RBRC02231]) with Ipf1-nlsCre mice. The genotypes of mice were determined by PCR analyses of genomic DNA samples extracted from tail biopsy specimens. For PCR primer sequences see Table S3. PCR primers Asna-exon2-196F, ASNA_G_WT-A and Asna-G4R were used to detect the Asna1+ (407bp), Asna1flox (525bp) and Asna1− (267bp) alleles. PCR primers Ipf1-5’3, IPF1-AR and CRE1 were used to detect the Ipf1-nlsCre transgene (950bp) and endogenous Ipf1 allele (700bp). The GFP-LC3 transgenic construct and the endogenous LC3 allele were detected by PCR primers GFP(LC3) and LC3*rc3 (400bp), and mLC3ex3GT and mLC3ex4AG (550bp), respectively. The PCR primers ATG5exon3-1, ATG5check and ATG5short were used together to amplify the Atg5flox (700bp) and Atg5WT (350bp) alleles. Genetically modified mice were kept on a mixed background. Embryonic WT tissue for in situ hybridization and explants were obtained from crossing CBA males with B6 females. The animal studies were approved by the Institutional Animal Care and Use Committee of Umeå University and were conducted in accordance with the guidelines for the care and use of laboratory animals.
Tissue isolation and preparation

Embryos were collected at selected stages. The day of the vaginal plug was considered embryonic day (E) 0.5. Embryonic tissue for immunohistochemical or in situ analysis were fixed in 4% PFA in PBS for 1h, equilibrated in 30% sucrose in PBS, frozen and sectioned. For qPCR analysis or ex vivo culture experiments, the dorsal pancreatic bud were isolated using tungsten needles to remove as much of the surrounding mesenchyme as possible. Alternatively, as indicated in text, the mesenchyme was removed after proteolytic degradation by Dispase II (Roche).

Immunohistochemistry and in situ hybridisation

Tissue sections or intact cells were incubated 20 min with blocking buffer (10% fetal bovine serum diluted in Tris-HCl pH7.4, 0.15M NaCl, 0.1% Triton-X100 [TBST]). For mouse monoclonal primary antibodies, section were additionally blocked with M.O.M-reagent (30-60 min, diluted 1:30 in TBST) (Vector labs #MKB-2213). Tissues were incubated with primary antibodies (Table S1) over night at +4°C followed by washes and fluorochrome labelled secondary antibodies for 1h. All antibodies were diluted in blocking buffer. Tunel assay to detect apoptosis was performed according to manufacturer’s recommendations (In situ cell death detection kit, Fluorescein, Roche #11684795). In situ hybridisation using digoxigenin labelled probes was performed as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993).

Transmission Electron Microscopy (TEM)

Tissue samples processed for TEM by Umeå Core Facility Electron Microscopy (UCEM). Briefly, tissue samples was fixed with 2.5% glutaraldehyde in 0.1 M
sodium cacodylate buffer pH 7.4, post fixed in 1% osmiumtetroxide, dehydrated and finally embedded in Spurr resin according to standard procedures. Sections were contrasted with uranyl acetate and lead citrate and examined in a Jeol 1230 TEM. Micrographs were acquired using a Gatan MSC 600CW. The presence of electron dense tight junctions between epithelial cells was used to distinguish them from mesenchymal cells.

**Ex vivo assays**

Explants were cultivated on Millicell CM culture plate inserts (Merk # PICM01250) in explant medium; DMEM [Gibco#21885) supplemented with 10% BCS, 10mM HEPES pH 7.4 and 10U/ml PEST (Gibco #15140-122). Explant medium was further supplemented with vehicle (DMSO), Retro-2 (Calbiochem, #554715), Pifithrin-α hydrobromide (PFT-α) (Tocris, #1267), Nutlin-3 (Tocris, #3984). To facilitate lentivirus infection, explants were split in half to expose the pancreatic epithelium and pre-incubated with concentrated virus particles for 2h at +4°C.

**Lentivirus expression.**

Asna1 mutant cDNA was cloned after the EF1-promoter of the lentivirus expression vector EF.CMV.RFP (gift from Linzhao Cheng: Addgene plasmid #17619), which also contains a separate CMV driven RFP reporter to monitor transduction efficiency (Yu et al., 2003). The various expression vectors were co-transfected with envelope and packaging plasmids, pMD2.G and psPax2 (gifts from Didier Trono: Addgene plasmid #12259 and #12260), to produce virus particles as previously described (Szulc et al., 2006). Infection efficiency of pancreatic epithelia was similar for all constructs (~1% of Ecad⁺ area) as monitored by the CMV-RFP expression. However,
due to imperfect correlation between expression from the EF1 and CMV promoters (Yu et al., 2003), the true efficiency of Asna1 re-expression is probably underestimated using this method.

**qRT PCR analyses**

Individual dorsal pancreatic buds were used to prepare total RNA (RNeasy Micro Kit, Qiagen #74004) and cDNA (SuperScript® III First-Strand Synthesis System, Life technologies # 18080-051). Expression of the TBP was used to normalize expression levels. To ensure comparable Cq values, equal amounts (5ng) of cDNA were used as template and Cq of the reference gene TBP was kept within +/-1 cycle. Quantitative PCR (qPCR) analysis was performed using an ABI Prism 7000 sequence detection system and SYBR Green PCR Master Mix (ABI). Oligonucleotide sequences used for qPCR are listed in Table S2.

**Western blot analysis**

Western blot expression data were normalized using α-tubulin expression. See Supplementary Methods for further details.

**Image analysis and quantification**

All comparisons were performed pairwise between Asna1<sup>Panc-/-</sup> and control tissue. For intensity comparisons and area quantification, images were captured using the same settings or modified to achieve similar background signal intensities. For quantification of immunopositive areas, the entire embryonic pancreas or pancreatic explant was sectioned (8μm sections), every 5<sup>th</sup>-15<sup>th</sup> section was collected for IHC (5-12 sections/IHC), all IHC sections were photographed and quantified using scripted
procedures in ImageJ software, including software based threshold algorithms to define immunopositive areas. Data from damaged sections were replaced by interpolation from adjacent sections.

**Statistical analyses**

All numerical data are presented as mean values +/- S.E.M. All statistical analyses were performed by heteroscedastic two-tailed Student $t$ test. P<0.05 was considered statistically significant and denoted with: *p<0.05, **p<0.01, ***p<0.001.

**Author contributions**

S.N. and V.P. contributed to the design and performance of experiments, and writing and editing of the manuscript. H.E. designed and directed the research, and secured funding. S.N. and H.E. analyzed and interpreted the data and wrote the manuscript.

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Competing interest

Helena Edlund is a co-founder, shareholder, and consultant of the unlisted biotech company Betagenon AB. Stefan Norlin and Vishal Parekh declare no competing financial interests.
References


Fig. 1. *Asna1*Panc−/− mice develop pancreatic and duodenal agenesis due to apoptosis.

(A) Upper gastrointestinal tract dissected from neonatal *Asna1*Panc−/− and control littermate showing pancreatic and duodenal agenesis.

(B) X-gal staining of E15.5 *Asna1*Panc:Ctrl and *Asna1*Panc−/− embryos on a *Rosa26loxP-stop-loxP-LacZ* background.

(C) Quantification of the dorsal pancreatic epithelia (E-cad+) area of *Asna1*Panc−/− and control littermates at E12.5 (n=5 and 7 respectively), E13.5 (n=6 and 7 respectively) and E15.5 (n=5).

(D) Representative immunohistochemistry of dorsal pancreatic sections from E13.5 *Asna1*Panc:Ctrl and *Asna1*Panc−/− embryos using antibodies against E-cadherin (E-cad; green), cleaved Caspase 3 (c.Casp.3; red) and phospho-Histone H3 (pH3; red). Inset show magnification of *Asna1*Panc−/− epithelia with cleaved Caspase 3+ cells at epithelial protrusions (arrows). Scale bar in (B), 100μm.
(E) Quantification of cleaved Caspase 3+ cells relative to E-cad+ epithelial area in the dorsal pancreatic epithelia of $Asna1^{Panc-/-}$ and control littermates at E13.5 (n=5 and 7 respectively) and E15.5 (n=5).

(F) Quantification of phospho-Histone H3+ (pH3+) cells relative to E-cad+ epithelial area in the dorsal pancreatic epithelia of $Asna1^{Panc-/-}$ and control littermates at E13.5 (n=5 and 7 respectively) and E15.5 (n=5).

dp, dorsal pancreas; du, duodenum; vp, ventral pancreas; sp, spleen; st, stomach. Data are presented as mean±SEM, *p<0.05, **p<0.01, ns=not significant (student t test).
Fig. 2. Impaired endocrine and acinar differentiation in Asna1\textsuperscript{Panc-/-} DPE

(A) Immunohistochemistry of dorsal pancreatic sections from E12.5 Asna1\textsuperscript{Panc:Ctrl} and Asna1\textsuperscript{Panc-/-} embryos (n=3) using antibodies against Sox9, Ptf1a, CPA and Ngn3 (all red), and E-cadherin (E-cad) and glucagon (both green). Insets show Ptf1a\textsuperscript{High} acinar progenitors (arrowheads) and Ptf1\textsuperscript{Low} putative MPCs (open arrowheads).

(B-C) Quantification of total pro-endocrine (Ngn3\textsuperscript{+}) area (B) and total endocrine (glucagon\textsuperscript{+}) area (C) in the DPE from Asna1\textsuperscript{Panc:Ctrl} and Asna1\textsuperscript{Panc-/-} embryos at E12.5 (n=3).
(D-F) Immunohistochemistry of dorsal pancreatic sections from E14.5 (D), E15.5 (E) and E16.5 (F) $Asna1^{Panc:Ctrl}$ and $Asna1^{Panc/-}$ embryos (n=3) using antibodies against Ptf1a, Sox9, Ipf1, Ngn3, insulin, amylase and phospho-Histone H3 (pH3) (all red), and E-cadherin (E-cad) and glucagon or DBA lectin (DBA) (all green).

(G-H) Quantification of total pro-endocrine (Ngn3$^+$) area (G) and total endocrine (glucagon$^+$ + insulin$^+$) area (H) in the DPE from $Asna1^{Panc:Ctrl}$ and $Asna1^{Panc/-}$ embryos at E13.5 and E15.5 (n=5 respectively).

Data are presented as mean±SEM, *p<0.05, **p<0.01, ns=not significant (student t test).
Fig. 3. Apoptosis of pro-acinar progenitors in Asna1Panc-/- DPE is associated with p53 activity and activation of the IRS.

(A) Representative immunohistochemistry of dorsal pancreas explants from E10.5 Asna1Panc:Crl and Asna1Panc-/- cultivated for 5 days exposed to vehicle (n=7 and 4 respectively) or PFT-α (30μM) (n=11 and 4 respectively), using antibodies against amylase (red) and insulin (green).

(B-C) Quantification of experiments described in (A); Relative glucagon+ (Glu) Ngn3+, insulin+ (Ins) and amylase+ (Amy) area over total E-cadherin+ area in explants from Asna1Panc:Crl (B) and Asna1Panc-/- (C) mice.

(D) Representative immunohistochemistry of dorsal pancreas explants from E12.5 WT embryos cultivated for 3 days-exposed to vehicle or Nutlin-3 (10μM), using antibodies against E-cadherin (E-cad) and insulin (both green), and amylase (red).

(E-F) Quantification of experiments described in (D) after 1 day (n=6 for each condition) (E) and 3 days (Vehicle: n=4, Nutlin-3:n=5) (F) of cultivation; E-cadherin+ Epithelial (Epi) and E-cadherin-
Mesenchymal (Mes) area; relative cleaved Caspase3+ (c.Casp.3) over E-cadherin+ area after 1 day; Absolute insulin+ (Ins) and amylase+ (Amy) area after 3 days; arbitrary units (a.u).

(G) qRT-PCR mRNA levels of the indicated ISR genes in dorsal pancreatic buds from E13.5 Asna1Panc:Ctrl (n=7) and Asna1Panc−/− (n=6) embryos.

(H) Immunohistochemistry of dorsal pancreatic sections from E12.5 and E13.5 Asna1Panc:Ctrl and Asna1Panc−/− embryos (n=5) using antibodies against ATF4 (red) and E-cadherin (E-cad; green). Arrowheads indicate ATF4+ tip cells.

(I) qRT-PCR mRNA levels of the indicated UPR genes in dorsal pancreatic buds from E13.5 Asna1Panc:Ctrl and Asna1Panc−/− (n=7) and Asna1Panc−/− (n=6) embryos. DAPI (blue) indicate nuclei in (A, C). Scalebars: 50µm in (F). Data are presented as mean±SEM, *p<0.05, **p<0.01, ***p<0.001, ns=not significant (student t test).
Fig. 4. Altered morphology and Stx5 and Stx6 expression in the Golgi apparatus of Asna1Panc−/− DPE cells.

(A) Immunohistochemistry of dorsal pancreatic sections from E12.5 Asna1Panc:Crl and Asna1Panc−/− embryos (n=3) using antibodies against Gm130 (green) and TGN46 (red). Insets show magnification of green and red color channel for selected areas (squares).

(B) Transmission electron micrograph (TEM) of dorsal pancreatic sections from E12.5 Asna1Panc:Crl and Asna1Panc−/− embryos (n=3) showing compact Golgi stacks (red arrowheads), distended Golgi stacks (open arrowheads).
(C-E) Immunohistochemistry of dorsal pancreatic sections from E11.5 (C, E) or E12.5 (D) Asna1\textsuperscript{Panc:Ctrl} and Asna1\textsuperscript{Panc-/−} embryos (n=3) (C,D) or Asna1\textsuperscript{null/Panc+} and Asna1\textsuperscript{null/Panc⁻} (E) embryos (n=3), using antibodies against Gm130 (green), Stx5 (red), Stx6 (green) and TGN46 (red).

(F) Immunohistochemistry of dorsal pancreatic sections from E16.5 Asna1\textsuperscript{Panc:Ctrl} and Asna1\textsuperscript{Panc-/−} embryos (n=3) using antibodies against Stx5 (red) and Gm130 (green) together with either glucagon, insulin or DBA-lectin (all white). Merged panel shows co-expression between Stx5 and Gm130 (yellow).

(G) Immunohistochemistry of dorsal pancreas explants from E10.5 Asna1\textsuperscript{Panc-/−} cultivated for 5 days (n=3) exposed to vehicle or PFT-α, using antibodies against Stx5 (red) and Gm130 (green) together with amylase (white). Merged panel shows co-expression between Stx5 and Gm130 (yellow).

DAPI (blue) indicate nuclei in (A, C-G). Scalebars: 50μm in (A), 0.5μm in (B), 10μm in (C-E) and 5μm in (F-G).
**Fig. 5. Accumulation of autophagy markers in Asna1Panc-/- DPE.**

(A) Immunohistochemistry of dorsal pancreatic sections from E13.5 Asna1Panc:Ctrl and Asna1Panc-/- embryos (n=5) using antibodies against p62 (green). Arrowheads indicate p62+ puncta.

(B) Dorsal pancreatic sections from E13.5 Asna1Panc:Ctrl and Asna1Panc-/- embryos on a LC3-GFP transgenic background, showing LC3-GFP fluorescence in green (n=2).

(C) Immunohistochemistry of dorsal pancreatic sections from E14.5 Atg5Panc+/- and Atg5Panc-/- embryos (n=3), using antibodies against p62 (green), E-cadherin (E-cad; green) and cleaved Caspase3 (c.Casp.3; red). Insets show magnification of selected trunk (t) and acinar (a) epithelia areas (orange squares, letters and arrows).

DAPI (blue) indicate nuclei in (A-B, C). Dashed lines delineate pancreatic epithelium (e) and mesenchyme (m). Scalebars: 5µm in (A,B and insets in C), 50µm in (C).

All image pairs were captured using the same settings.
Fig. 6. Normal Golgi morphology, Stx5 distribution, and acinar differentiation requires the ATPase domain and CXXC(285-288) di-cysteine motif of Asna1.

(A) Representative immunohistochemistry of lentivirus infected dorsal pancreas explants using antibodies against Gm130 (green), Stx5 (red), E-cadherin (E-cad; green) and amylase (red). Dorsal pancreas explants from E11.5 Asna1\textsuperscript{Panc:Ctrl} or Asna1\textsuperscript{Panc/-} embryos were transduced with lentivirus
particles carrying either empty expression vector (Vector) (n=6 and 5 respectively) or constructs encoding wildtype Asna1 (WT) (n=5), Asna1 (D74N) (n=4), Asna1 (C246S-X-C248S) (n=13) or Asna1 (C285S-XX-C288S) (n=4), as indicated, and grown for 5 days.

(B) Quantification of relative amylase\(^+\) epithelial area (amylase\(^+\)/total E-cad\(^+\)) from experiments described in (A).

(C) Quantification of relative RFP\(^+\) epithelial area (RFP\(^+\)/Ecad\(^+\)) in control explants infected with the different lentivirus expression constructs from experiments described in (A).

Dashed lines delineate pancreatic epithelium. Scalebars: 25\(\mu\)m in (A). Data are presented as mean±SEM, **p<0.01, ***p<0.001, (student \(t\) test).
Fig. 7. Inhibition of retrograde transport by Retro-2 disrupts Golgi morphology and Stx5 distribution but allows for acinar differentiation.

(A-B) Immunohistochemistry of E11.5 dorsal pancreas explants from WT embryos grown 48 hours exposed to vehicle (n=4) or Retro-2 (50μM) (n=5), using antibodies against Gm130, Stx6 and E-cad (all green), and Stx5, TGN46 and ATF4 (all red). Insets in (A) show magnification of green and red color channel for selected areas (dashed squares).

(C-E) Quantification of total epithelial (Epi) and mesenchymal (Mes) area (C), relative endocrine (Ngn3+, glucagon+ and insulin+) and exocrine (amylase+) area (% of total E-cad+ area) (D), and relative apoptotic cell area (% cleaved Caspase-3+ [c.Casp.3+] of E-cad+ epithelium [Epi] and E-cad− mesenchyme [Mes]) (E) in E10.5 dorsal pancreas explants grown for 5 days exposed to vehicle (n=8) or Retro-2 (50μM) (n=7).

Dashed lines delineate pancreatic epithelium. Scalebars: 10μm in (A), 50μm in (B). Data are presented as mean±SEM, *p<0.05, ns=not significant (student t test).
SUPPLEMENTARY INFORMATION

Supplementary Methods

Western blot analysis

Tissue were collected in lysis buffer (2% SDS in 100mM Tris-HCl pH6.8), sonicated, and cleared by centrifugation. Protein expression was probed on western blots using antibodies (Table S1) diluted in blocking buffer (2.5% BSA in TBSTw [20mM Tris-HCl pH7.4, 0.15M NaCl, 0.1% Tween-20]) and quantified using chemiluminescent detection in ChemiDoc™ MP system (BioRad, #170-8280) followed by densitometry analyses of bands on Image Lab 4.1 using expression of α-tubulin to normalize expression levels.
Table S1. Primary antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
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<tr>
<td>Amylase</td>
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<td>Atf4</td>
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<td>α-tubulin</td>
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* dilution for western blot
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<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
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<td>XBP1-spliced</td>
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### Table S3. Primers used for PCR genotyping.

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<td>Asna-G4R</td>
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<td>IPF1-5’3</td>
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<td>IPF1-AR</td>
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<td>CRE1</td>
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<td>mLc3ex4AG</td>
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<td>ATG5short</td>
<td>GTACTGCTATAATGGTTAATCTCTTGC</td>
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**Figure S1 (related to Figure 1)**

(A) In situ hybridization using Asna1 antisense and sense probes showing expression of Asna1 in the pancreatic epithelium of E10.5-E13.5 wildtype embryos.

(B) Schematic presentation of the conditional Asna1 allele (Asna1^flox) and the Ipf1-nlsCre transgenic construct used to generate Asna1^{Panc-/-} mice.

(C) X-gal staining of E10.5 Ipf1-nlsCre:Rosa26^{loxP-stop-loxP-LacZ+/-} (Soriano, 1999) embryos showing Ipf1/CRE mediated reporter, i.e. LacZ, expression in pancreatic and duodenal progenitor epithelia.

(D) qRT-PCR analysis of Asna1(exon2) expression in dorsal pancreatic epithelium stripped of mesenchyme (DPE) isolated from E11.5 Asna1^{Panc+/+}, Asna1^{Panc+/-} and Asna1^{Panc-/-} mice (n=4).

Scale bar in (A), 50µm. Dashed lines in (A) delimit pancreatic epithelia. Arrows in (A) indicate pro-acinar tip cells. dp, dorsal pancreas; du, duodenum; m, mesenchyme; pe, pancreatic epithelium; vp, ventral pancreas.

**Fig. S1.**

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**Figure S2 (related to Figure 1)**

(A) Combined tunel staining and immunohistochemistry of dorsal pancreatic sections from E13.5 *Asna1^Panc:Ctrl* and *Asna1^Panc^-/-* embryos (n=2) using tunel assay to detect DNA fragmentation (red) in apoptotic cells combined with antibodies against E-cadherin (E-cad; green) to detect pancreatic epithelium.

(B) Immunohistochemistry of E13.5 ventral pancreas and E14.5 duodenum from *Asna1^Panc:Ctrl* and *Asna1^Panc^-/-* embryos (n=3) using antibodies against E-cadherin (E-cad; green) and cleaved Caspase3 (c.Caspase3; red).

Scalebar, 100μm in (A and B)
**Figure S3 (related to Figure 2)**

Quantification of total glucagon$^+$ (Gluc) and insulin$^+$ (Ins) area in the DPE from Asna1$^{Panc:Crl}$ and Asna1$^{Panc-/-}$ embryos at E15.5 ($n=5$ respectively). Data are presented as mean±SEM, *p<0.05, **p<0.01, (student $t$ test).
**Figure S4 (related to Figure 3)**

Western blot quantification of p53 protein levels relative to α-tubulin in E13.5 dorsal pancreas buds from $Asna1^{Panc:Ctrl}$ (n=6) and $Asna1^{Panc-/}$ embryos (n=3). Data are presented as mean±SEM, ns=not significant (student t test).
Figure S5 (related to Figure 4)

(A) Immunohistochemistry of dorsal pancreatic sections from E12.5 Asna1Panc:Ctrl and Asna1Panc-/- embryos (n=3) using antibodies against KDEL (green), ERGIC (red), EEA1 (green) and Lamp1 (red).

(B) Transmission electron micrograph (TEM) of dorsal pancreatic sections from E12.5 Asna1Panc:Ctrl and Asna1Panc-/- embryos (n=3) showing mitochondria (m) and rough ER (yellow arrows).

(C) Western blot quantification of the 35kDa and 42kDa isoforms of Stx5 protein levels relative α-tubulin in E12.5 dorsal pancreas buds from Asna1Panc:Ctrl (n=3) and Asna1Panc-/- embryos (n=3).

(D) Representative immunohistochemistry and quantification of dorsal pancreas from E12.5 Asna1null/Panc+ and Asna1null/Panc- (n=3) using antibodies against E-cadherin (E-cad; green) and cleaved Caspase 3 (c.Caspase3; red).

(E) Immunohistochemistry of ventral pancreas and duodenum from E12.5 Asna1Panc:Ctrl and Asna1Panc-/- embryos (n=3) using antibodies against Gm130 (green) and Stx5 (red). Insets show magnification of selected areas (squares). Stx5 expression is reduced in the Golgi of E12.5 Asna1Panc-/- ventral pancreas and duodenum. To illustrate different degrees of Golgi fragmentation (Gm130 in green) at this stage, the ventral pancreas from two different animals are shown and two regions of the Asna1Panc-/- duodenal epithelium are magnified (insets).

(F) Immunohistochemistry of ventral pancreas and duodenum from E12.5, E13.5 and E14.5 Asna1Panc:Ctrl and Asna1Panc-/- embryos (n=3) using antibodies against E-cadherin (E-cad; green) and cleaved Caspase 3 (c.Caspase3; red). Increased number of c.Caspase3+ apoptotic cells are observed at E13.5 in the ventral pancreas and at E14.5 in the duodenum, which is 1-2 days after the first signs of perturbed Golgi morphology and Stx5 expression (compare with panel [E]).

DAPI (blue) indicate nuclei in (A). Dashed lines in (E) delimit pancreatic or duodenal epithelia. Scalebars: 10µm in (A), 0.5µm in (B), 50µm in (D and F), and 25µm in (E).

Fig. S5.

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