



Pancreatic Neuroendocrine Tumor Mouse Models

A thesis submitted for the degree of

Doctor of Philosophy (PhD)

in the subject of *Molecular Medicine*

by

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June 2021

Hannover Medical School

International MD/PhD program "Molecular Medicine"

at Hannover Biomedical Research School (HBRS)

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Day of final exam / public defense: June 11th 2021

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Abstract

Pancreatic neuroendocrine tumors are rare, highly heterogeneous, and a complex class of tumors. Their survival rate varies depending on their stage and grade at diagnosis (Christodoulos P Pipinikas, 2019). Even though new approaches like targeted molecular therapy are also opening new opportunities for precision oncology, surgical excision is still the only curative therapy in PanNETs patients (Amit Akirov V. L., 2019). NGS data has advanced our understanding of cancer genomes' mutation, and it has revealed a large amount of genetics and epigenetics mutations and alterations in PanNETs, including mutation in *MEN1*, *DAXX*, and *PTEN* (Scarpa, 2019).

Genetically Engineered Mouse Models play an essential role in the understanding of gene' function and the molecular mechanisms of cancer development and progression (Lukas E. Dow, 2014). Using an inducible and reversible shRNA system to generate transgenic ES-cell-based mouse models enables the generation of biallelic or even multiallelic chimeras in a more time-effective way than traditional transgenic mouse models, and it provides a system with temporal control and reversibility of gene expression (Joerg Heyer, 2010), (Kelly Kersten K. E., 2017).

We demonstrated that Menin, Daxx, and Pten shRNAs expression resulted in a substantial reduction of protein expression *in vitro*; however, because of the mosaic expression pattern of *pdx1*-Cre and having a mixed population of cells with and without shRNA expression, we could not show strong proteins knock-down *in vivo*. We demonstrated that reduction of Daxx and Pten next to Menin affected exocrine compartments more than endocrine tissues and led to mostly fibrosis and acinar to ductal metaplasia but not PanNET.

1 Introduction

1.1 Pancreas

The pancreas is an abdominal gland organ with dual functions and consists of three main cell types (Crosta, 2017). Most of the pancreas consists of acinar tissue that produces and secrete digestive enzymes (elastase, gelatinase, protease, pancreatic lipase, amylase, ribonuclease) and bicarbonate to support digestion (Joel F Habener, 2005). The rest of the pancreas be made up of ductal cells and endocrine or islet of Langerhans that secrete hormones to maintain blood glucose levels such as insulin (β cells), glucagon (α cells), somatostatin (δ cells), and pp cell (Maria E Wilson, 2003) (Figure 1)

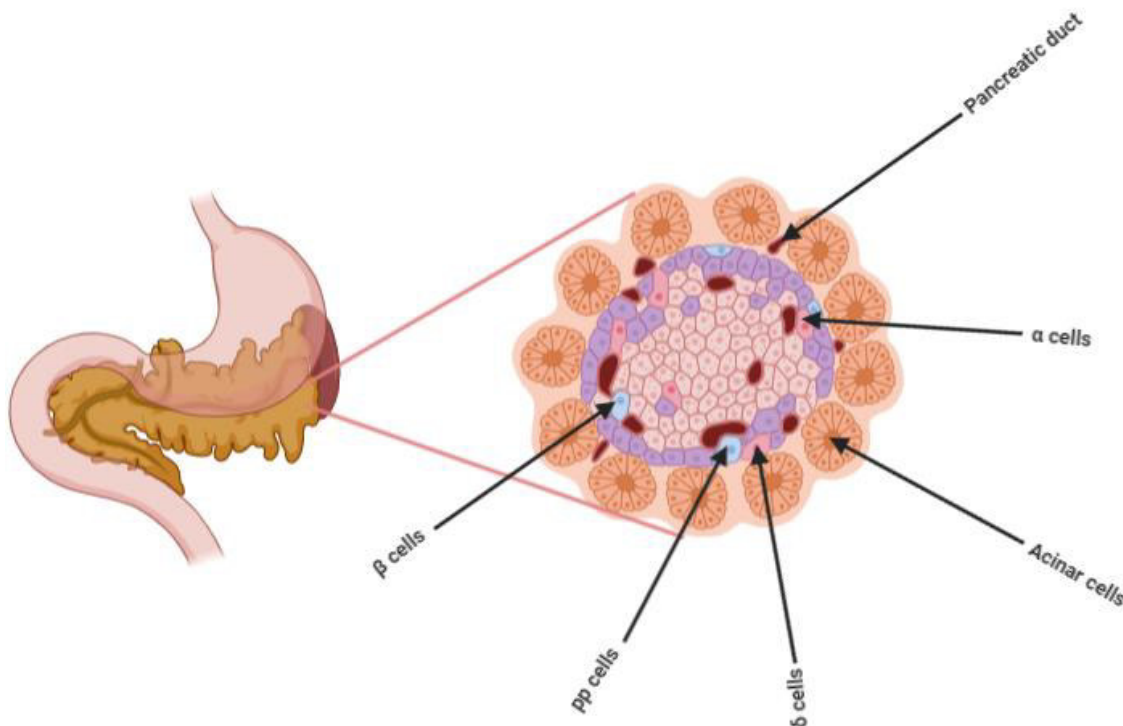


Figure 1 Schematic representation of all pancreas cell types. (Biorender)

Transcriptional factors (TF) play a fundamental role in pancreas development, differentiation, and function. Most transcription factor expression patterns at different developmental stages in mice are similar in humans (Rachel E. Jennings, 2020). In general, pancreas development can be separated into three steps. (Pin, 2017)

In the mouse pancreas, development initiates at embryonic day 8.5 to 9.5 from the distal part of the endodermal foregut and proximal part of the midgut (Pin, 2017). All mature pancreatic cells are derived from pancreatic progenitor cells that express *Pdx1* and/or *Ptf1a*. The primary transition is the step at which pancreatic progenitor cells actively proliferate and form stratified epithelia (Reshmi Dassaye S. N., 2016). Transcriptional factors such as *Hb9* and *Pdx1* are required for primary transition at embryonic day 8.5-12.5. (Figure 2)

In secondary transition, active growth and epithelial remodeling shape the future pancreas. In this stage, most of the cells have been specified to become endocrine or exocrine cells. *Ngn3*, *Hnf6*, *Pdx1* are three essential transcription factors involved in secondary transition at embryonic day 12.5 to 16.5. (Pin, 2017) (Figure 2)

Tertiary transition (embryonic day 16.5 to postnatal) (Murtaugh, 2007) is the stage at which differentiated pancreatic cells go through further remodelings such as replication and apoptosis to form a mature pancreas. (Reshmi Dassaye S. N., 2016) (Figure 2)

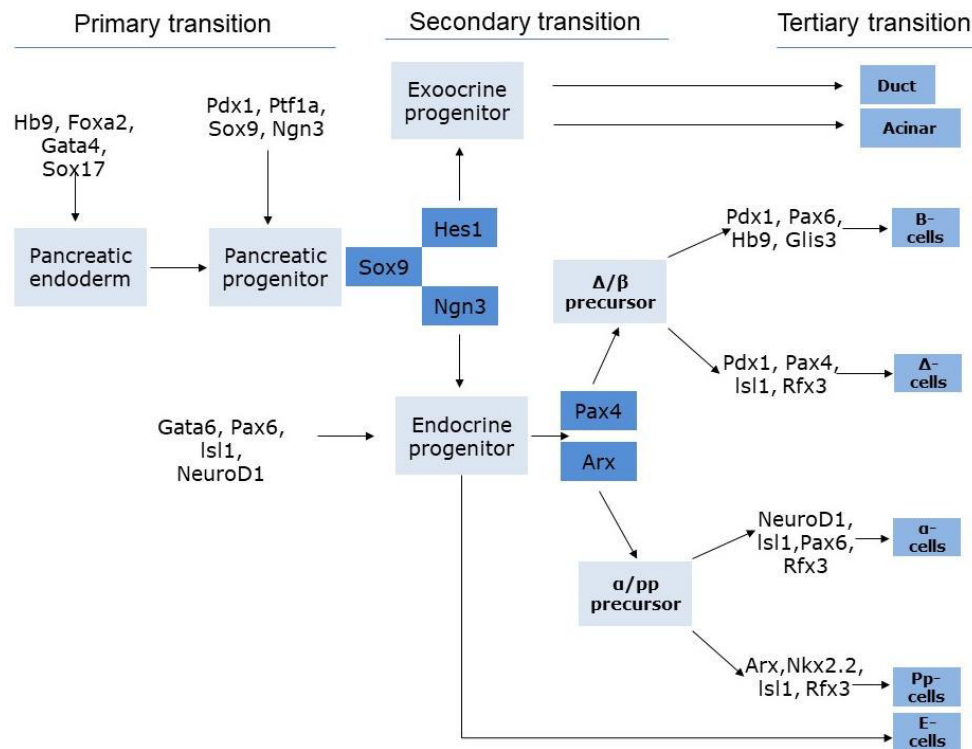


Figure 2 TFs involved in pancreatic organogenesis, differentiation, and maturation (Edited version) (Reshmi Dassaye S. N., 2016). Primary, secondary and tertiary transitions are three stages of pancreas development. Several TFs are essential for the development, differentiation, and maturation of the pancreas into a functional organ in each stage.

1.2 Common pancreatic disorder

Pancreatitis and pancreatic cancer are two major types of pancreatic disorders. Evaluation and diagnostics of pancreatic disease are complex because of organ inaccessibility. Physical examination, blood test, CAT scan, MRI, and endoscopic ultrasound are all pancreatic disorder diagnostic methods (Pavan Tummala, 2011). There are also some occurrences in which surgical assessment is the only way to approve the diagnosis (M.I. COSTACHE, 2017).

1.2.1 Pancreatitis

Inflammation of the pancreas is called pancreatitis and can be hereditary (HP), acute (AP), or chronic (CP). Hereditary pancreatitis is an autosomal dominant genetic disorder of the pancreas characterized by repeated pancreatic inflammation incidents and recurrent acute and progression to chronic pancreatitis (Celeste A Shelton, 2016) (Gapp & Chandra., 2020).

The incidence rate of AP has been rising in the United States and the rest of the world (Gapp & Chandra., 2020) (Whitcomb, 2010). Some of acute pancreatitis' causes are alcohol use, hypertriglyceridemia, drug-induced pancreatitis, gallstones, and idiopathic (Julia B Greer, 2009) (Gapp & Chandra., 2020).

Chronic pancreatitis is a progressive disorder involving fibrosis, loss of acinar cells, inflammation, and loss of islet cells, leading to the pancreas' destruction (Angela Pham, 2018). CP is caused by multiple factors such as acute pancreatitis that damages pancreatic ducts, cystic fibrosis, a blockage of pancreatic ducts, and smoking. (Vv Ravi Kanth, 2014).

1.2.2 Pancreatic cancer

Pancreatic cancer is one of the main causes of cancer-related deaths (Prashanth Rawl, 2019) and is mainly diagnosed in patients around 70 years old. The five-year survival rate is around 3% because patients are mostly diagnosed at an advanced stage (Hongcheng Wang, 2020) (Rebecca L Siegel, Cancer statistics, 2018) (Rebecca L Siegel, 2019).

Pancreatic cancer can be divided into two major groups; neuroendocrine pancreatic cancer and exocrine pancreatic cancer.

1.2.2.1 Exocrine pancreatic cancer

Exocrine pancreatic cancers arise from exocrine glands and ductal epithelial cells (Trevor J. Grant, 2016). 95% of all pancreatic cancers belong to different types of exocrine pancreatic cancers (Melissa Oliveira-Cunha, 2019), including Pancreatic Ductal Adeno Carcinoma (PDAC), Squamous Cell Carcinoma, Colloid Carcinoma, and Adenosquamous Carcinoma.

Pancreatic ductal adeno carcinoma is the fourth common cause of cancer-related death (Elvira Pelosi, 2017) and contributes to more than 85% of all pancreatic cancers (Ilic, 2016). The five-year survival rate among patients with PDAC is approximately 10% (Elvira Pelosi, 2017). Several studies using sequencing technology reported that *KRAS* is the most frequently mutated gene in PDAC (Lennerz, 2015) (Andrew G Stephen 1, 2014) (G Garcea 1, 2015). Andrew V. et al. pointed out that ~ 93% of PDAC samples carry a *KRAS* mutation (Andrew V Biankin, 2012). It has also been reported that the cooperation of *CDKN2A*, *TP53*, and *SMAD4* mutations in combination with *KRAS* mutations causes aggressive PDAC tumor growth (Simeon Springer 1, 2015) (Trevor J. Grant, 2016) (Figure 3 Genetic landscape of pancreatic cancer (edited version)). In the upper part, there are germline mutations and copy number alteration involved in pancreatic cancer. Pancreatic intraepithelial neoplasia and intraductal papillary mucinous neoplasms are shown in the middle part (dotted line). They are the pre-neoplastic stage. And lower part shows the most frequent mutated genes in pancreatic neuroendocrine tumors. (Figure 3).

1.2.2.2 Neuroendocrine pancreatic cancer

Pancreatic Neuroendocrine Tumors (PanNETs) belong to a rare class of neoplasm, highly heterogeneous and complex tumors that show diverse

clinical behaviors from the rate of development, progression, and metastasis, to treatment responses and resistance (Christodoulos P Pipinikas, 2019).

While PanNETs incidence is ≥ 1 case per 100000 individuals per year and comprises 1% to 2% of pancreatic neoplasms, their incidence rate has been rising in the last few decades (Zu-Yi Ma, 2020) (Julie Hallet, 2015).

The survival rate varies depending on the stage and grade at diagnosis, with a five-year survival rate of around 65% (Christodoulos P Pipinikas, 2019) (Hanbaro Kim, 2019). Based on WHO classification criteria, PanNETs are divided into well-differentiated tumors Grade I-III and poorly differentiated neuroendocrine carcinoma Grade III. While the GI and GII can be functional or non-functional, Grade III is mostly non-functional due to the loss of expression of hormone-secreting genes.

PanNETs are divided into functional and non-functional tumors (NF). Most PanNETs are non-functional tumors (Jennifer H Kuo, 2014). Since there is no hormone production in NF-PanNETS, they are defined as being asymptomatic, and they are usually found incidentally after metastasis in very advanced stages (Neda Nozari, 2014) (Jennifer H Kuo, 2014).

Functional tumors demonstrate various clinical behaviors leading to earlier diagnosis than NF due to the clinical symptom (PietroCaglià, 2015). They are divided into insulinomas, gastrinomas, glucagonomas, VIPomas, and somatostatinomas (Cynthia Ro, 2013). However, functional tumors pose a significant diagnostic challenge since the symptoms become evident later in a disease case due to growing tumor mass. (Tetsuhide Ito, 2012)

Pancreatic neuroendocrine tumors are mostly sporadic. They also arise due to inherited germline mutation in tumor suppressor genes that increase

organ susceptibility to tumor developments (Antonio Pea, 2015). These cancer predisposition syndromes such as *MEN1*, *TSC*, *VHL*, *NF1* account for less than 10% of PanNETs. (Oberg, 2013) (Figure 3)

So far, surgery is the only treatment option that can cure a PanNET (Amit Akirov V. L., 2019). Furthermore, surgical resection can be used to improve symptoms due to excess hormone levels (Amit Akirov V. L., 2019). For patients who are not ideal candidates for surgery, medical treatment such as symptoms-directed therapy or control of tumor growth such as molecular targeted treatment and chemotherapy is required (Amit Akirov V. L., 2019).

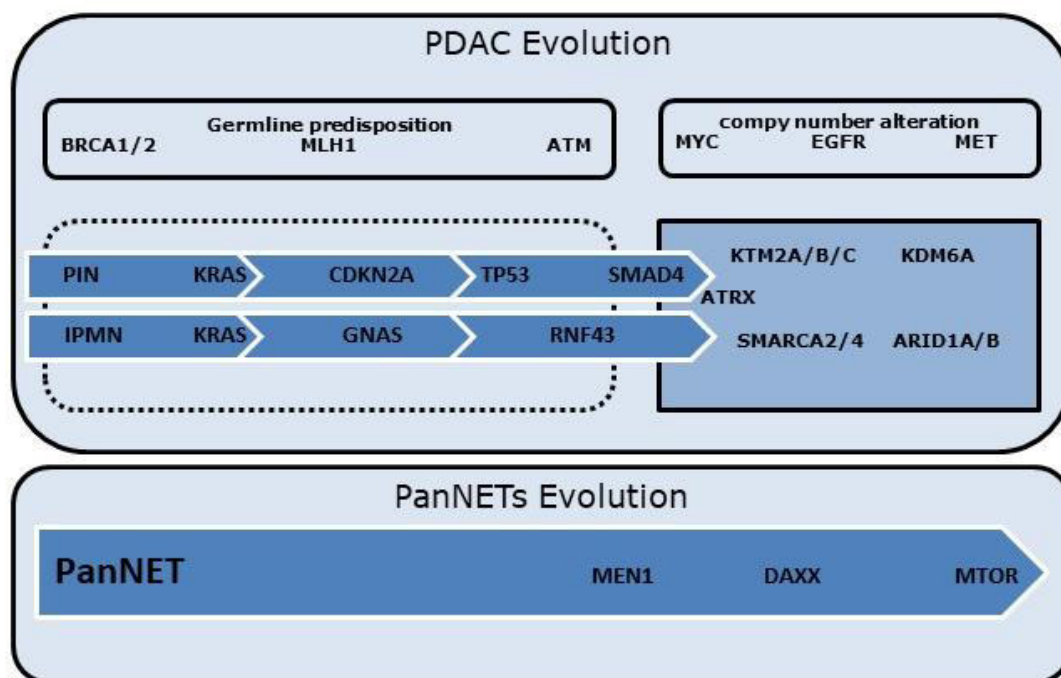


Figure 3 Genetic landscape of pancreatic cancer (edited version) (Francesco Natale, 2019). in the upper part, there are germline mutations and copy number alteration involved in pancreatic cancer. Pancreatic intraepithelial neoplasia and intraductal papillary mucinous neoplasms are shown in the middle part (dotted line). They are the pre-neoplastic stage. And lower part shows the most frequent mutated genes in pancreatic neuroendocrine tumors.

1.3 Genomic landscape, implicated pathways and mechanisms of PanNETs

Whole-genome sequencing of human tumor specimens have shown the most frequently mutated genes in sporadic PanNETs. *MEN1*, *DAXX*, and *PTEN* are three essential mutated genes that co-mutation of these genes also occurs in some patients(Figure 4) (YUCHEN JIAO C. S., 2011)(SCARPA, 2017).

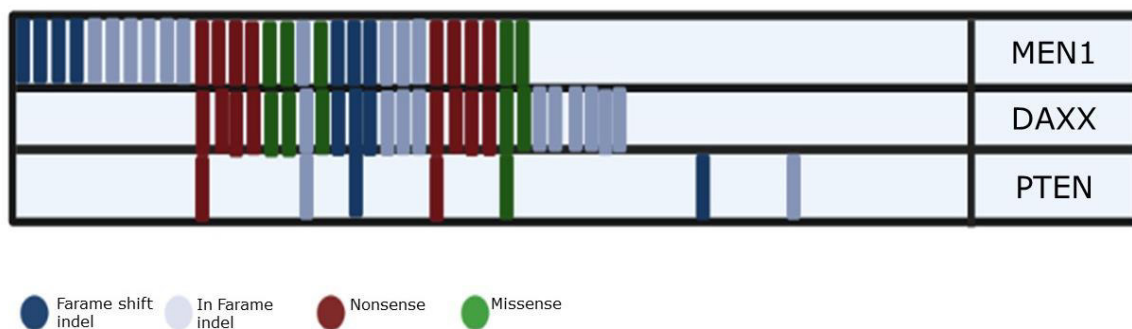


Figure 4 Schematic representation of somatic mutations in three essential genes that have been identified as significantly mutated genes in PanNETs (edited version) (Aldo Scarpa, 2017). Each line represents one mutation in one patient.

The most frequently altered pathways in PanNETs are chromatin remodeling, DNA damage and repair, telomere maintenance, and activation of mTOR signaling (Figure 5).

41% mutation frequency for *MEN1* confirms its significant role in PanNETs development. *MEN1* gene product Menin is a scaffold protein that regulates more than 40 proteins to control several vital processes such as DNA damage repair, transcriptional regulation, cell cycle, and chromatin remodeling (Smita Matkar, 2013). The delay in tumor development in *MEN1*

patients proposes a probability of cooperation of other mutations with *MEN1* to induce PanNETs (Chung Wong, 2020).

Approximately 22% of tumors were found to carry a mutation in *DAXX*. *DAXX* is a histone chaperon and plays roles in apoptosis, chromatin remodeling, and telomerase maintenance (Roeland F de Wilde, 2012).

Mutation in the tumor suppressor phosphatase and tensin homolog (*PTEN*) occurs in a high number of tumors. It's a negative regulator of the mTOR pathway that limits proliferation and prevents cancer development, and mutation of *PTEN* causes tumorigenesis and tumor progression (Nicola Fusco, 2020).

Animal models of PanNETs should be used to evaluate the cooperation of these genetic lesions' in the induction of PanNETs. They help researchers to study early tumor detection and novel targeted drug therapies (Varsha Babu 1, 2013).

Several PanNETs animal models are available, but not all of those models resemble human PanNETs. Mouse models of *Men1* deficiency are not ideal models for pre-clinical testing of drug candidates. Therefore, using genes that function in conjugation with *Men1* could help us develop better pre-clinical PanNETs animal models that resemble the histology and behavior of human PanNETs.

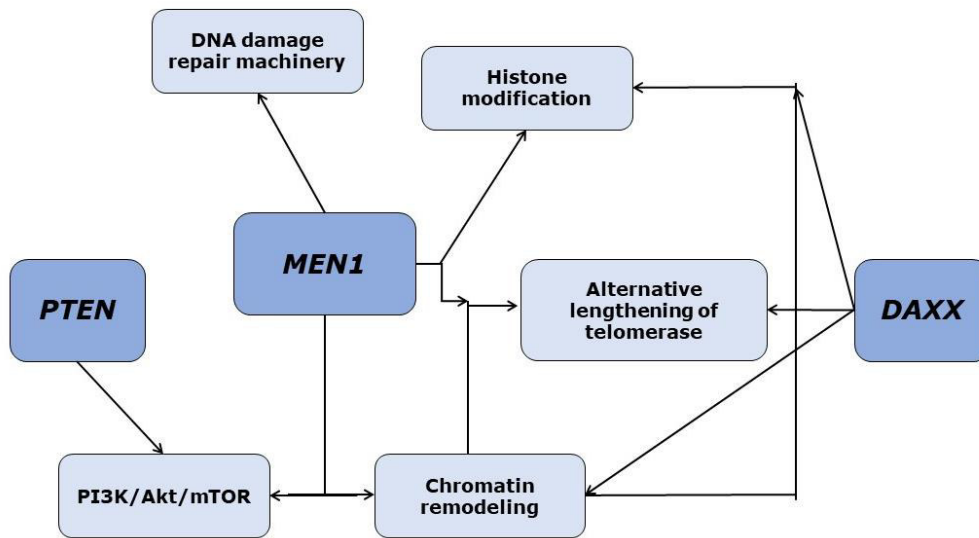


Figure 5 Schematic of the most frequently altered pathways and genes in PanNETs. *Men1* is involved in several essential cell functions such as DNA damage repair machinery, chromatin remodeling, and histone modification (W Karges, 2000). *DAXX* plays a vital role in chromatin remodeling, and histone modification and mutation are *DAXX* is associated with ALT phenotype (Iqbal Mahmud, 2019). *PTEN* controls cell death and survival by negatively regulating (Hon Yan K Yip, 2020).

1.4 Pancreatic Neuroendocrine tumor mouse models

Several PanNETs mouse models have been developed in order to study the molecular mechanism of PanNETs development, progression, metastasis, and treatment options. Among those models, *Men1* inactivation in pancreatic β cells, α cells, and progenitor cells (*Pdx1*) is the closest to the human counterpart. *Men1* mouse models are not suitable models for testing candidate drugs because of their tumorigenic latency. Hence, novel murine models that closely reflect the histology and behavior of human PanNETs are needed (Several PanNETs animal model has been shown in Table 1) Therefore, using the genes that function in conjugation with *Men1* could help develop better pre-clinical PanNETs, mouse models.

Model	Strain	Mechanism	Age	PNET	Tumor behavior	Reference
RIP-Tag	B6D2F1	Insulin promoter-driven expression of large T antigen	2.5–5	Insulinoma	Aggressive	Hanahan, 1985
Glu2-Tag	C57Bl/6J	Preproglucagon promoter (860-bp)-driven expression of large T antigen	9–12	Glucagonoma	Moderate	Efrat et al., 1988
Glu-Tag	CD-1	Preproglucagon promoter (2.1-kb)-driven expression of large T antigen	3	Glucagonoma	Aggressive	Lee et al., 1992
Men1 ^{TSM/+} or Men1 ^{ΔN3–8/+}	NIH Black Swiss or 129/SvEv TacFBR	General heterozygous <i>Men1</i> deletion	9–16	Insulinoma	Indolent	Crabtree et al., 2001
Men1 ^{+/-}	C57BL/6 or 129/Sv	General heterozygous <i>Men1</i> deletion	8–12	Insulinoma, gastrinoma, glucagonoma, and mixed tumors	Indolent	Bertolino et al., 2003b
Men1 ^{ΔN/ΔN} ; RIP-Cre	B6; FVB; 129Sv	β cell-specific homozygous <i>Men1</i> deletion	5–7	Insulinoma	Indolent	Crabtree et al., 2003
Men1 ^{F/F-} RipCre ⁺	C57BL/6J	β cell-specific homozygous <i>Men1</i> deletion	6–10	Insulinoma	Moderate	Bertolino et al., 2003c
Men1 ^{LoxP/LoxP} -RipCre ⁺	Not specified	β cell-specific homozygous <i>Men1</i> deletion	12	Insulinoma	Cannot be assessed	Biondi et al., 2004
Men1 ^{F/F-} GluCre ⁺	Not specified	α cell-specific homozygous <i>Men1</i> deletion	8	Glucagonoma first, insulinoma later	Cannot be assessed	Lu et al., 2010
Glu-Cre; Men1f/f	Not specified	α cell-specific homozygous <i>Men1</i> deletion	13–14	Insulinoma	Cannot be assessed	Shen et al., 2010
Pdx1-Cre; Men1f/f	FVB; 129Sv	Pancreas-specific homozygous <i>Men1</i> deletion	10–12	Insulinoma	Moderate	Shen et al., 2009a

Table 1 Pancreatic Neuroendocrine tumors animal model. Several PanNETs mouse models have been generated by knocking out different genes in different pancreatic cells. It has been shown that different types of PanNETs arise from deleting the same gene in different pancreatic cells or impairing different gene functions in the same pancreatic cells.

1.5 Genetically Engineered Mouse Models in cancer

Cancer results from the accumulation of several genetic alterations that transform cell faith. Genetically Engineered Mouse Models (GEMMs) are the best tools to better understand the molecular mechanism of cancer development and progression and are critical to identify and evaluate new treatment strategies (Lukas E Dow, 2012) (Joerg Heyer, 2010). Compared to the traditional germline-derived mouse models, making shRNA transgenic GEMMs is faster because multiple genes can be targeted directly. The time and duration of gene suppression can be inducible, reversible, and controlled (Kelly Kersten K. E., 2017).

There are two technical approaches to produce GEMMs. The first one is the pronuclear injection of modified ESCs into a single cell of the mouse embryo. The second is injecting modified embryonic stem cells into the mouse's blastocyst. (Figure 6)

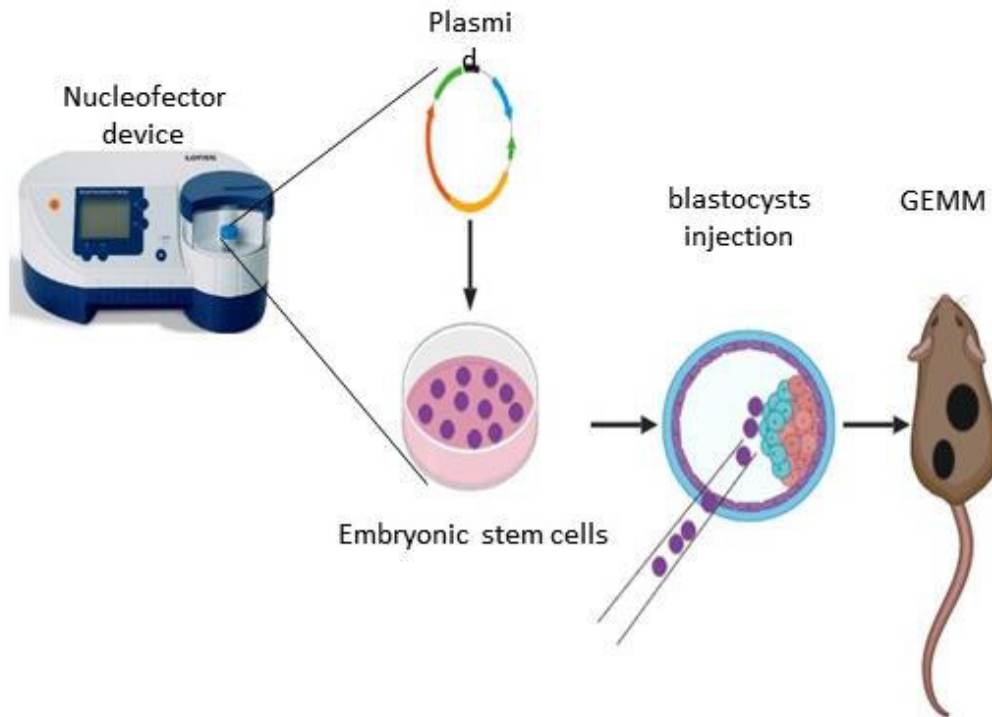


Figure 6 Schematic overview of non-germline GEMMs. To generate the chimeras with blastocysts injection, first, the Embryonic Stem Cells need to be targeted with plasmid using a nucleofector device.

1.6 Organoids

In vitro studies are still popular and widely used in cancer studies despite the availability of developing genetically engineered mouse models. To develop targeted therapies, evaluating the impact of oncogenes and tumor suppressor mutations on signaling pathways is crucial. Before organoids, 2D cell culture and genetically engineered mouse models were the only tools (Tomohiro Mizutani, 2017) (Wadosky, 2019). The newly developed method of culturing epithelial-derived cells to model organ systems *in vitro* enables scientists to replicate many organ-of-origin characteristics, recapitulate disease, and allow personalized drug screening (Else Driehuis, 2019).

1.7 Recombinase technologies

One of the most potent aspects of GEMMs is the ability to control gene expression (Yueju Wang, 2011). Recombinase technologies are based on the excision or inversion of DNA sequences between defined sites. Cre-loxP (B Sauer, 1990), FLP-FRT (K G Golic, 1989), and R-RS (H Onouchi, 1991) are examples of recombinase technologies systems where Cre and Flp are recombinases, and loxP and Frt are the identical DNA recognition sites (Yueju Wang, 2011).

1.7.1 Cre-LoxP system

Cre recombinase was first discovered as a DNA recombinase formed from the cyclization recombinase gene of bacteriophage P1 (Hyeonhui Kim ,. M.-K., 2018) (N Sternberg, 1978). Cre recombinase mediates site-specific insertions, deletions, inversions, and translocations of specific DNA sequences between two DNA fragment sequences called LoxP (Hyeonhui Kim ,. M.-K., 2018). When loxP cells express Cre, recombination occurs between the loxP sites. The Cre binds to the regions of a lox site and forms a dimer. The dimer binds to a dimer on another lox site and creates a tetramer. Then, Cre cut the ds DNA at both loxP sites (Maizels, 2013). Finally, the strands rejoin with DNA ligase. Based on loxP sites' orientation combination leads to deletion, insertion, inversion, or translocation (John C. Marecki, 2014). Cre-LoxP system is a potent system for controlling gene expression for mammalian gene editing (Micheal A McLellan, 2017). Tissue specificity for the Cre-Lox system was first achieved by the adenoviral or retroviral mediated delivery of Cre-recombinase to any particular tissue and is still a widely used method for specific tissues like lungs (H Shibata, 1997).

To achieve tissue-specific knock-down or knock-out, there is also the possibility of expressing Cre-recombinase under specific-tissue promoters

(HYEONHUI KIM M. K.-K., 2018) (Jared S. Burlison, 2008) (Figure 7). In addition, to get temporal control, the Cre-LoxP system can be combined with an inducible promoter to create an advanced inducible tissue-specific Cre system like tet-On Cre (inducible by Tetracycline or Doxycycline) (Cristina M Bäckman, 2009) (Baojie Jiang, 2016).

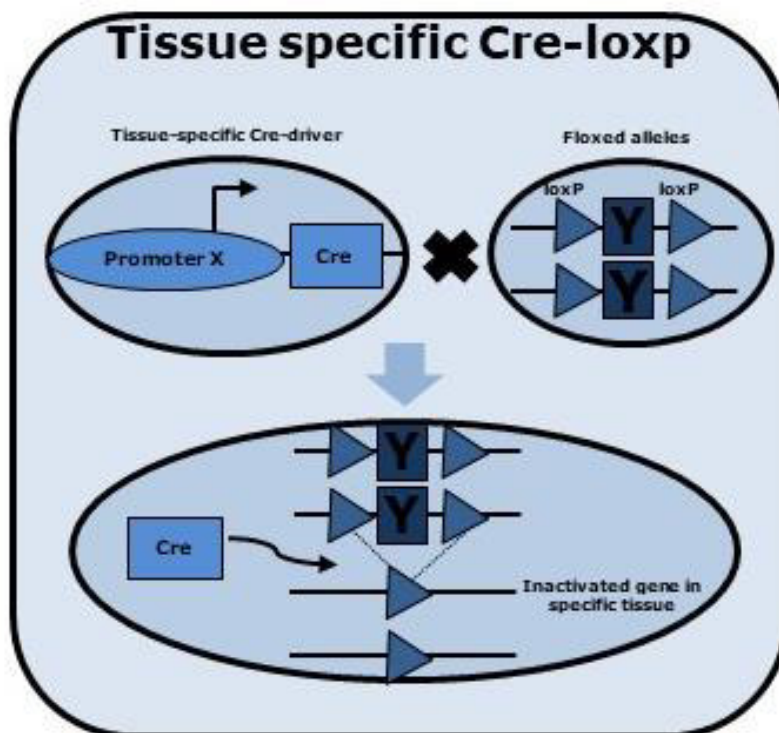


Figure 7 Schematic mechanism of tissue-specific Cre-loxP system in vivo. (edited version) (Hyeonhui Kim M. K.-K., 2018). One mouse must have a tissue-specific driven Cre gene, and the other one has loxP alleles of the gene of interest. Expression of Cre excises floxed loci and inactivates the gene of interest.

1.7.2 Flp-Frt system

Flp-Frt system is also site-specific recombinase technology like Cre-LoxP in genetics. Flp recombinase identifies target sites (Frt) that should flank the gene of interest (Yang-Nim Park, 2011)(Figure 8). Recombination can lead to excision, inversion, and DNA translocation similar to loxP sites and performs an effective recombination system in ESCs and transgenic animals (Michael Saborowski, 2014) (Nina Schönhuber, 2014).

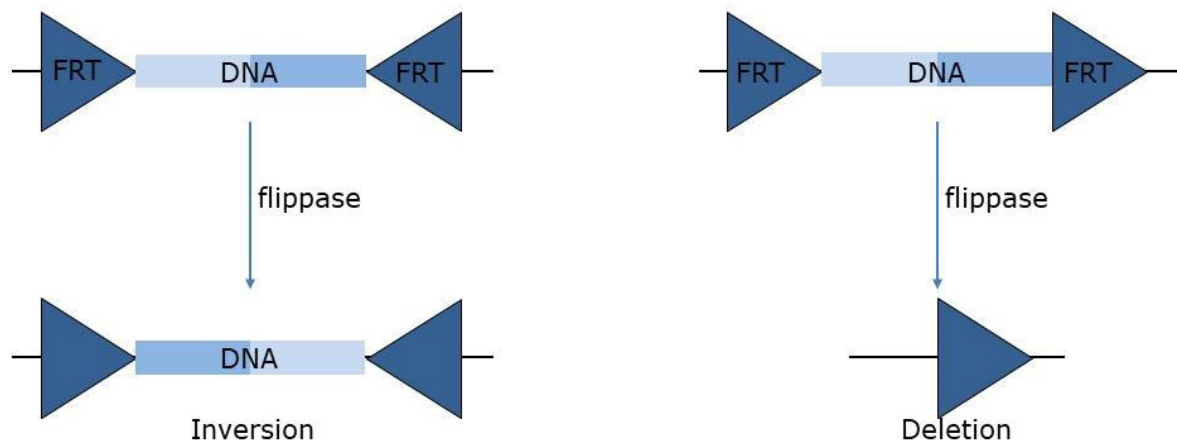


Figure 8 Schematic representation of inversion and deletion by Flp-Frt system. The left side shows two frt sites in different orientations and flp recombinase that can cause inversion. On the right side, two frt sites face the same direction, and flippase can excise the DNA in between, and only one frt site stays in the DNA (Metzler, 2014).

1.8 RNA-mediated interference

1.8.1 RNAi system

RNA interference is a cellular defense mechanism that has been discovered by Andrew Z. Fire and Craig C. Mello (A Fire, 1998). It is a rapid and effective gene silencing method in which small double-stranded RNA molecules prevent gene expression or translation with specificity and selectivity by degrading targeted mRNA. RNAi works through two types of molecules; short hairpin RNA and small interfering RNA (Rossi, 2009). The silencing process initiates when short double-stranded RNA (dsRNA) enters a cell's cytoplasm and interacts with Argonaute (AGO) protein, a component of catalytic RNA-induced silencing complex (RISC) (Doudna, 2018) (Jin'en Wu, 2020). Dicer binds to dsRNA and cleaves it into short fragments. Subsequently, an Argonaute protein cuts the passenger strand, and the guide strand stays linked to the RISC (Ronald P. van Rij, 2006). Then, the guide strand guides the RISC to the targeted mRNA for cleavage of the mRNA by AGO protein. Perfect complementarity leads to cleavage of the mRNA by RISC, and imperfect complementarity represses the mRNA's translation via RISC. In both cases, the shRNA leads to target gene silencing (Neema Agrawal, 2003).

The RNAi system can be used in antiviral responses, gene regulation, potential therapeutics for genetic diseases, and cancer treatment (Anghesom Ambesajir, 2012).

1.8.2 Inducible RNAi system

Inducible RNAi systems allow avoiding embryonic lethality or any interruption of organ development since the shRNA expression can be started after embryogenesis has been completed. Using the inducible RNAi system prevents obstacles such as non-spatial, non-temporal, off-target effect, and triggering type I immune responses. shRNA expression in non-viral or viral vectors has the advantage of longer gene silencing than synthetic siRNAs. Inducible shRNA systems are divided into irreversible and reversible, and reversible control systems are frequently used (Yi Liao, 2016). In irreversible systems, recombinases such as Cre or Flp perform one-time excision. Accordingly, the shRNA process goes under permanent expression.

In contrast, using the Doxycycline response element, the gene expression can be precisely regulated by temporal control of shRNA expression. The reversible system's expression is switched on and off at any desired point in time (Joana A. Vidigal, 2010) (Figure 9).

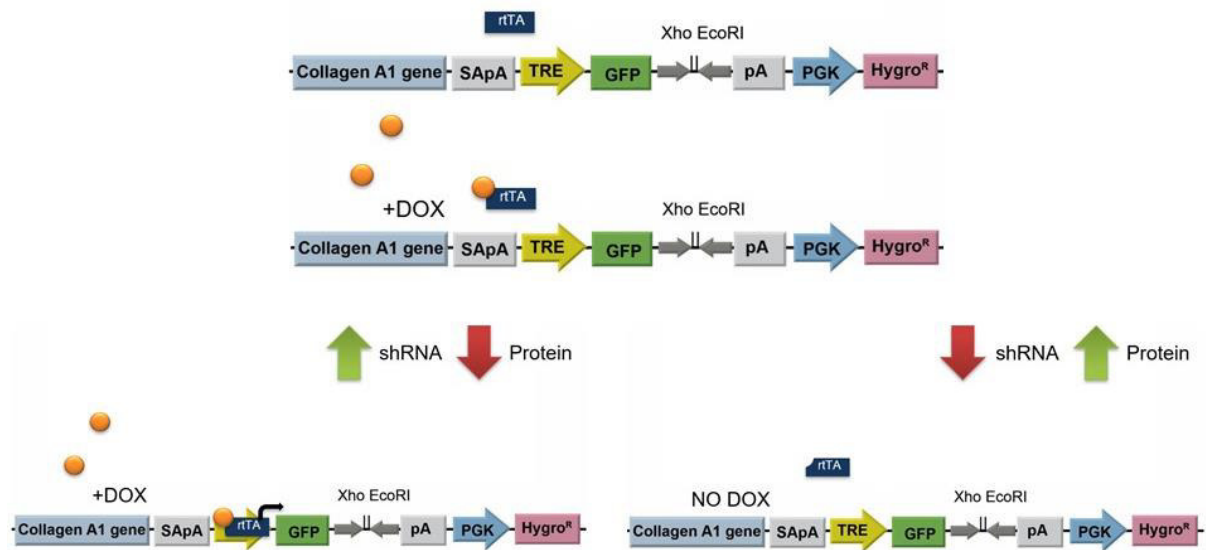


Figure 9 Schematic representation of inducible and reversible RNAi system in GEMMs. rtTA promoter is only activated in the presence of Doxycycline and has strong induction on TRE promoter. After induction of Doxycycline, rtTA induces TRE expression. TRE expression leads to GFP and shRNA expression and subsequently to protein knock-down. In the absence of Doxycycline, rtTA, TRE, and shRNA do not express, and protein expression level returns to normal. (Lukas E. Dow, 2014)

1.8.3 Application of the RNAi system in cancer

RNAi is a valuable tool in analyzing cancer's molecular mechanism and allows researchers to silence the desired genes' expression and personalize cancer therapy. So far, only two approvals of siRNA therapeutics have been achieved. One was ONPATTRO® (patisiran, ALN-TTR02) for the treatment of hereditary amyloidogenic transthyretin amyloidosis with polyneuropathy in 2018 (YuhuaWeng, 2019) (Huang, 2019). The second one was GIVLAARI™ (givosiran, ALN-AS1) for the treatment of acute hepatic porphyria (Eliane Sardh, 2019) (Manisha Balwani 1, 2019).

Tumors can be divided into five stages, and due to the post-translational gene silencing ability, the RNAi system can be effective at any stage (Catalina Méndez, 2015). CIS or stage 0 is the cancer development stage where normal cells start transforming into neoplastic cells. The main reason for this

altered behavior is the disruption of RNAi pathways (Jeffrey T DeSano, 2009). Hence, by taking advantage of the RNAi system, there might be a chance to cure cancer in the CIS stage (Maduri, 2015).

In cancer stage I-III, RNAi can prevent the formation of tumor vascularity by blocking Angiopoietin-1 (Xian-bao Liu, 2008) or by preventing cancer nutrient uptake by cancer cells or protein that support cancer cell proliferation. RNAi can be utilized to stop tumor growth by induction of apoptosis in cancer cells. Recently, it is also possible to inhibit more than one gene at the same time with the RNAi system, which paved the way for combinatorial RNAi (coRNAi) therapy. As the name indicates, it uses several shRNAs to simultaneously silence a set of genes simultaneously (Glen J McIntyre, 2011).

As stage IV and metastatic tumors are the deadliest and most complex tumors to treat, it is vital to block metastasis in many different steps such as cellular invasion, migration, and epithelial-mesenchymal transition (EMT) by blocking the responsible gene.

2 2 Materials and Methods

2.1 shRNA design, cloning, and validation

2.1.1 Design oligos for each targeted mRNA

Between 5-10, different shRNAmir have been designed using the SplashRNA website (<http://splashrna.mskcc.org/>). Oligos were ordered as 4nmol from Sigma Aldrich, and they were dissolved in 120 μ l, which gives a stock concentration of 1 μ g/ μ l. (4 nmol of 97mers have an MW of $\approx$ 30 kDa = 120 μ g). Oligos were amplified with primers containing XhoI and EcoRI restriction sites. 4 μ l of Amplified oligos in single PCRs have been confirmed by running them on 2% agarose gel. Pool 25 μ l from each PCR reaction for subsequent column purification. Use the QIAquick PCR purification kit according to the manufacturer's instructions.

Temperature (°C)	Time	Cycles
94 (Initial Denaturing)	2 min	1
94 (Denaturing)	15 s	
54 (Annealing)	30 s	33
68 (Extension)	25 s	
68 (Final Extension)	5 min	1
4 (Storage)	∞	-

Reagent	Volume (µl)	Final
ddH ₂ O	33,0	
10x PCR Buffer	5,0	1X
10x PCR Enhancer	5,0	1X
MgSO ₄ (50 mM)	1,0	1 mM
dNTP (10 mM)	1,5	0.25 mM
For Primer (10 µM)	1,5	0.3 µM
Rev Primer (10 µM)	1,5	0.3 µM
Template 0.01 -0.1 ng/µl	1,0	
Pfx DNA polymerase	0.5	1.25 U
Total	50,0	

2.2 Cloning shRNAs into the MlpE (viral vector) vector to check protein knock-down efficiency

Amplified shRNAs with *XhoI*/*EcoRI* restriction sites were digested with *XhoI*-HF and *EcoRI*-HF restriction enzymes for 3-4 hours at 37°C. According to the manufacturer's protocol, the digest is resolved on a 2% (wt/vol) agarose gel, and the desired 125 bp band is gel purified using the QIAquick Gel Extraction Kit.

The targeted vector was digested with *EcoRI*-HF and *XhoI* for four hours following heat inactivation of restriction enzymes at 70 °C for 15 min and treatment with 1U (1 µl) CIP Alkaline Phosphatase for one hour and column purification was done using Qiagen PCR purification kit.

The ligation was set up overnight at 16°C using high-concentrated T4 Ligase with an Insert (4 ng): target Vector (100 ng). After transforming the ligation into fast and go bacteria and isolating the plasmid DNA, the correct extracted plasmid was verified using the ZUB-SEQ-SH sequencing primer (5'TGTTTGAATGAGGCTTCAGTAC-3').

2.2.1 Test shRNA protein knock-down efficiency

2.2.1.1 Calcium-phosphate transfection

Calcium phosphate transfection was used for transfecting and testing shRNAs in the *PlatE* cell lines. "Solution A" was mixed with 20 µg of plasmid DNA, 62.5 µl of 2M CaCl₂, and H₂O, and "Solution B" (2XHBS) was kept in a 15ml tube. Solution A was then added to solution B dropwise while blowing bubbles into the solution. The mixture was mixed well by blowing bubbles. The mixture was incubated at room temperature for 15-20 mins and then added to packaging cells dropwise. The media was changed on the plate gently after the next day.

2.2.1.2 Viral infection

The viral supernatant was harvested 36hrs after transfection by passing the virus through a 45µm syringe filter and adding Polybrene to transduced cells. For each shRNA, chose a transduced population that contains 5–20% GFP-positive cells and used Puromycin for positive selection until they reached the 98-100% GFP-positive cells.

2.2.1.3 Measuring protein knock-down efficacy by western blot

RIPA buffer (0.5% sodium deoxycholate, 0.1% SDS, 1M 50mM Tris-HCl, 5M 150mM NaCl, 1% Triton x100 or Np-40) was used to isolated protein from infected NIH3T3 cells and 40-50 µg protein was loaded into 8-12% SDS gels to measure shRNA protein knockdown.

2.3 subcloning of shRNAs into cTGM vector for targeting ES cells

Cloning of vector for targeting into ES cells was done in CTGM vector at *XhoI/EcoRI* for the first shRNA and *MluI/BssHI* to insert the second shRNA.

1. For the first shRNA, the shRNA was cut out with *XhoI/EcoRI* from the MlpE vector. For the second shRNAs, shRNA in the MlpE vector was amplified with *BssHI* and *MluI* primers and respectively digested with the *BssHI*-HF and *MluI*-HF enzymes.
2. The ligations were set up overnight at 16°C using high-concentrated T4 Ligase with an Insert (4 ng): target Vector (100 ng).
3. After transforming ligations into fast and go bacteria and isolating the plasmid DNA, the correct extracted plasmid DNAs were verified using a primer in the GFP region.

2.4 Cloning

2.4.1 Polymerase chain reaction

Q5 High-Fidelity DNA Polymerase (NEB) kit was used for all cloning. The reactions were in an Eppendorf thermal cycler, and for all PCR purifications, the Qiagen PCR purification kit or gel extraction kit was used.

2.4.2 Restriction digestion

For all restriction digestions, 20U NEB enzymes in the total volume of 25 or 50 μ l with 1 μ g of PCR product or 4-6 μ g of plasmid DNA for 3-4hrs in proper temperature (following manufacture protocol) in heat block was used.

2.4.3 Dephosphorylating

All the digested plasmids were dephosphorylated to prevent plasmid religation by adding NEB Alkaline Phosphatase Calf Intestinal (CIP) into the digestion mix and incubating for 1hr at 37°C.

2.4.4 Ligation

Ligations were set up with NEB 20U of T4 DNA ligase and the buffer in the total volume of 20 μ l with 100ng of the plasmid and 4ng of the insert at 16°C.

2.4.5 Transformation

All the transformations 50-100 μ l of bacteria prepared using "Mix & Go" E.coli transformation kit and buffer set and 5-10 μ l of the ligation were mixed and plated onto the LB agar plates. Plates were incubated at 37°C, and the colonies were picked the following day.

2.4.6 Mini preparation for plasmid DNA isolation

The picked colonies from LB agar plates were inoculated into 3-4 ml of LB media containing ampicillin. This mixture was incubated overnight on a

shaking incubator at 37°C at 200rpm. The next day Zyppy Plasmid Miniprep Kit was used to isolate plasmid DNA according to the manufacturer's protocol.

2.4.7 Midi preparation for plasmid DNA isolation

Plasmid midiprep was done using endotoxin-free plasmid DNA purification according to the manufacturer's protocol.

2.5 Embryonic Stem cell (ESC) culture

ESCs were maintained in M15 + leukemia inhibiting factor media on irradiated mouse embryonic fibroblast (MEF) according to established protocol. (Lukas E Dow, 2012)

2.5.1 ESC cell plating

ES cells were cultured in 6 well plates coated with gelatin and mouse embryonic fibroblasts. For gelatin coating, 2ml of 0.1% gelatin was incubated for 30 minutes at 37°C on 6 well plates in an incubator. Gamma-Irradiated DR4 MEF was plated on the 6 well plates to cover the plate's whole surface with mouse embryonic fibroblasts. Two days following the plating of MEF, MEF plates were washed with PBS, and ES cells were thawed and plated over the MEFs. ES cell media was changed every day.

2.5.2 Targeting of ES cells

For targeting ESCs, well-formed colonies were used. Trypsin, PBS, and ESC media were warmed up to room temperature before use. Supplemented Mouse ESC Nucleofector Solution was Pre-warmed to room temperature.

Also, tubes containing the targeting plasmid DNA and FlpE plasmid DNA were prepared. For resuspension of ESCs after targeting ES cell media in a Falcon tube was Prewarmed to 37°C for each targeting plasmid. To separate MEFs from the ESCs before targeting, ESCs were trypsinized, and trypsin was neutralized with ESC media.

Cells were collected in a tube and were plated onto another feeder cells coated plate. The plate of ESCs with MEF was kept in the incubator for 30 min to allow MEF cells to reattach to the plate. Nonattached ESCs were then collected by gentle pipetting. Collected ESCs were mixed in a Falcon tube and were counted. 2 million cells were used for each target. ESCs were centrifuged down for 5 min at 250xg, and cells were washed twice with PBS. They were then re-suspended in supplemented mouse ESC nucleofection solution. They were mixed, and the cell suspension was added to plasmid DNA in mouse ESC nucleofection solution and was mixed by pipetting. The mix was transferred into the nucleofection cuvette carefully to avoid air bubbles between the electrodes. Nucleofection was done using program A-023 on the nucleofector device. Pre-warmed ESC media was added to the cuvette immediately but slowly and transferred to the pre-warmed media tube.

ESCs were plated in MEF coated 6 well-plate. The medium was changed 24 hrs after nucleofection. 48 hours after nucleofection, hygromycin B was added to the ESC medium. ESCs were selected on Hygromycin for 8-10 days. ESC colonies with a compact, regular 3D morphology were picked. For picking colonies, ESCs were washed with PBS once and kept in 2 ml of PBS. 96 well plate with trypsin was prepared. Colonies were picked carefully and transferred to one well of the 96 well-plate with trypsin. Colonies were trypsinized for 7 minutes at 37°C, and single cells were made. Each clone

was plated onto 24 well plates containing 1ml ESC media with Hygromycin. The next day media was changed to ESC media without Hygromycin.

2.6 Blastocyst injection

Blastocyst injection was performed by our collaborator Helmholtz-Zentrum für Infektionsforschung Braunschweig.

2.7 Genomic DNA

Genomic DNA isolation was done using tail lysis buffer with proteinase K. Tissue/tail, or cell pellet was collected, and a 255µl tail lysis master mix was added to each sample. Samples were incubated overnight at 56°C. The following day, samples were heated for 5 min at 96°C, and saturated NaCl was added to the samples and was mixed. Samples were centrifuged at maximum speed for 10 minutes. The supernatant was transferred into a new tube containing isopropanol and was mixed by inverting. Samples were again centrifuged at maximum speed for 10 minutes. The supernatant was then discarded, and they were washed with cold 70% ethanol. DNA pellets were dissolved in an appropriate volume of Tris-EDTA buffer. 2.8 Genotyping PCR

To identify the correct genotype, PCR was performed on the extracted DNA samples with a G2 master mix with the following primers was prepared.

PCR products were analyzed by running on 2% agarose gel. Mutant 295bp; Wild type 220bp.

Primer	Sequence
Col1A-For	AATCATCCCAGGTGCACAGCATTGCGG
Col1A-Rev	ACTTGAGGGCTCATGAACCTCCCAGG
SAdpA-Rev	ATCAAGGAAACCCTGGACTACTGCG

Component	Volume
2X G2 Green master	12.5 μ l
Primers	0.5 μ l
DNA template	2.0 μ l
H2O	up to 25 μ l

Temperature ($^{\circ}$ C)	Time	Cycles
94 (Initial Denaturing)	2 min	1
94 (Denaturing)	25 s	35
58 (Annealing)	25 s	
72 (Extension)	30 s	
72 (Final Extension)	5 min	1
4 (Storage)	∞	-

2.9 Flow cytometry

After cell trypsinization, a small aliquot was taken for FACS analysis. Cells were analyzed.

2.10 Histology

2.10.1 Sample preparation, paraffinization, and blocking

Mouse organs were harvested, and after fixation in 4% paraformaldehyde solution for 24 hrs, they have stored another 24 hrs in PBS at 4 $^{\circ}$ C. Then,

paraffin embedding was done using a Leica TP1020 automated embedding machine. For paraffinization, samples were kept in a cassette in hot liquid paraffin for one hour. Samples were embedded in paraffin, and slides of tissues were cut for histological investigations.

2.10.2 Hematoxylin and Eosin staining

All the H&E stainings were done with our collaborator in Heidelberg (CMCP & wissenschaft.-histologisches Labor, Pathologisches Institut)

2.10.3 Immunohistochemistry staining

Deparaffinization started by washing the section in the following solutions for five min: two times in xylene, two times in 100% ethanol, once in 95% ethanol, once in 70% ethanol, once in 50% ethanol, and once in distilled water. Once deparaffinization was done, samples were immersed in sodium citrate antigen retrieval buffer at 95°C for 20 min. Then, samples were taken out and let cool for 20 min. Slides were washed for 5 min with TBST on the shaker. Endogenous peroxidase was deactivated by putting the slide in freshly prepared 3% H₂O₂ in H₂O for 10 min. Afterward, slides were washed three times with TBST for 3 min per wash on a shaker. The papPen was used to circle the cutting samples on the slides. Next, samples were blocked with the blocking solution for 1 hr. samples were incubated with primary antibody (in 5% serum TBST) at 4°C overnight in a humidified chamber. The following day, the slides were washed three times with TBST for three min per wash on the shaker. Subsequently, a secondary antibody (in 2% serum TBST) was applied for 30 minutes at room temperature. Slides were washed three times with TBST for three min per wash on the shaker. Then, slides were incubated with HRP-Streptavidin plus for 30 minutes at room temperature. Washing steps were repeated three times

with TBST for three min. DAB solution was added to the slide, and the color-changing was monitored under the microscope. Before counterstaining the slides with hematoxylin for 30 seconds, they were rinsed with dH₂O two times for five min. Then Slides were rinsed once in running lukewarm water for 5 min and again in dH₂O for 5 min. The last step was dehydrating the slides by incubating them for 30 seconds in the following solutions: EtOH 40%, EtOH 70%, EtOH 96%, and 5 min EtOH 100%, EtOH 100%, xylol, and xylol. Finally, the slides were mounted with entellan.

2.11 Cell culture

2.11.1 Thawing cell

Fast thawing of cells at 37°C was followed by immediately transferring the freezing vial into the tube containing warm. The cells were pelleted and were kept in a CO₂ incubator.

2.11.2 Freezing cells

After Spinning down the trypsinized cells, the pellet was re-suspended into the freezing media, and after two days in -80, they were transferred into liquid nitrogen.

2.11.3 Cell cultivation

All the cells were cultured using DMEM media with FBS and Penicillin/Streptomycin in dishes in a CO₂ incubator at 37°C. The cells were sub-culture when they were 80-90% confluent. For sub-culturing, trypsin was incubated with the cell for 5-8min at 37°C and neutralized by adding full media. Afterward, cells were plated in an appropriate ratio.

2.11.4 Generation of pancreas 3D cells line (organoid)

A small piece of the pancreas was cut with a razor blade and rinsed with cold PBS several times to eliminate digestive enzymes. After aspirating the PBS, small pieces of the pancreas were re-suspended in 100µl of Matrigel and plated as two 50µl drops in a pre-warmed 24 suspension well-plate. The plate was kept in at 37°C incubator until the Matrigel solidified. 500 µL initiation media was added to each well for the first 3-4 days or until the organoid formed a sphere.

Component	Stock conc.	Working concentration	Amount for 1ml
DMEM/F12 Adv.			833ul
HEPES	1M	10mM	10ul
Pen/Strep/Ampho	100x	1x	9ul
Glutamin	100x (200mM)	1x	9ul
A8301	500uM	0,5uM	1ul
hEGF	50ug/ml	50ng/ml	1ul
rhFGF-10	50ug/ml	100ng/ml	2ul
Gastrin I	10uM	10nM	1ul
rhNoggin	100ug/ml	0,1ug/ml	1ul
NAC	0,5M	1mM	2ul
Nicotinamid	1M	10mM	10ul
R-Spondin I	1,0	0,1	100ul
B27 Supplement	50x	1x	20ul
Wnt	100ug/ml	100ng/ml	1ul
Y-27632	10mM	10uM	1ul

2.11.5 Splitting organoids

After aspirating the media from each well, organoids were washed twice with PBS. Then, 500 μ L TrypLE was added to each well, and the Matrigel was disrupted by pipetting up and down at least and keeping them into 37°C incubator three min. The disruption step was repeated three times. To neutralize TrypLE, organoids with TrypLE were collected and added to DMEM/F12 Adv. Media and spun it down at 300g for five min. After washing the pellets twice with PBS, they were used for further culture or freezing.

2.11.6 Freezing and thawing organoids

After aspirating the media from each well, organoids were washed twice with PBS. Then, 500 μ L TrypLE was added to each well, and the Matrigel was disrupted by pipetting up and down at least and keeping them into 37°C incubator three min. The disruption step was repeated three times. To neutralize TrypLE, organoids with TrypLE were collected and added to full DMEM/F12 Adv. Media and cells were pelleted by centrifugation at 300g for five min. After washing the pellets twice with PBS, they were re-suspended in organoid freezing media and were kept two days in -80°C.

For thawing, the frozen vial was kept at 37°C and waited until the ice was fully thawed. Then, they were transferred in a falcon tube filled with cold DMEM media, and the last step was, spinning them down at 300g at 4°C for 5 minutes.

Component	Final
DMEM/F12 Adv.	40,0%
FCS	50,0%
DMSO	10,0%

2.11.7 Orgnoid transfection

Two days before transfection, organoids were plated to be confluent at the time of transfection. After aspirating the media from each well, organoids were washed with PBS. Then, TrypLE was added to each well, and the matrigel was disrupted was kept at 37°C. The disruption step was repeated three times. Organoids with TrypLE were collected and added to full DMEM/F12 Adv. Media and spun it down at 300g for five min

During the centrifugation, the transfection mixtures were mixed following the Lipofectamine 2000 protocol:

1. One tube with 50ul Optimum plus DNA, mix well.
2. One tube with 50ul Optimum plus lipo2000, mix well.

Both mixtures were incubated for about 5 min at room temperature. Subsequently, they were mixed by adding the DNA mix to Lipo-mix. For confluent organoid well of 24 well-plate, 1ug DNA plus 2ul lipo2000 is safe for the cells.

After centrifugation, the supernatant was removed, and pellets were re-suspended in 300ul full organoid media and were transferred to a pre-warmed 48 well-suspension plate. Transfection mixtures were added

dropwise to the cells, and the plate was wrapped with parafilm and centrifuged at 600g, 32°C for 1 hr.

After centrifugation, they were incubated in a 37°C incubator for 1 hr. Subsequently, the pellets were re-suspended in Matrigel after washing the cells with DMED F12 and spinning them down at 4°C, 300 g for 5 min.

2.11.8 Organoid's Blasticidin selection

48 hrs after transfection, Blasticidin was added to the media. Organoids were splitted 1:1, and fresh media was added with Blasticidin for at least 72 hours.

2.12 Animal experiment

All animal experiments were performed according to protocols approved by the local authorities (The Lower Saxony State Office for Consumer Protection and Food Safety, LAVES). Animals were maintained under standard housing conditions with a 12-hour day-night cycle and access to food and water. All interventions were conducted during the day cycle. All the experiments were planned according to animal protocol with authorization number 33.12.42502-04-18/2909.

Chimeric mice were generated by blastocyst injection at the Helmholtz-Zentrum für Infektionsforschung in Braunschweig

1. CAGs-LSL-rtTA3-IRES-mKate2:tm/wt:PGK-TGMshMenin- TRE-Hygrot - m/wt.
2. CAGs-LSL-rtTA3-IRES-mKate2:tm/wt:PGK-TGMshMenin-shDaxx-TRE -Hygrot -m/wt.

3. CAGs-LSL-rtTA3-IRES-mKate2:tm/wt:PGK-TGMshMenin-shPten- TRE-Hygrot-m/wt.

B6. FVB-Tg (Pdx1-cre) 6Tuv/J (Stock# 014647) mice were purchased from The Jackson Laboratory.

After cross-breeding following mice, pups' diet was switched to doxycycline-enriched food (625 mg/kg, Altromin, Lage, Germany) 5-7 days after birth. Endpoint criteria included sacrificing at certain ages, signs of ill health and/or palpable tumors. Mice were monitored twice a week to measure the weight or any signs of ill health.

1. CAGs-LSL-rtTA3-IRES-mKate2:tm/wt: PGK-TGMshMenin-TRE-Hygro-tm/wt, Pdx1:tg/wt
2. CAGs-LSL-rtTA3-IRES-mKate2:tm/wt: PGK TGMshMenin; shDaxx-TRE-Hygro-tm/wt, Pdx1:tg/wt
3. CAGs-LSL-rtTA3-IRES-mKate2:tm/wt: PGK-TGMshMeninshPten-TRE-Hygro-tm/wt, Pdx1:tg/wt

2.13 Glucose measurement

A drop of tail blood after mouse euthanization was used to measure the blood glucose level using ACCU-CHEK.

2.13 Serum insulin measurement

After euthanizing the mice, whole-body blood was collected directly from the heart. Blood serum was separated by centrifugation at 4°C, 2000 g for 20 min. Serum insulin was measured by following the Ultra-Sensitive Mouse Insulin Kit (CRYSTAL CHEM # 90080), the manufacturer's protocol.

2.14 Statistical analysis

To analyse the data and create comparison graphs and charts, GraphPad Prism 7 software was used. Adobe Photoshop was used to crop the images, and the pictures were modified and assembled in Adobe Illustrator (Adobe; San Jose). FlowJo (FlowJo LLC; Oregon) was used to analyze flow cytometry data. ImageJ (ImageJ 1.50i, NIH; Bethesda) was used to measure islet cell size and the percentage of GFP and CPA positive cells.

2.15 Reagents

2.15.1 Western blot

2.15.1.1 12% SDS resolving gel buffer

2.15.1.2 SDS stacking gel buffer

Component	Volume
30% Acrylamid	4 mL
H ₂ O	3.4 mL
1.5M Tris HCl pH 8.8	2.6 mL
10% SDS	98 µL
TEMED	25 µL
10% APS	74 µL

Component	Volume
30% Acrylamid	352.5 μ L
H ₂ O	1410.1 μ L
0.5M Tris HCl pH 6.8	585.5 μ L
10% SDS	22.6 μ L
TEMED	7.1 μ L
10% APS	22.6 μ L

2.15.1.3 Running buffer

- 190 mM Glycine
- 25 mM Tris
- 0.1% SDS
- pH 8.3

2.15.1.4 Transfer buffer

- 190 mM Glycine
- 25 mM Tris
- 20% Methanol
- pH 8.3

2.15.1.5 Blocking buffer

- 5gram powdered mil in 100ml TBST

2.15.1.6 RIPA buffer

- 10 mM Tris-HCl pH 8
- 1 mM EDTA
- 0,5 mM EGTA
- 1% Triton X-100

- 0,1 Sodium deoxycholate
- 0,1% SDS
- 140 mM NaCl

2.15.2 Es-Cell culture

2.15.2.1 Es-Cell culture media (M15 media)

- To make the 100x β -ME, 37 μ l of β -ME was added to 50 ml of PBS.

Component	Volume
Knockout DMEM	500ml
ES-Cell Serum	90ml
100X Penicillin- Streptomycin- Glutamine	6ml
100x beta- mercaptoethanol (β -ME)	6ml
ESGRO-LIF	60 μ l

2.15.2.2 Es-cells freezing media

- M15 medium 50%, Es-cell serum 40% and DMSO 10%.

2.15.2.3 Feeders cells media

Component	Volume
DMEM	500ml
Serum	50ml
100x Penicillin-Streptomycin	5.5ml

2.15.3 DNA isolation buffers

Component	Volume
Lysis buffer (for DNA isolation)	10mM Tris/ HCl
	400mM NaCl
	2mM EDTA
	pH 8.2
Tail Lysis (DNA isolation) master mix	200µl Lysis buffer
	22.6 µl
	50µl 10% SDS
	5µl Protein kinase K (20U)

2.16 Antibodies

2.16.1 Primary antibodies

Antibody	Supplier	Cat No.
Menin	Biomol	#A300-105A
Daxx	MyBioSource	#MBS8509410
Pten	cell signaling	9188S
GFP	cell signaling	2956
tRFP	Evrogen	#AB233-234
CK19	Abcam	#ab133496
CPA1	R&D	# AF2765
Chromogranin A	Abcam	#AB45179
Synaptophysin	Invitrogen	#MA5-14532
Insulin	cell signaling	#4590S
Glucagon	cell signaling	#27060S

2.16.2 Secondary antibodies

Antibody	Supplier	Cat No.
goat anti-rabbit IgG	Life technolo- gies	#B2770
goat anti-mouse IgG-HRP	Santa Cruz Biotechnology	#sc-2005
mouse anti-mouse IgG-HRP	Santa Cruz Biotechnology	#sc-2357

2.17 Oligoe sequences

Oligo Name	Oligo Sequence
Men1-1	CCACCCTCATCCTCTAATTCAATAGTGAAGCCACAGATGTATTGAATTAGAGGATGAGGGTGA
Men1-2	CCCTGTTTTGTACATAGATTTATAGTGAAGCCACAGATGTATAAATCTATGTACAAAACAGGA
Men1-3	CCACCCTCATCCTCTAATTCAATAGTGAAGCCACAGATGTATTGAATTAGAGGATGAGGGTGA
Men1-4	CTAAATGCATTTTGTAAAGAAATAGTGAAGCCACAGATGTATTTCTTAACAAAATGCATTTAT
Men1-5	CAAGATAAATGCATTTTGTAAATAGTGAAGCCACAGATGTATTAACAAAATGCATTTATCTTT
Men1-6	AAAAGATAAATGCATTTTGTATAGTGAAGCCACAGATGTATAACAAAATGCATTTATCTTTC
Men1-7	CCCTGTTTTGTACATAGATTTATAGTGAAGCCACAGATGTATAAATCTATGTACAAAACAGGA
Daxx-1	ATAAGATCTATGTGTACATTAATAGTGAAGCCACAGATGTATTAATGTACACATAGATCTTAG
Daxx-2	CCAGCATCATTGACTTGATGATAGTGAAGCCACAGATGTATCATCAAGTACAATGATGCTGT
Daxx-3	ATAAGATCTATGTGTACATTAATAGTGAAGCCACAGATGTATTAATGTACACATAGATCTTAG
Daxx-4	CCAGCATCATTGACTTGATGATAGTGAAGCCACAGATGTATCATCAAGTACAATGATGCTGT
Daxx-5	ATGTTAGGAAACAGCTATATAATAGTGAAGCCACAGATGTATTATATAGCTGTTTCCTAACAG
Daxx-6	CGATGGTGTTCCTTTTGACAAATAGTGAAGCCACAGATGTATTTGTCAAAAGAAACACCATCA
Daxx-7	ATCCGGCAGTGTATTTATAAGATAGTGAAGCCACAGATGTATCTTATAAATACACTGCCGGAG

2.18 Enzymes

Enzyme	Company
DNaseI	Sigma-Aldrich®
Q5® High-Fidelity DNA Polymerase	New England Biolabs
Collagenase IV	Sigma-Aldrich®
Restriction endonucleases	New England Biolabs
T4 DNA-Ligase	New England Biolabs
T4 Polynucleotide kinase	New England Biolabs

2.19 Softwares

Software	
GraphPad prism 7	GraphPad Software; San Diego
SnapGene	03.03.2004
FlowJo	FlowJo LLC; Oregon
ImageJ	
Adobe Illustrator	Adobe; San Jose
Adobe Photoshop	

2.20 Instruments

Bacterial incubator BM 600	Memmert
Ice machine	Ziegra
Analytical digital weighing balance (BP 221 S)	Sartorius
Flow cytometer FACS Canto™ II	Becton Dickinson
Flow cytometer FACS LSR II	Becton Dickinson
GelDoc 1000	BioRad
Laboratory Fridge	LIEBHERR
Ligation bath (Kälte-Umwälzthermostat F12)	JULABO
Magnetic stirrer IKAMAG® RCT	IKA®-Werke
Micropipette (10 µl, 100 µl, 1000 µl)	Eppendorf
Microscope (Wilvert S)	Hund
Micro-tabletop centrifuge (5415 C)	Eppendorf
A microtome (Rotationsmikrotom HM335E)	MICROM
Multifuge™ X1 centrifuge	Heraeus™
Automated tissue embedding system TP 1020	Leica Biosystems
Cooling centrifuge (Avanti J-25)	Beckman Coulter
Cooling centrifuge (5417 R, with Rotor FA-45-24-11)	Eppendorf
Cooling centrifuge (Megafuge 1.0R)	Heraeus
Rotor (JA-10, JA-25.50)	Beckman Coulter
Sterilbank 164 HERA safe	Heraeus
NanoDrop™ Lite Spectrophotometer	Thermo
ProFlex PCR System	Applied Biosystems

SDS-PAGE apparatus	BioRad
Thermocycler (Thermomixer Comfort 1,5 ml)	Eppendorf
Nalgene centrifuge tubes /-for cooling centrifuge (Avanti J-25)	Nalge Nunc International
Nalgene® Cryo 1°C "Mr. Frosty" Freezing Container	Nalge Nunc International
Paraffin-Embedding system (TES 99)	MEDITE
Forceps	Carl Roth®
Pipetting aid	Eppendorf
Cell culture incubator (Heracell™ VIOS 160i)	Thermo
AMAXA Nucleofector II	Lonza
Bacterial shaker (SM – 30 and TH 30)	Edmund Bühler
Vortex-Genie® 2 2 Shaker	Scientific Industries
Weighing balance (LC 220 S)	Sartorius
Waterbath	GFL
Waterbath for mounting paraffin sections	Electrothermal
Hamacytometer	Brand

2.21 Consumables

Aspiration pipette	Sarstedt
Costar® 96 well Platte, UV-transparent, (Flat bottom)	Corning®
Coverslip (24×50 mm)	Menzel-Gläser
Embedding Cassette	MEDITE
Filter paper	Whatman
SuperSignal™ West Femto Maximum Sensitivity Substrate	ThermoFisher
Amersham ECL Western Blotting Detection Reagent	GE
Blotting membranes, Amersham™ Hybond™-N+	GE
Flow cytometry tubes (5 ml, PS)	Sarstedt
Serological Pipettes (5 ml, 10 ml, 25 ml)	Sarstedt
Cell culture plate (96 well)	Sarstedt
Cell culture plate (6 well, flatbottom)	Sarstedt
Centrifuge tubes (50 ml) (PP)	Greiner Bio-One

Centrifuge tubes (15 ml) (PP)	Sarstedt
Nylon-cell filter (40 µm, 100 µm)	Becton Dickinson
Coverslip (76×26 mm)	Menzel-Gläser
Parafilm M (PM-996)	Bemis
Pipette tips (10 µl, 100 µl, 1000 µl, mit und ohne Filter)	Sarstedt
Reaction microtube (1,5 ml, 2 ml)	Sarstedt

2.22 Chemicals and kits

Acrylamid/Bisacrylamid,(30%/0,8%) Rotiphorese 30	Carl Roth®
Adenosine triphosphate (ATP)	Sigma-Aldrich®
Agarose (UltraPure™)	Gibco®
Ammonium persulfate (APS)	Sigma-Aldrich®
Ampicillin	Sigma-Aldrich®
β-Mercaptoethanol (2-Mercaptoethanol)	Sigma-Aldrich®
DMSO (Hybrimax)	Sigma-Aldrich®
dNTPs	New England Biolabs
DTT (Dithiothreitol)	Sigma-Aldrich®
EDTA (Ethylendiamintetraacetat)	Sigma-Aldrich®
Eosin Y Lösung	Sigma-Aldrich
DMEM + GlutaMAX™-I	Life Technologies (Gibco®)
BSA (Bovines Serumalbumin) (10 mg/ml)	Sigma-Aldrich®
ddH ₂ O (Ampuwa®)	Fresenius Kabi Deutschland
DNase	Sigma-Aldrich®
Dextrose	Sigma-Aldrich®
DMEM + GlutaMAX™-I	Life Technologies (Gibco®)
Glycine	AppliChem
HCl	Merck
Yeast extract	Carl Roth®
Isopropanol (2-Propanol, absolute)	J. T. Baker
K ₂ HPO ₄	Merck
KCl	Merck

KHCO ₃	Sigma-Aldrich
Roti®-Mark BI-PINK protein ladder	Carl Roth
DMSO (Hybrimax)	Sigma-Aldrich®
dNTPs	New England Biolabs
DTT (Dithiothreitol)	Sigma-Aldrich®
EDTA (Ethyldiamintetraacetat)	Sigma-Aldrich®
Eosin Y Lösung	Sigma-Aldrich
Acetic acid (99%)	J. T. Baker
Ethanol (absolute)	J. T. Baker
Midori Green	Nippon genetics
FCS (fetal calf serum)	Life Technologies (Gibco®)
Glycerol	Sigma-Aldrich®
1 Kb Plus DNA Ladder	Invitrogen
Lipofectamine® 2000 Transfection Reagent	Invitrogen
Mouse ES Cell Nucleofector® Kit (25 RCT)	Lonza
Puromycin	Sigma Aldrich
6X DNA gel loading dye	Thermo
EmbryoMax® 0.1% Gelatin Solution	Epicenter
GoTaq® G2 Master Mix	Promega
MgCl ₂ (Magnesium chloride)	Merck
MgSO ₄ (Magnesium sulfate)	Merck
Dry milk powder	Serva
Qiagen PCR purification kit	Qiagen
Qiagen gel extraction kit	Qiagen
Histostain-Plus Streptavidin-Peroxidase	Life technologies
Mix & Go! (competent bacteria kit)	Zymo research
Mayers Hematoxylin	Merck
MES (2-(N-Morpholino)ethansulfonsäure)	Sigma-Aldrich®
Methanol	J. T. Baker
NaCl	Th. Geyer
Na ₂ CO ₃ (Sodium carbonate)	Merck
Na ₂ HPO ₄ (di-Sodium hydrogen phosphate)	Carl Roth®
NaH ₂ PO ₄ (Sodium dihydrogen phosphate)	Merck
NaN ₃ (Sodium azide)	Merck

NaOH	Merck
Tween 20	Serva
ESGRO- LIF (ESG1107)	Merck
Dox-enriched food (625 mg/kg)	Altromin
180k mouse arrays (#AMADID 027411)	Agilent Technologies
NucleoSpin Tissue Kit (#740952.50)	Macherey-Nagel
ZymoPURE™ II Plasmid Midiprep	Zymo research
NH ₄ Cl (Ammonium chloride)	Sigma-Aldrich®
Nonidet-P40 (NP-40)	Sigma-Aldrich®
Paraformaldehyde (PFA)	Applichem
PBS (10×)	Life Technologies (Gibco®)
Penicillin-Streptomycin	Biochrom
Penicillin-Streptomycin-Glutamine	Life Technologies (Gibco®)
Peptone, trypsin extract	Carl Roth®
Quick Start™ Bradford 1× Dye Reagent	Biorad
DMEM	Life Technologies (Gibco®)
SDS (sodium dodecyl sulfate)	Sigma-Aldrich®
Select Agar	Invitrogen
Knockout-DMEM	Life Technologies (Gibco®)
TE-buffer (TRIS/EDTA-Puffer), pH8	Thermo Fisher Scientific
TEMED (N,N,N',N'-Tetraethylethylendiamin)	Sigma-Aldrich®
TRIS (Tris(hydroxymethyl)-aminomethane)	Applichem
Triton X 100	Sigma-Aldrich®
Trypanblue-stain (0,4%)	Sigma-Aldrich®
Trypsin	Life Technologies (Gibco®)

3 Results

3.1 Design and validation of the shRNAs for the generation of transgenic mice.

Designing and validating a panel of oligos against *Men1*, *Daxx*, and *Pten* were the first steps of generating shRNA transgenic mice. NIH3T3 cells were transduced with retroviral vectors in which the expression of shRNAs was linked to GFP. To choose the most potent shRNAs low MOI (5-20%) transductions were followed by puromycin selection for five to seven days until the GFP expressed cells showed more than 95% expression (Figure 10 A). Knock-down efficiency was determined by western blot. Menin shRNA-2, Daxx shRNA 1, and Pten shRNA resulted in the lowest protein expression than non-silencing control and have been chosen for the next step. (Figure 10 B)

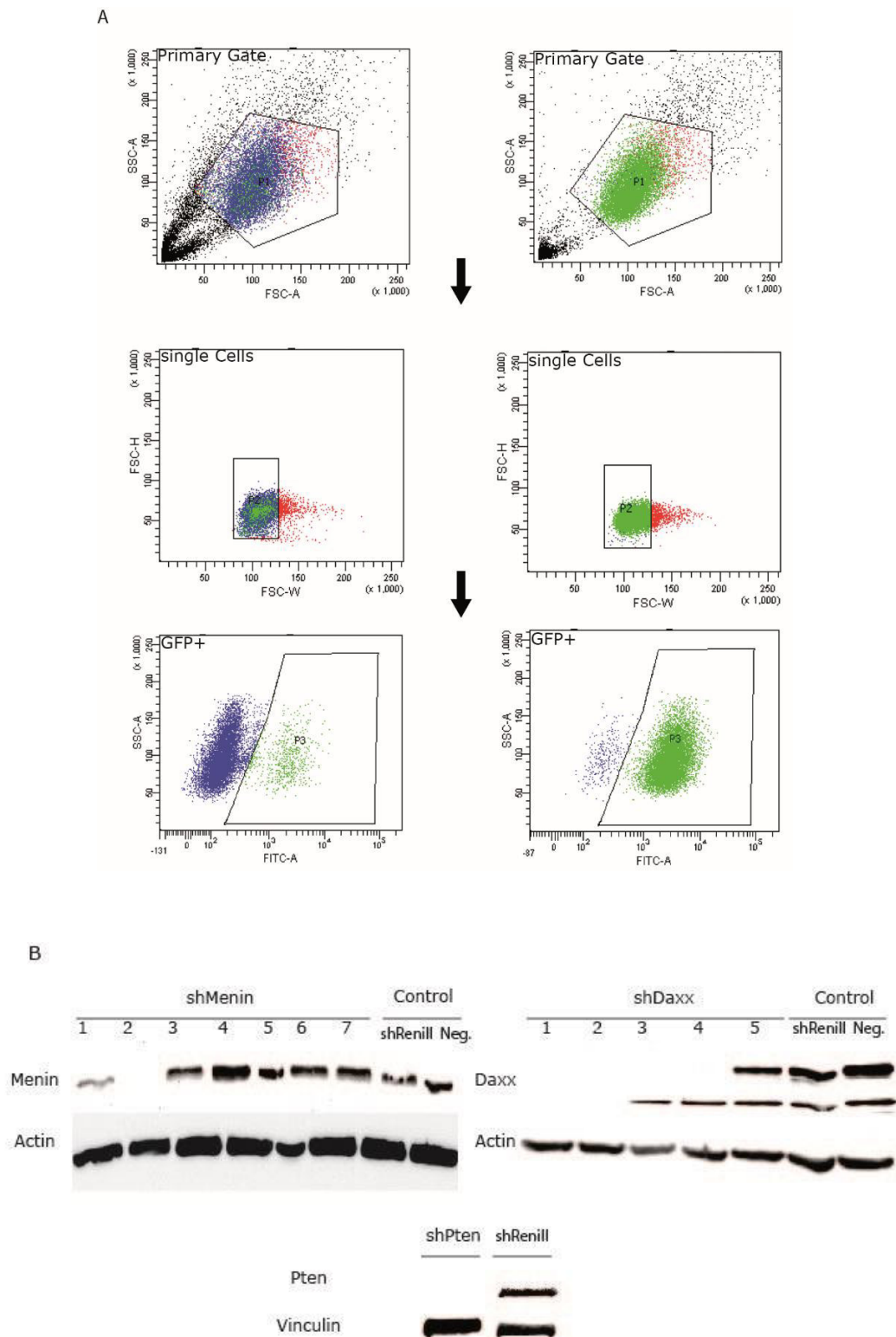
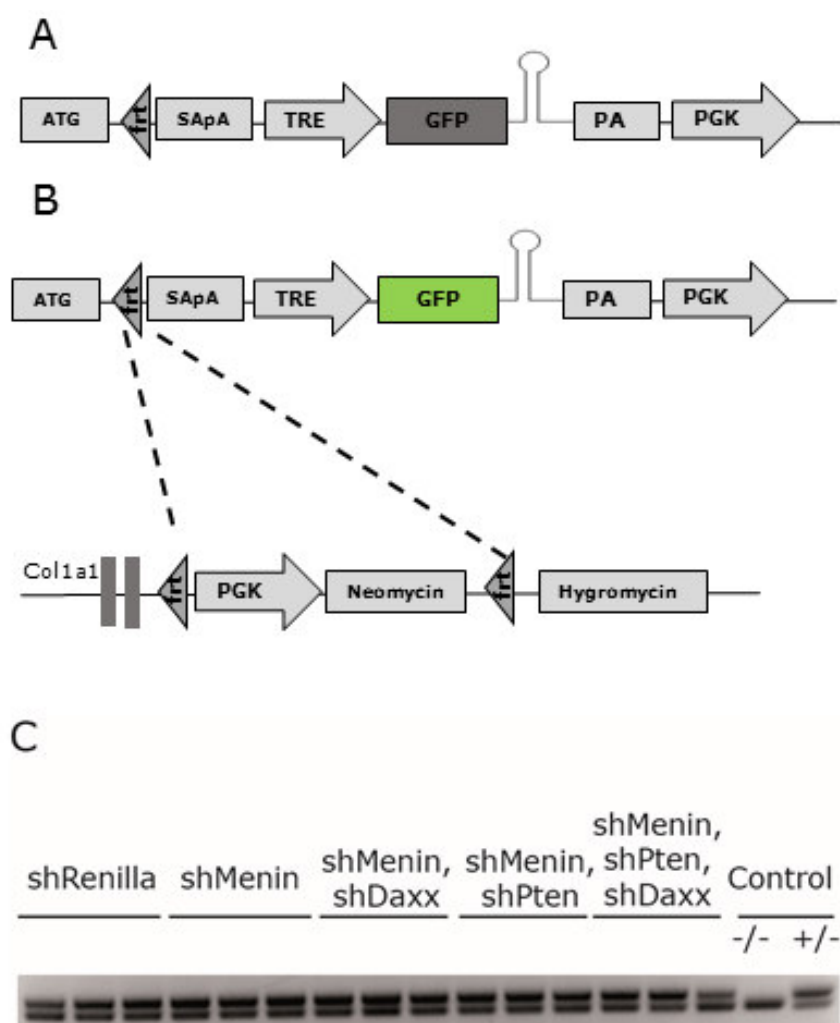


Figure 10 (A) FACS analysis of NIH3T3 cells after retroviral transduction (MlpE was used as a backbone for shRNAs) on the left and seven days after puromycin selection on the right side. (B) Immunoblot demonstrating different knock-down efficiency in different shRNAs compare to non-silencing control.

3.2 *In vitro* validation of single, tandem, and tridem vectors.

To have single, double, and triple knock-down, the most potent shRNAs were subcloned into the cTGM targeting vector to make a single, tandem, and tridem vector (Figure 11 A). A previously reported ESC line KH2 (Caroline Beard, 2006) was chosen to further validate Menin, Daxx, and Pten knock-down in another cellular context and driven by the TRE promoter. The Col1a1 locus has been modified in KH2 ES cells that enable the insertion and consecutive expression of tet-regulatable shRNAs in the presence of Doxycycline. (Figure 11 B)

After targeting ESCs, hygromycin selection took place for 8 to 10 days, and the best-looking clones were chosen. The correctly targeted clones were confirmed by genotyping the Col1a1 locus (Figure 11 C), and they were treated with Doxycycline for 72 hrs. FACS analysis was performed in order to check shRNAs functionality. Since shRNAs expression was linked to EGFP, measuring EGFP expression with FACS analysis as a surrogate for shRNA expression showed ~95% GFP positive cells (Figure 11 D). Immunoblotting exhibited a strong knock-down efficiency for Menin in all constructs, for Daxx in shMenin;shDaxx and shMenin;shPten;shDaxx for Pten in shMenin;shPten and shMenin;shPten;shDaxx in the presence of Doxycycline and GFP only expressed when Doxycycline were induced (Figure 11 F). Knock-down efficiency of Menin, Daxx, and Pten were similar in KH2 ES cells as seen in NIH3T3 cells.



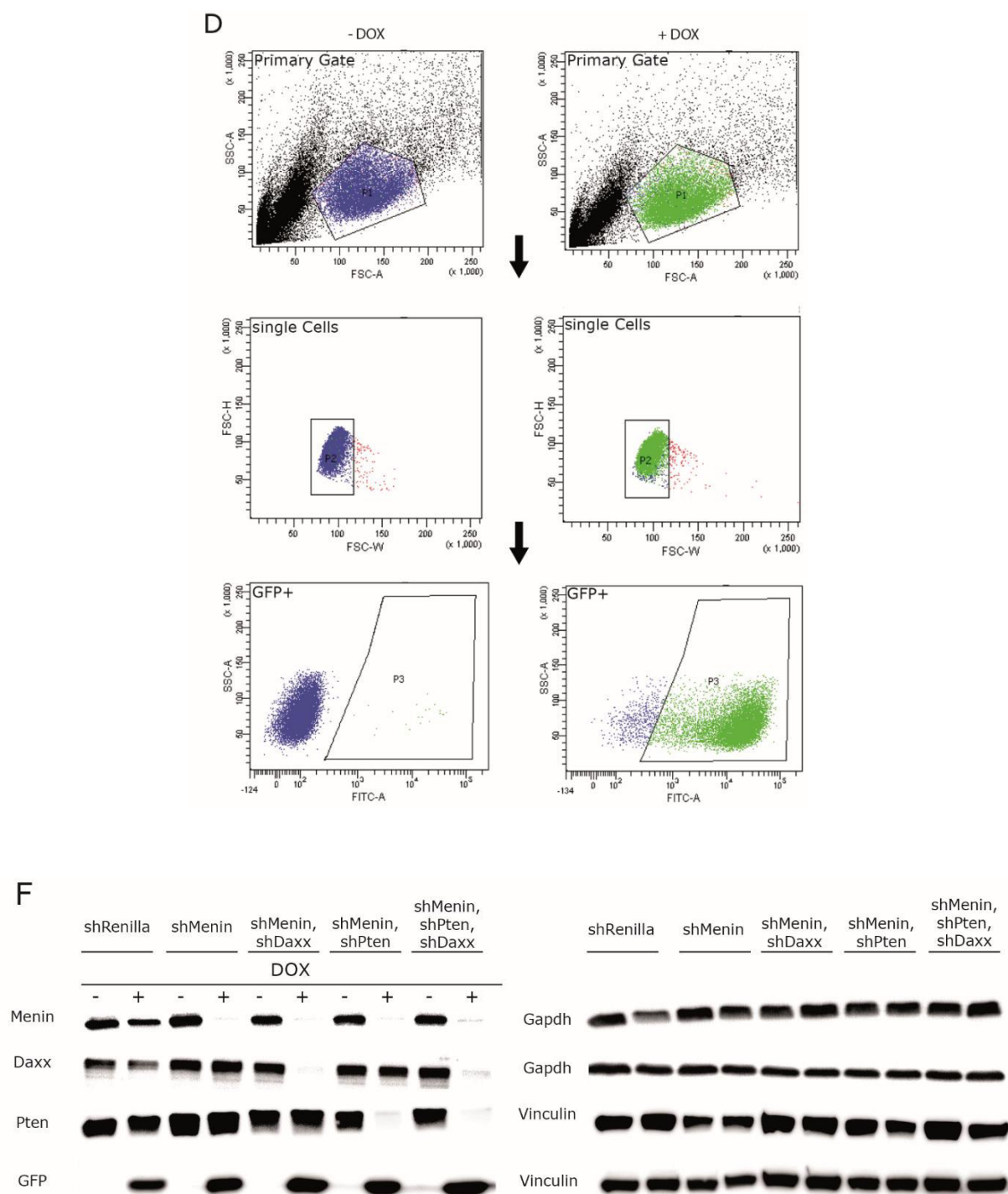


Figure 11 (A) Schematic of the cTGM targeting vector. (B) Schematic of a *Col1a1* modified locus in KH2 ESC and outline ES cell targeting strategy (Cornelius Miething, 2014). (C) In *Col1a1* genotyping wild-type product shows a band at 220 bp and the mutant at 295bp. (E) Flow cytometric analysis of EGFP in KH2 ESC. (F) Western blot analysis for Menin, Daxx, Pten, and GFP protein.

3.3 Generation of inducible and reversible ESC-based shRNA transgenic mice

To generate Genetically Engineered Mouse Models (GEMM), previously reported D34 ESC (Lukas E. Dow, 2014) (Figure 12 A) was targeted with single, double, and triple shRNAs in the cTGM targeting vector. After puromycin selection, the best-looking ESC clones were used for blastocyst injection (Figure 12 B). According to standard procedures, the Transgenic Mouse Platform performed the blastocyst injections at the Helmholtz Centre for Infection Research (head Prof Dr. Wirth). ES cells were injected into C57BL/6N blastocysts and transplanted into foster mothers. Chimeras were identified by coat colour and confirmed by genotyping for *Col1a1* and *Rosa26* loci. (Figure 12 C)

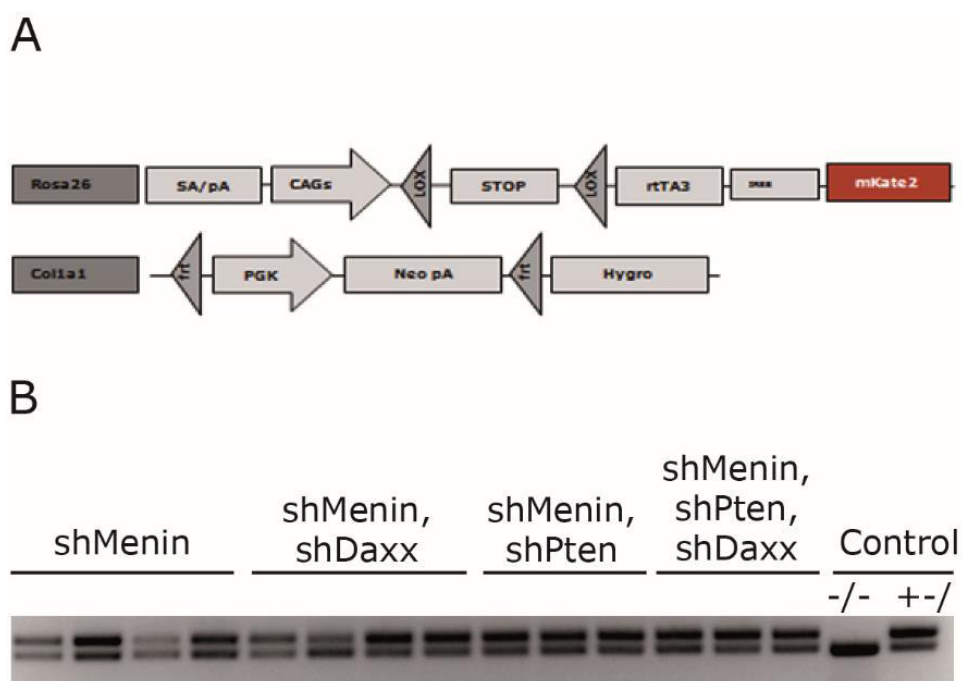
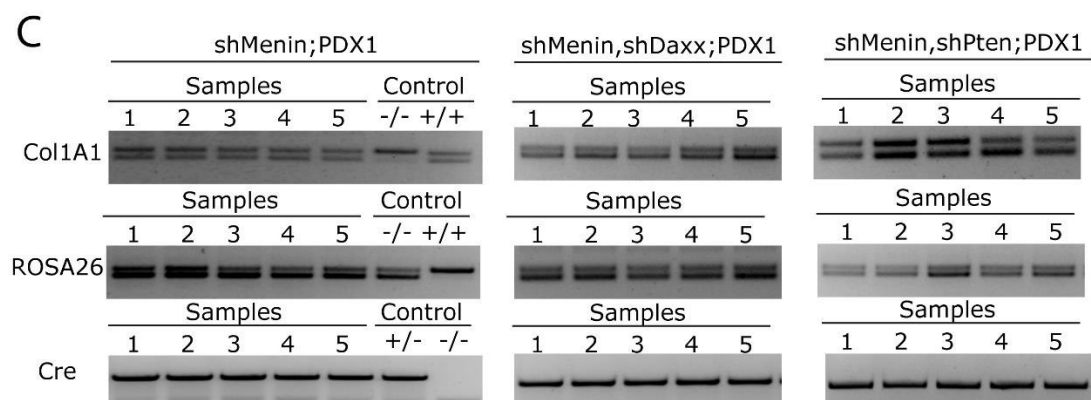
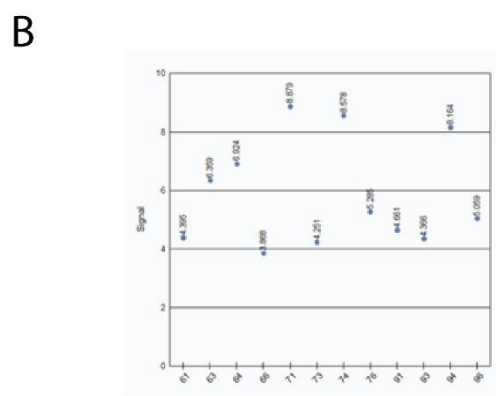
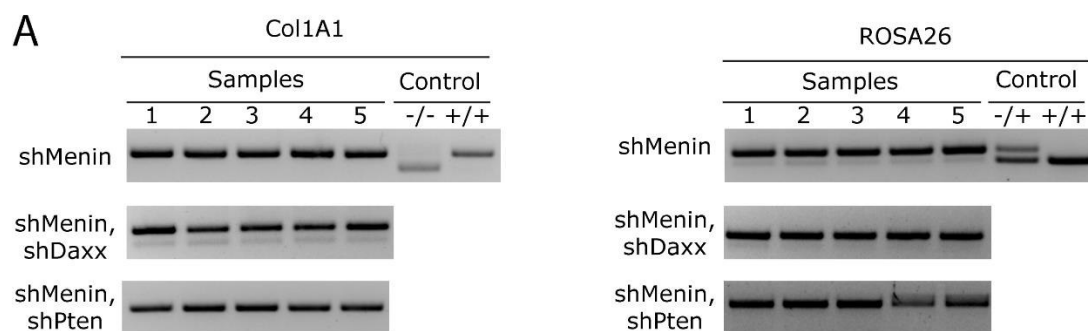


Figure 12 (A) Schematic of two modified loci in D34 ESC. (B) *Col1a1* genotyping to confirm that D34 ESC was correctly targeted. Wild-type product shows a band at 220 bp and the mutant at 295bp.

3.4 Generation of shMenin, shDaxx, and shPten transgenic mice using the D34 ES cells

To generate PanNETs mouse models, homozygous mice for both the targeted *Col1a1* locus and the CAGs-RIK allele (Figure 13 A) were cross-bred to homozygous *Pdx1*-Cre mice (Figure 13 B). To activate shRNA expression, 5-7 days after birth, pups' diet has changed to Doxycycline enriched food. After 23 days, their genotypes were confirmed by checking heterozygous alleles for *Col1a1*, *Rosa26*, and Cre. (Figure 13 C)

Our GEMMs works in this way; mKate2 is a reporter of Cre recombinase activity and rtTA3 expression. After *pdx1*-Cre excises the Lox-stop-Lox site in the targeted *Rosa26* (CAGs-LSL-rtTA3-IRES-mKate2), locus mKate2 starts to express. Reverse tet-Trans Activator (rtTA3) promotes gene expression from *TRE* promoters (in targeting vector), only in the presence of Tetracyclin, or its analog, Doxycycline (Dox). GFP is a reporter of TRE promoter activity and shRNA expression; after activation of TRE promoter, shRNAs and GFP start to express (Figure 13 D).



D

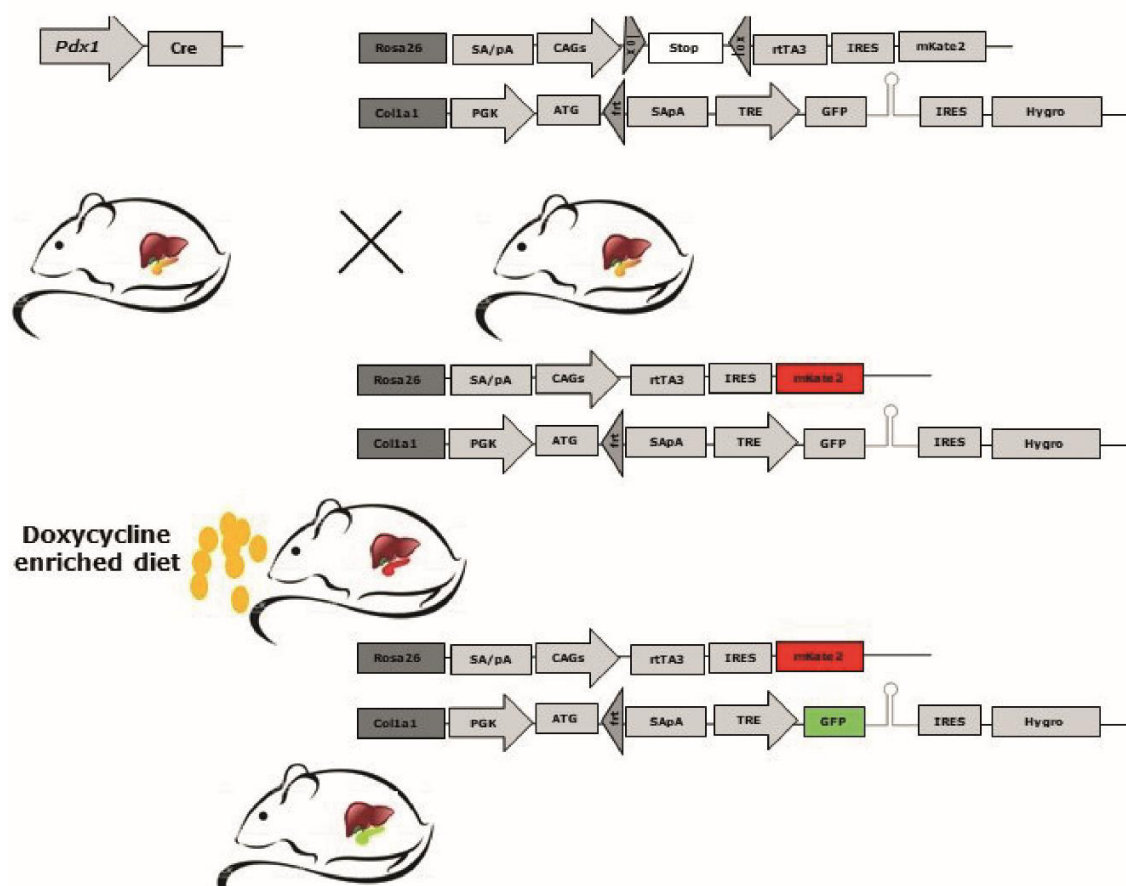
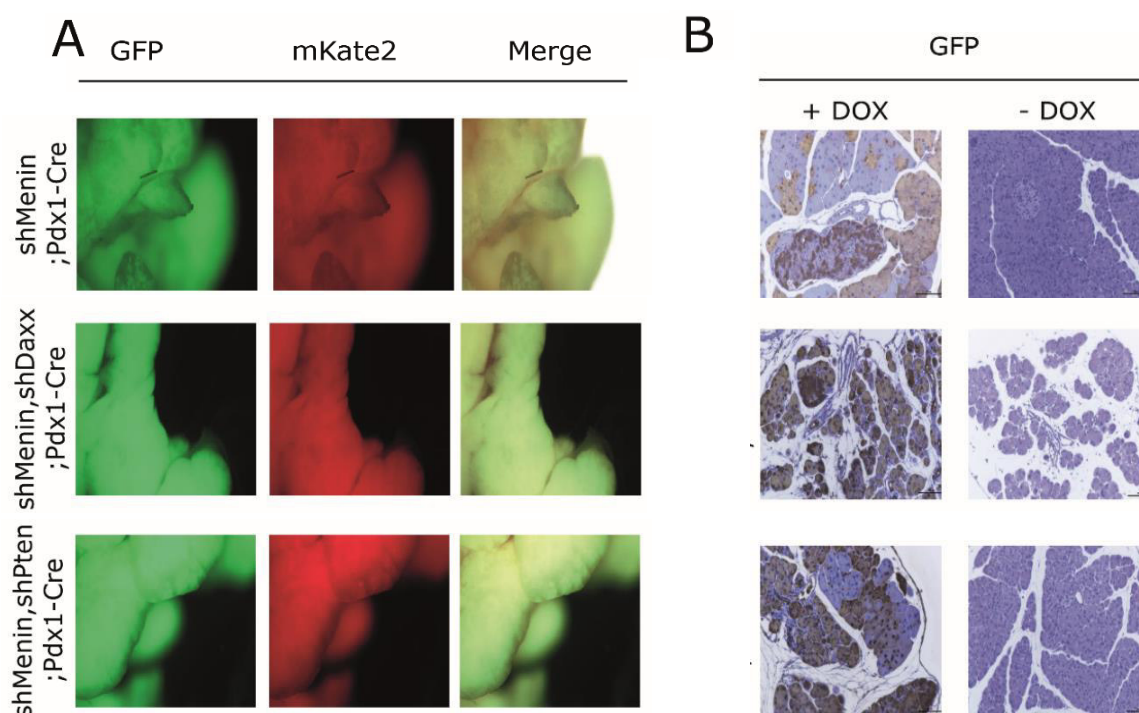


Figure 13 (A) Confirming the chimerism with *Col1a1* and *Rosa26* genotyping. (B) *PdX1*-Cre genotyping was done by Transnetyx company. (C) Genotyping for *Col1a1*, *Rosa26*, and Cre after cross-breeding chimeras to *pdx1*-Cre mice. (D) Schematic representation of inducible and reversible RNAi system in GEMMs. mKate2 is a reporter of Cre recombinase activity. When *Pdx1*-Cre excises lox-stop-lox sites in pancreatic progenitor cells, mKate2 starts to express. Tet Regulatable Element (TRE) is an inducible promoter for shRNA expression, and it only starts to express in the presence of Tetracycline or Doxycycline. Since the shRNA expression is linked to GFP, after starting Doxycycline enriched food, all the cells that express shRNAs also express GFP. (Lukas E. Dow, 2014)

3.5 Transgenic RNA interference technology showed the proper function *in vivo*

In order to determine the TRE promoter's efficiency for the expression of shRNAs within the pancreas and to induce protein knock-down, we used small pieces of the pancreas. Imaging of the whole pancreas from these mice showed the expression of mKate2 (red) and EGFP (green) under a fluorescence microscope (Figure 14 A). Immunohistochemistry (IHC) for EGFP on tissue sections showed a mosaic expression pattern of EGFP in the pancreas. (Figure 14 B)

Furthermore, using Doxycycline treated and non-treated total pancreatic lysate, we confirmed a reduction of Menin, Daxx, and Pten protein expression in shMenin; *Pdx1*, shMenin,shDaxx; *Pdx1*, and shMenin,shPten; *Pdx1* mice. However, as was expected, *in vivo* knock-down efficiency was not as strong as *in vitro* because of the *Pdx1*-Cre mosaic expression pattern. (Figure 14 C)



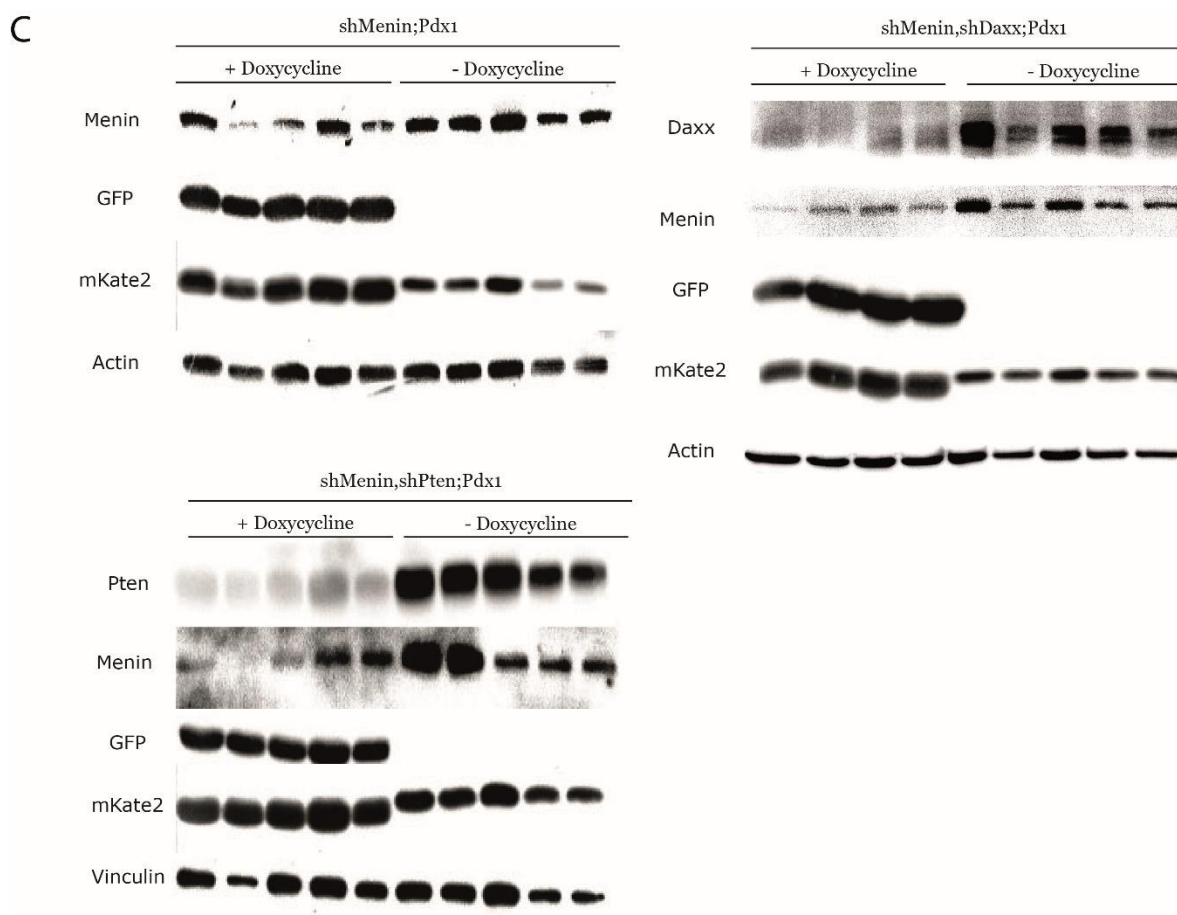


Figure 14 (A) The fluorescence image of the whole pancreas shows the expression of Cre recombinase reporter mKate2, shRNAs reporter EGFP, and the merged image after the induction of Doxycycline. (B) Immunohistochemistry staining for GFP on pancreas sections showed a mosaic expression pattern. (C) Immunoblotting for Menin, Daxx, and Pten on shMenin; Pdx1, shMenin,shDaxx; Pdx1, shMenin, shPten; Pdx1 before and after Doxycyclin induction.

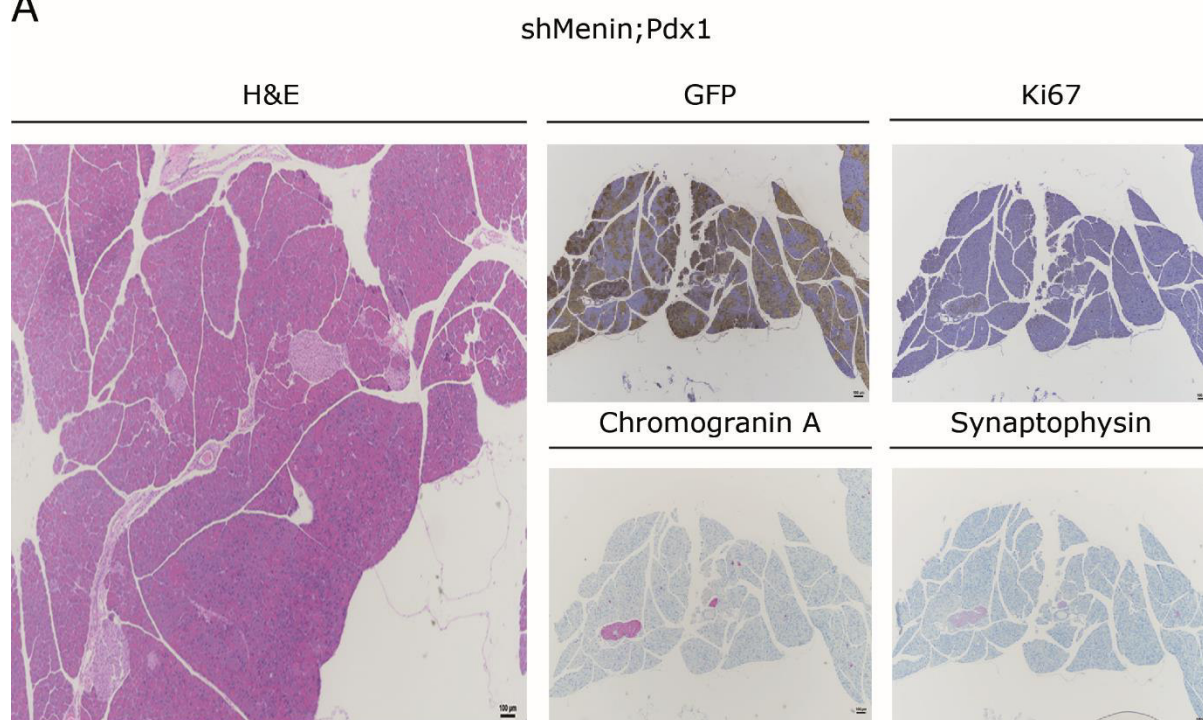
3.6 Menin, Menin; Daxx, and Menin; Pten deficiencies were well tolerated in the pancreas but altered cell state

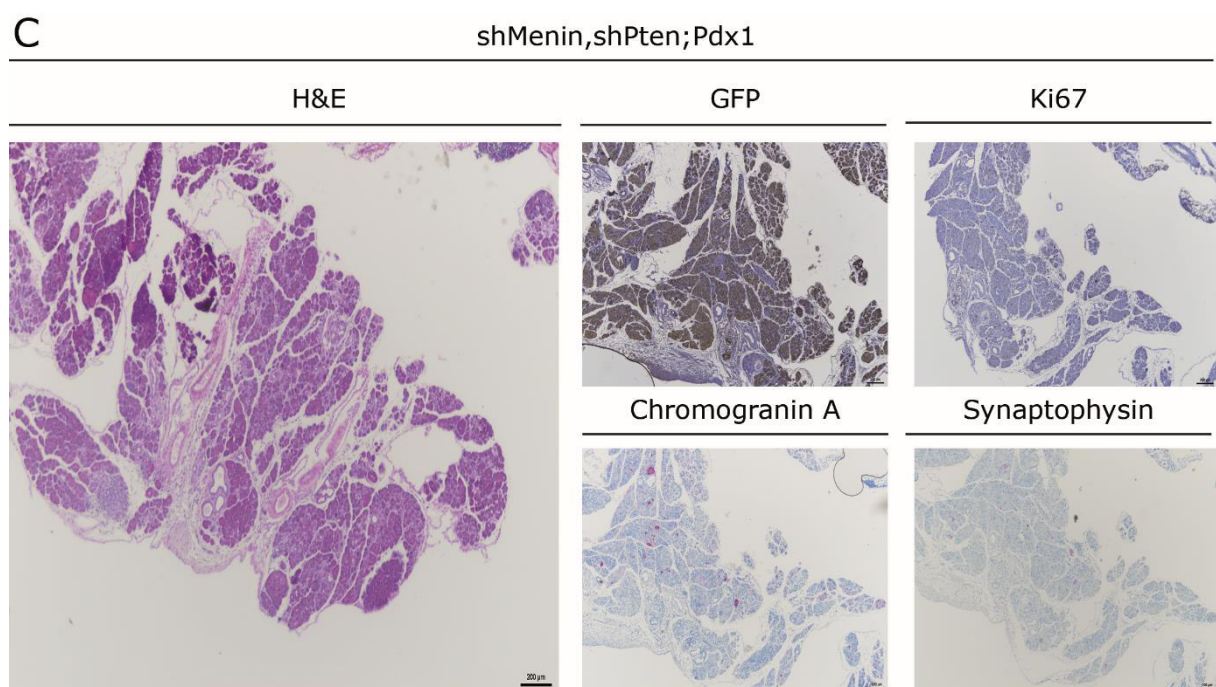
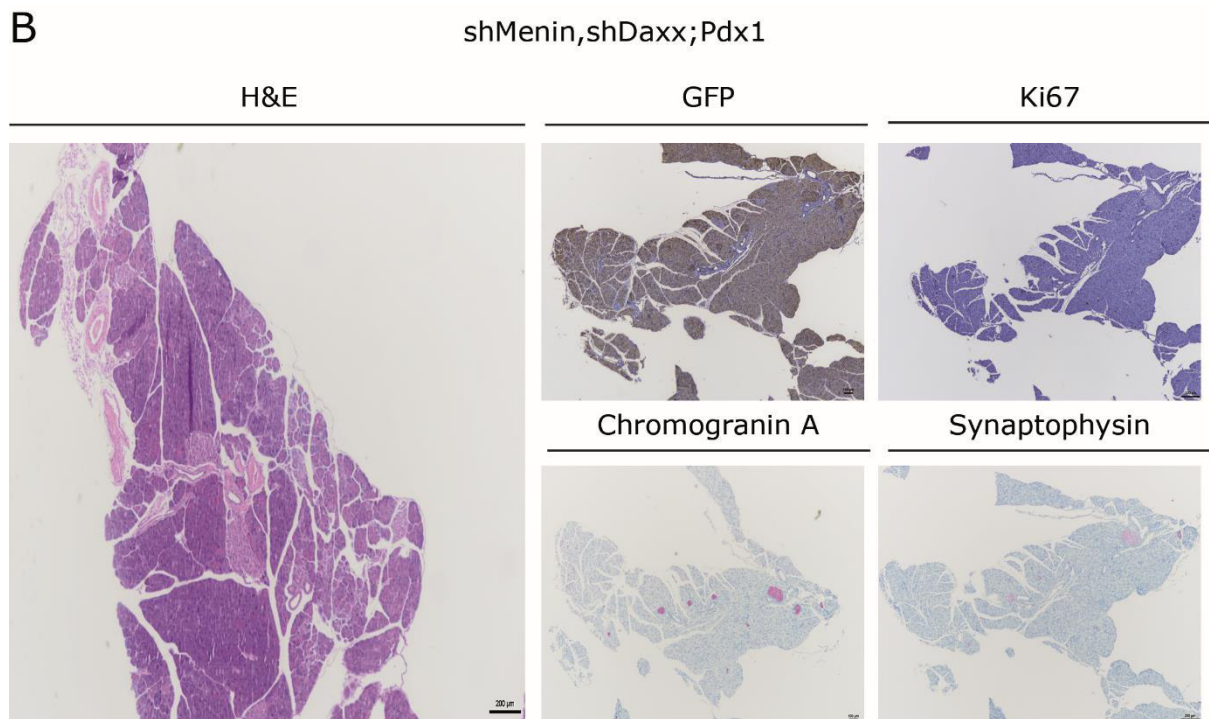
To examine the impact of proteins deficiency on pancreas phenotype in shMenin; *Pdx1* (MP), shMenin,shDaxx; *Pdx1* (MDP), and shMenin,shPten; *Pdx1* (MPP), we assessed pancreas histology for the first time on five mice per group at the age of 12 weeks. In macroscopic observation of the pancreas, no remarkable differences were noticed between the three experimental groups. Histopathological analysis of pancreatic tissues at early time points (12 weeks) showed normal endocrine and exocrine components in shMenin; *Pdx1*. It has been reported that mutation of *Men1* boosted the proliferation of endocrine cells and mutated *Men1* mice develop functional PanNETs (Pellegata, 2017). We tested whether Menin deficiency in *Pdx1* expressing cells would increase proliferation and cause hormone production in our model or not. We used Ki67 as a proliferation marker, insulin as a β -cells marker, and chromogranin A for neuroendocrine secretory granules (Figure 15 A). Although Menin downregulation was confirmed by immunoblotting, no significant difference in the number of proliferating cells or other markers were detected in the pancreas except a few islet cells bigger than standard size (Figure 15 D).

Genetic alteration can induce pancreas cancers. To evaluate and compare the effect of the combination of Daxx, the histone H3.3 chaperone (Pascal Drané1, 2010), and Menin knock-down in the pancreas, we performed the same histopathological analysis. We stained the pancreas with the same markers (Figure 15 B). According to our pathologist (Dr. Hartleben), shMenin,shDaxx; *Pdx1* mice showed fibrosis, pancreas parenchyma atrophy, and chronic pancreatitis, but no significant difference was observed in any of the other PanNET specific markers compare to the shMenin; *Pdx1* mice (Figure 15 E).

According to human PanNETs NGS data and based on tumorigenesis latency in *Men1*^{-/+} mice, cooperation of another genetic lesion is most probably involved in PanNETs development (Scarpa, 2019). We hypothesized that Pten deficiency combined with Menin downregulation accelerates tumorigenesis (Figure 15 C). Histopathological assessment of pancreatic tissues of shMenin,shPten; *Pdx1* mice at early time points exhibited enlarged lymph nodes, fibrosis, especially fibrosis around the ducts, inflammation, pancreatic parenchyma atrophy, loss of pancreatic parenchyma, and pancreatitis, according to Dr. Hartleben. (Figure 15 F)

A





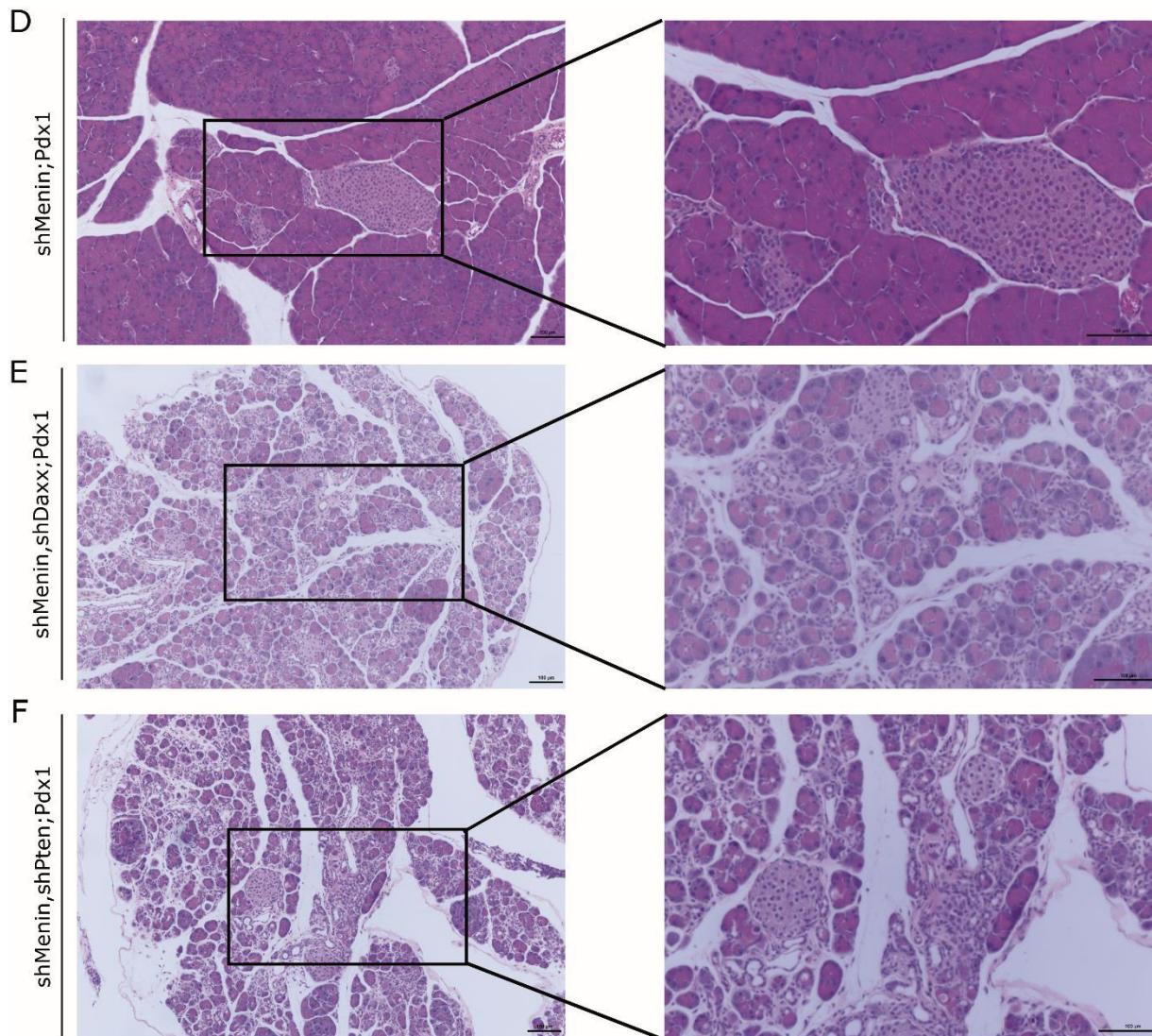
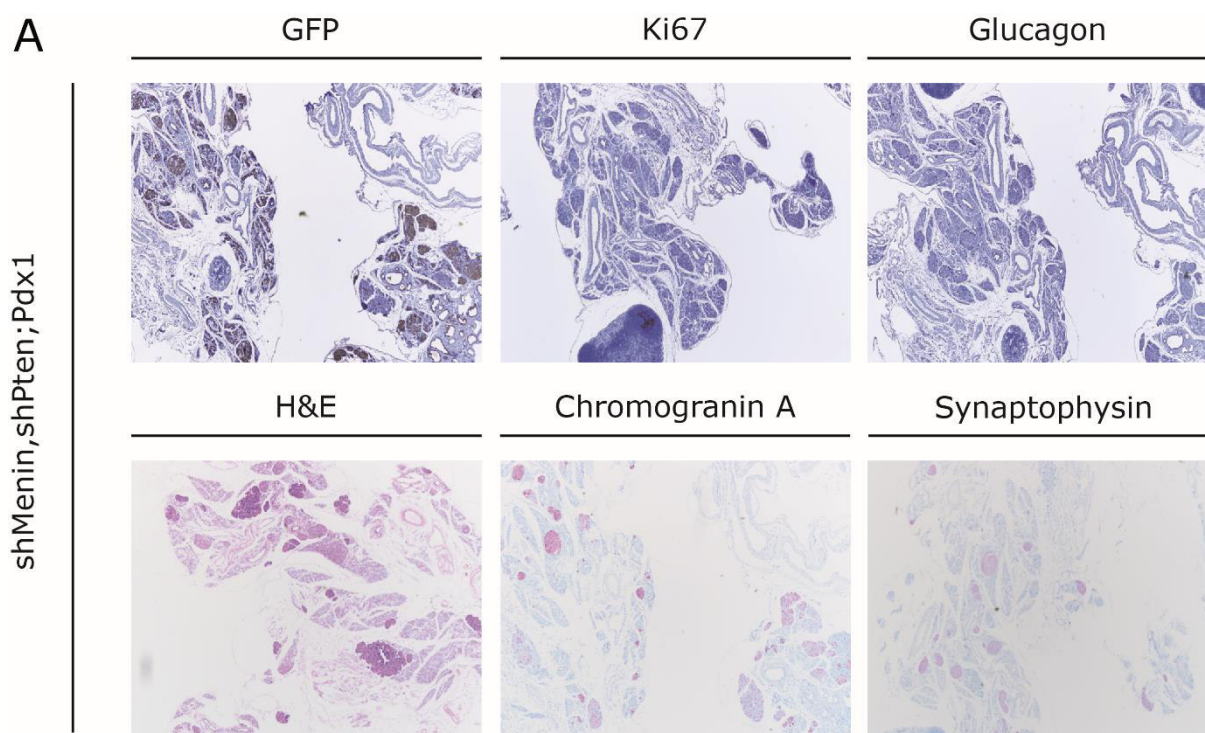


Figure 15 H&E staining and immunohistochemistry of GFP, Ki67, Chromogranin A, and Synaptophysin staining on FFPE sections of (A) shMenin; *Pdx1*. (B) shMenin,shDaxx; *Pdx1*. (C) shMenin,shPten; *Pdx1*. (F) A big islet cell $\leq 1000\mu\text{m}$ in shMenin; *Pdx1*. (E) Area with fibrosis, inflammation and parenchyma atrophy, and duct dysplasia in shMenin,shDaxx. (F) fibrosis around the ducts, inflammation, parenchyma atrophy in shMenin,shPten; *Pdx1*.

3.7 Knocking-down Pten and Menin together did not induce PanNETs at 18 weeks of age

To assess the Pten tumor suppressor's role in the cooperation of Menin tumor suppressor at later time points, we harvested five pancreases at the age of 18 weeks old of shMenin,shPten; *Pdx1* (MPP) mice. We could not detect any PanNETs within the pancreas. All histology sections were evaluated by an experienced pathologist (Dr.Hartleben) (Figure 16 A). Based on the pathologist's interpretation of H&E staining, 18 weeks old mice exhibited dilated ducts, loss of acinar cells, fibrosis, chronic inflammation, ductal dysplasia, and parenchymal atrophy. (Figure 16 B)



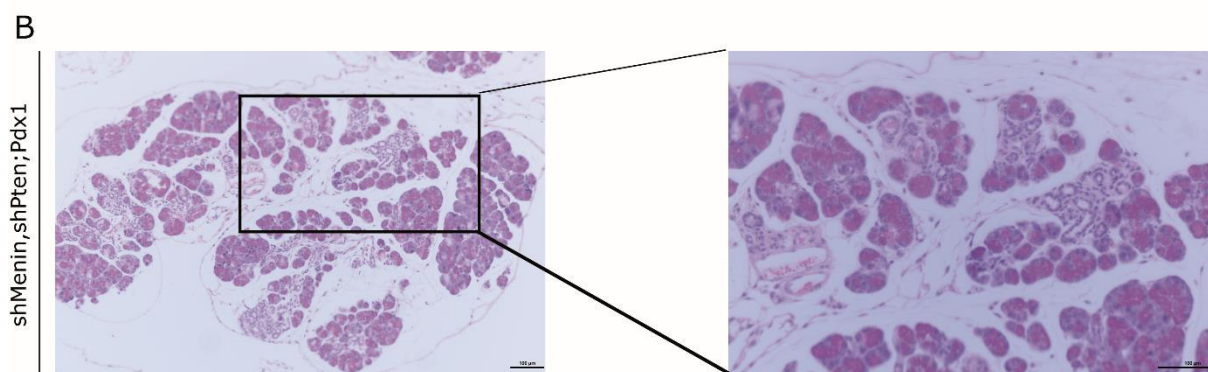


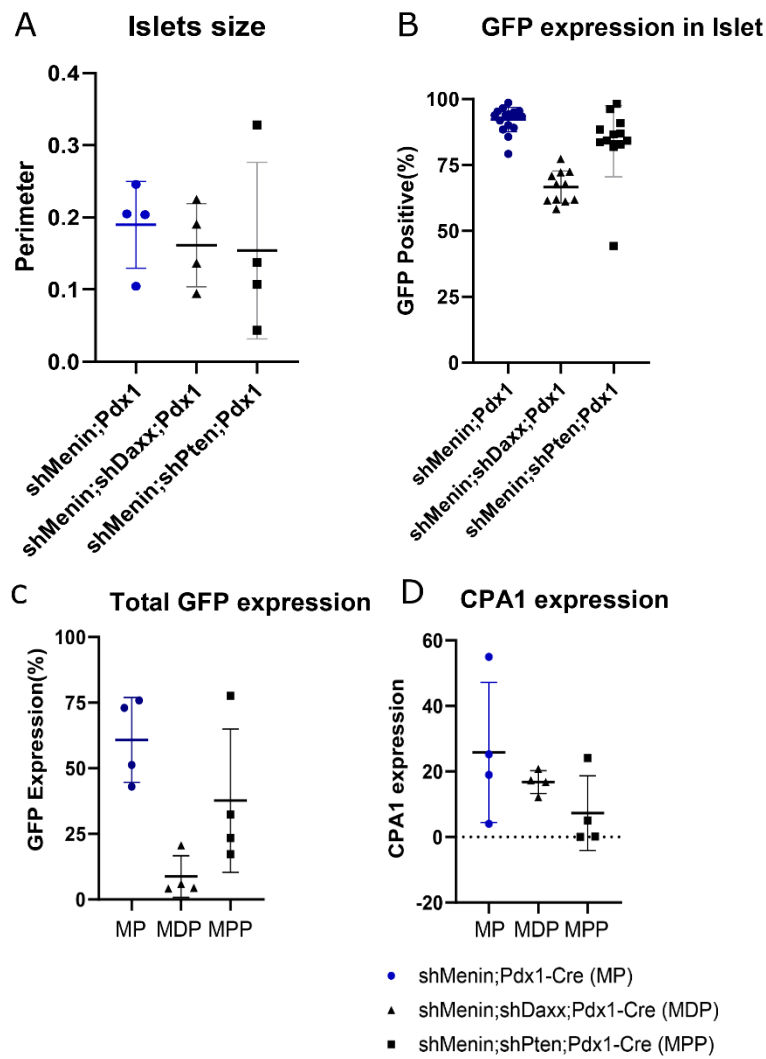
Figure 16 (A) H&E staining and Immunohistochemistry for GFP, Ki67, Chromogranin A, Synaptophysin, and Glucagon of 18 weeks old shMenin,shPten; *Pdx1* FFPE pancreas sections. (B) dilated ducts, loss of acinar cells, fibrosis, chronic inflammation, ductal dysplasia, and parenchymal atrophy at 18 weeks of age.

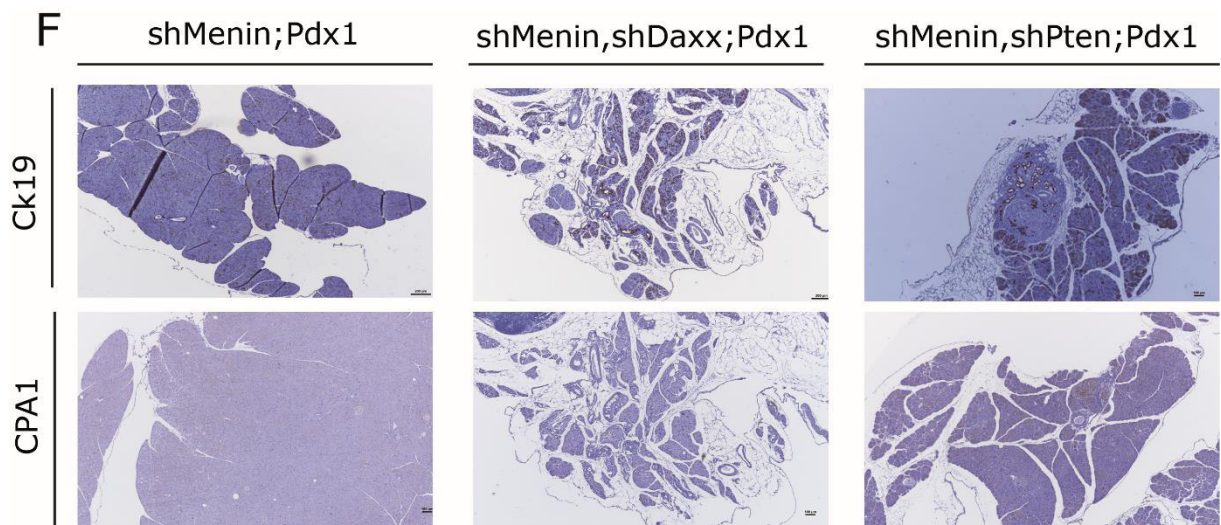
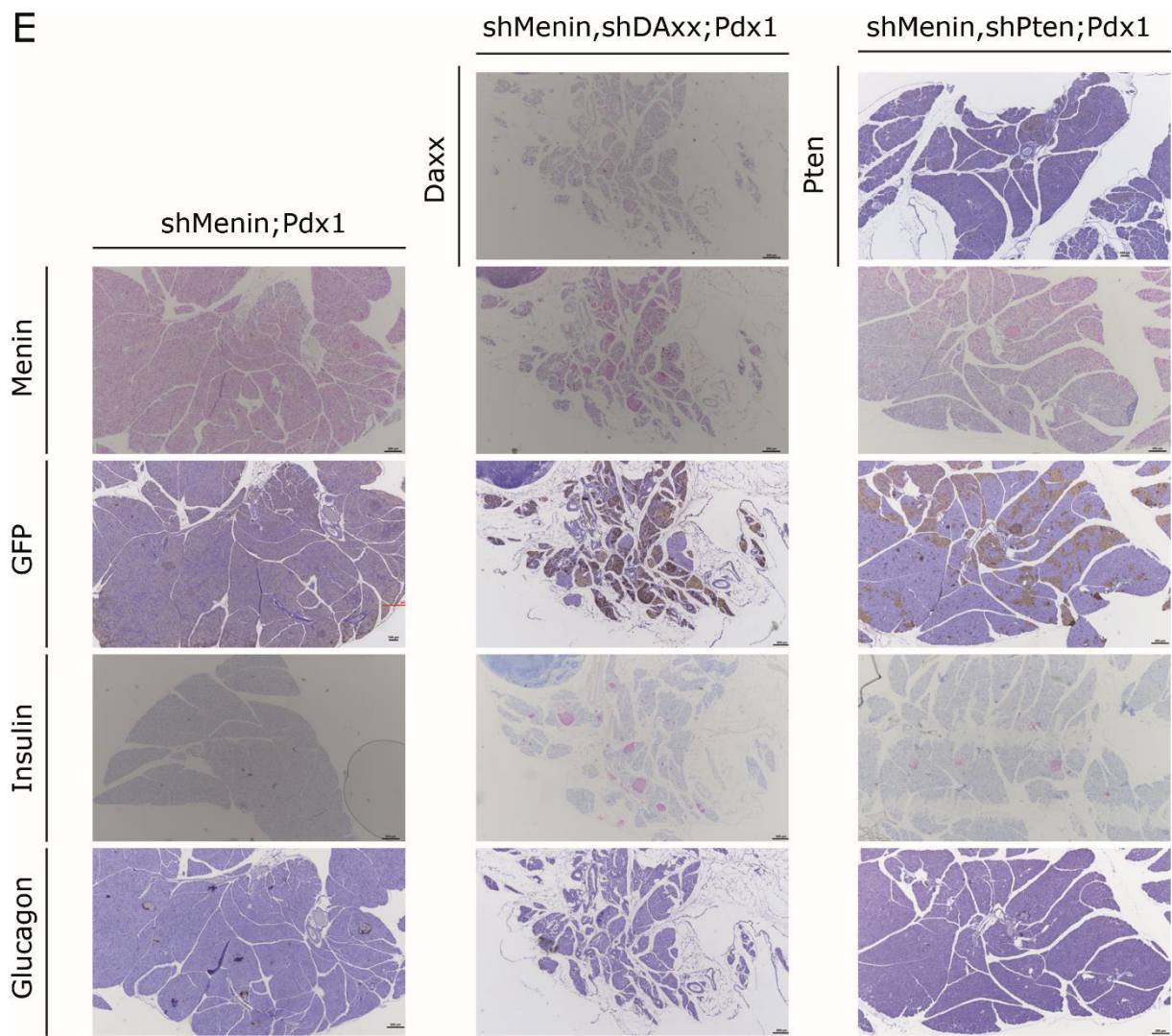
3.8 Effect of Menin, Menin;Daxx, and Menin;Pten deficiency on endocrine and exocrine tissue at 24 weeks of age

To further characterize the effect of Menin, Daxx, and Pten deficiency in shMenin; *Pdx1*, shMenin,shDaxx; *Pdx1*, and shMenin,shPten; *Pdx1* mice, we used older cohorts of mice. Quantification of the endocrine compartment (islets) in H&E staining revealed no significant difference in islet size between the different groups (Figure 17 A). To further check the effect of shRNAs expression in endocrine cells, we used serial sections and stained them for GFP, Menin, Daxx, Pten, Insulin, and Glucagon. Chung Wong et al. considered one or more islets of equal or bigger than 1 mm in diameter with loss of α -cells and proliferation of β -cells as PanNET (Chung Wong, 2020). Since the expression of shRNAs was linked to GFP, we compared each marker's expression to GFP to see the effect of protein knock-down. Comparing GFP staining with Insulin and Glucagon did not show any significant difference in endocrine tissues. To deeper the analysis, we quantified GFP expression in islet cells (Figure 17 B). With this measurement, we quantified the percentage of EGFP expression in endocrine cells in each group as a

surrogate for shRNA expression. Even though the shMenin; *Pdx1* group had the highest GFP percentage, they showed the least phenotypes. Quantifying the percentage of GFP positive cells in the whole pancreas showed shMenin; *Pdx1* had the highest and shMenin,shDaxx; *Pdx1* had the lowest GFP percentage in the whole pancreas sections (Figure 17 C).

To check the effect of protein knock-down on exocrine cells, we stained the pancreas sections with Ck19 and CPA1 antibodies (Figure 17 F). with Ck19 staining, we observed ductal structure in shMenin,shDaxx; *Pdx1* and shMenin,shPten; *Pdx1* groups, and then we quantified CPA1 expression in pancreas sections of all three groups, but no statistical difference was observed between groups (Figure 17 D). To summarize, we did not see any significant difference in terms of CPA1 expression as a surrogate for the intact acinar area or massive effect of ADM development or remodeling of the exocrine pancreas (Figure 17 E), but we did see under the scope the spots that we had fibrosis, dilated ducts, and acinar to ductal metaplasia in 50% of shMenin,shDaxx; *Pdx1* pancreas sections (Figure 17 G), and shMenin,shPten; *Pdx1* in all pancreas sections (Figure 17 H) but not in shMenin; *Pdx1* (Figure 17 I).





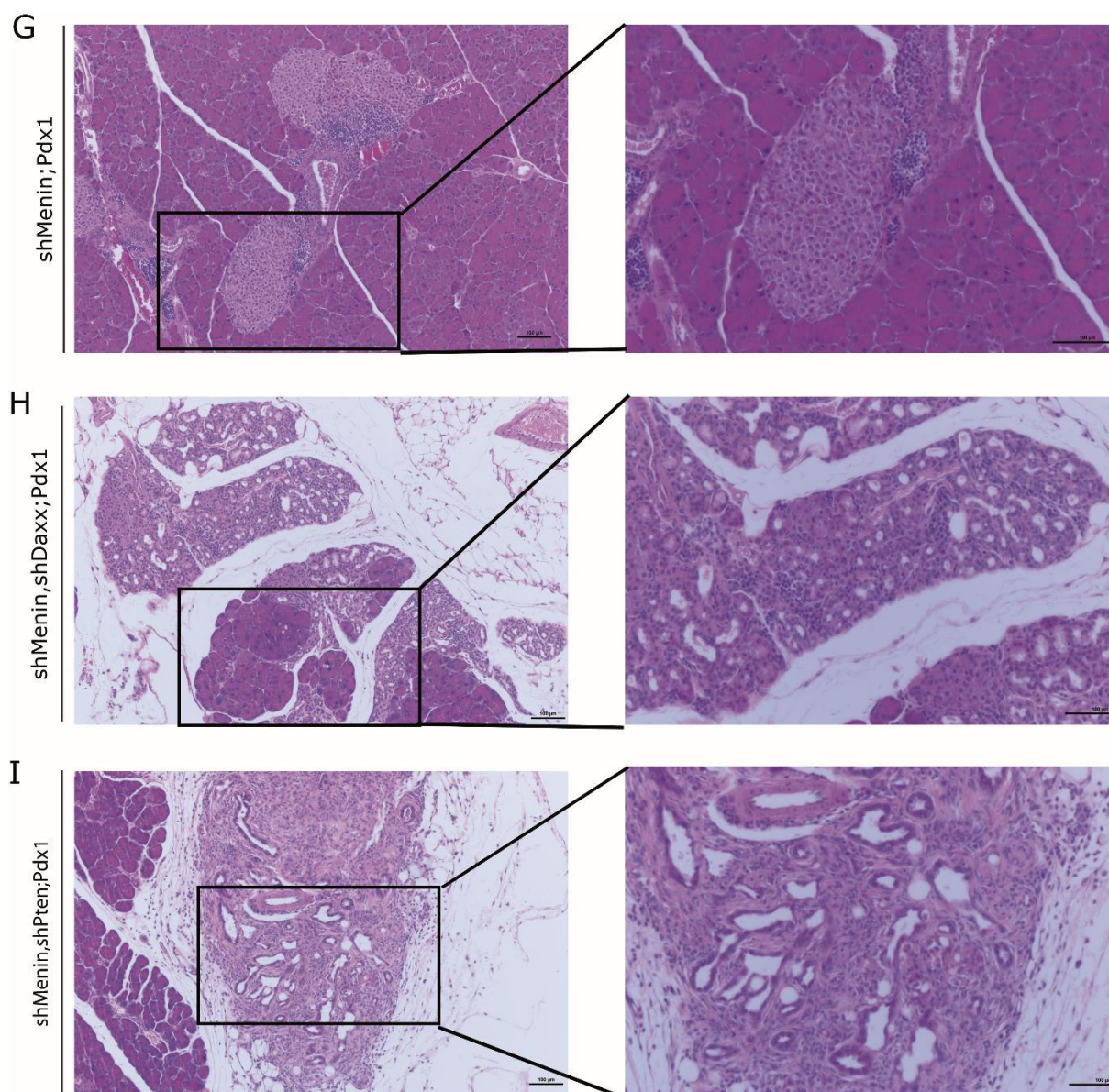
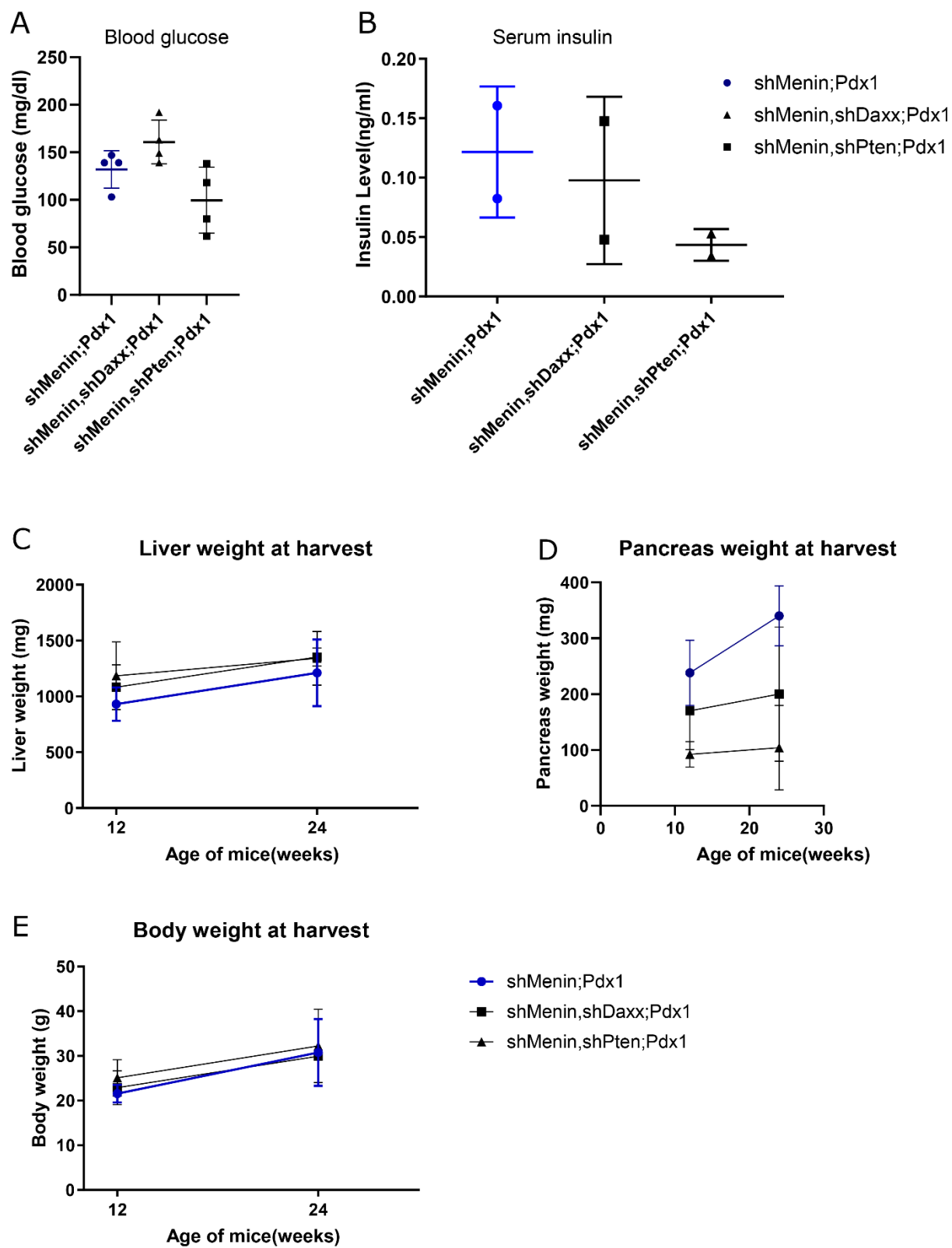


Figure 17 (A) Average islet size from all three groups, determined by dividing all islet's average size by the total pancreas area. (B) GFP expression in Islet cells, determined by dividing the GFP signal in each islet cell by the same total islet area. (C) GFP expression quantification in the whole pancreas. (D) CPA1 expression quantification. (E) ICH staining for Menin, Daxx, Pten, and endocrine cells markers insulin and glucagon. (F) IHC staining for exocrine cells Ck19 and CPA1. (G) 2 islet cells ~ 1000mm in diameter in *shMenin; Pdx1* (H) Acinar to ductal metaplasia, fibrosis, and inflammation in 50% of *shMenin,shDaxx; Pdx1* pancreas sections (I) Acinar to ductal metaplasia, fibrosis, and inflammation in all *shMenin,shPten; Pdx1* pancreas sections.

3.9 Menin, Menin;Daxx, and Menin;Pten deficiencies are neither associated with bodyweight loss, liver and pancreas weight nor the level of blood glucose or serum insulin

It has been shown that lack of Menin protein is associated with insulinomas development (Viveka P. Jyotsna, 2015). Measuring the blood glucose level (Figure 18 A) and serum insulin (Figure 18 B) at the harvest time did not show any significant difference between groups. Liver weight (Figure 18 C), pancreas weight (Figure 18 D), and body weight (Figure 18 E) at the time of sacrifice in 12 and 24 weeks of age also did not exhibit any significant differences. Mice body weight was measured twice per week from 23 days after birth until the end of the study. Their body growth rate did not suggest any particular difference between the groups (Figure 18 F).



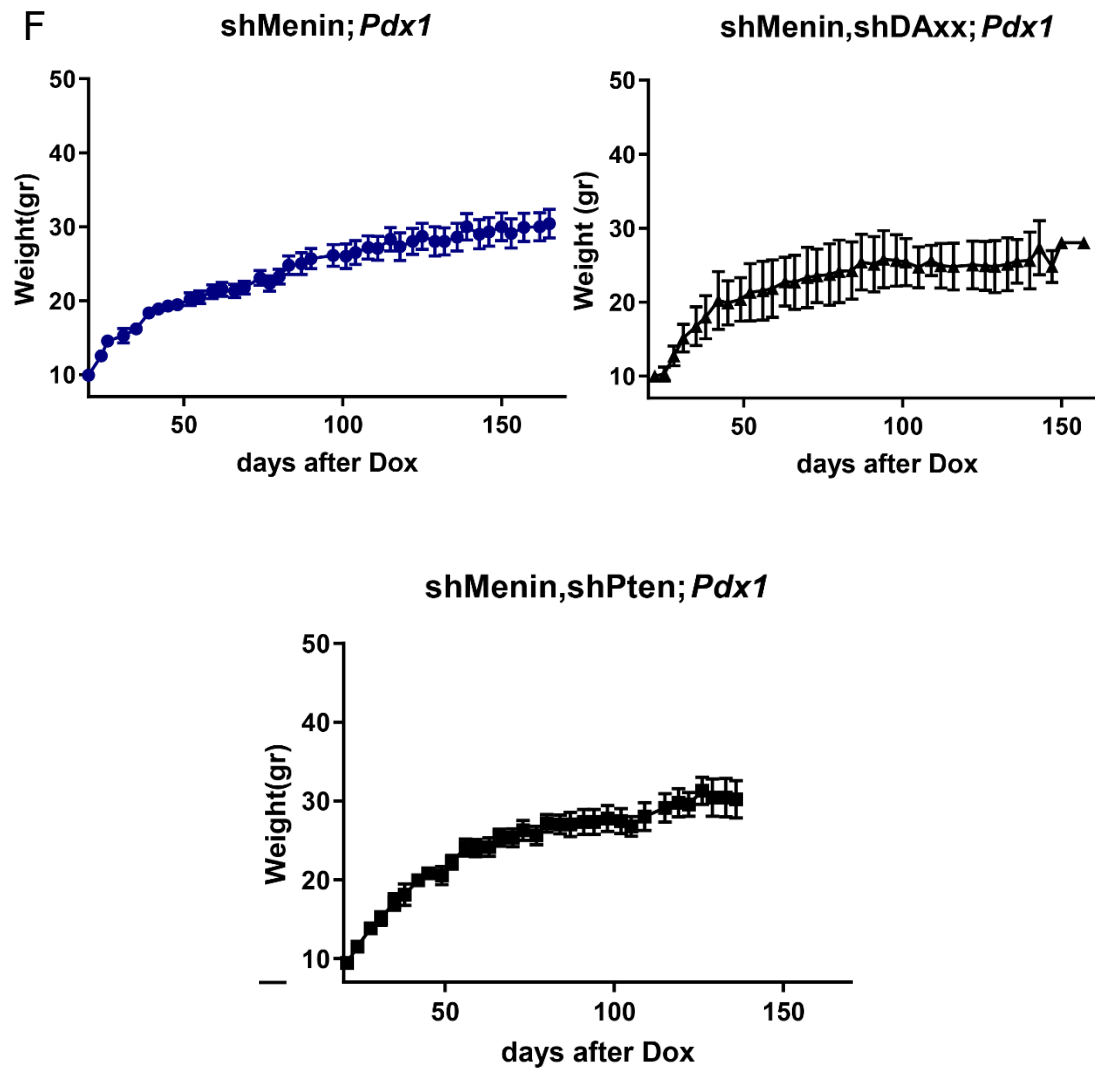


Figure 18 (A) Blood glucose. (B) Serum insulin. (C) liver weight. (D) Pancreas weight. (E) Body weight at harvest. (F) Bodyweight growth curve from 23 days after birth until the end of the study.

3.10 Organoid generation from GEMMs

The newly developed three-dimensional method of epithelial-derived cell culture to model organ systems is easy to manipulate and has facilitated the analysis of molecular mechanisms behind the diseases (Meritxell Huch, 2017). We detailed a 3D cell culture method to generate pancreas organoids from our GEMMs first, to validate the *in vivo* system *in vitro*, and to be able to keep the GEMM's pancreas for further analysis. We generated organoids from the pancreas of cTRE GFP.shMenin; CAGs-lox stop lox rtTA3 IRES mKate2, cTRE GFP.shMenin.shDaxx; CAGs-lox stop lox rtTA3 IRES mKate2, and cTRE GFP.shMenin.shPten; CAGs-lox stop lox rtTA3 IRES mKate2 (Figure 19 A). To induce shRNAs expression and test protein knock-down efficiency, we excised the L-S-L site by co-transfecting the organoid with PGK-Cre plasmid and PTA-GFP-Blastocystin. This transfection resulted in mKate2 expression, and after Doxycyclin induction and activating the rtTA3 and TRE promoters (Figure 19 B). By immunoblotting, we confirmed substantial protein downregulation in shMenin,shDaxx organoids (Figure 19 C). However, Knock-down in organoids was not as strong as KH2 ESC, and the residual protein was detected most likely because of incomplete selection.

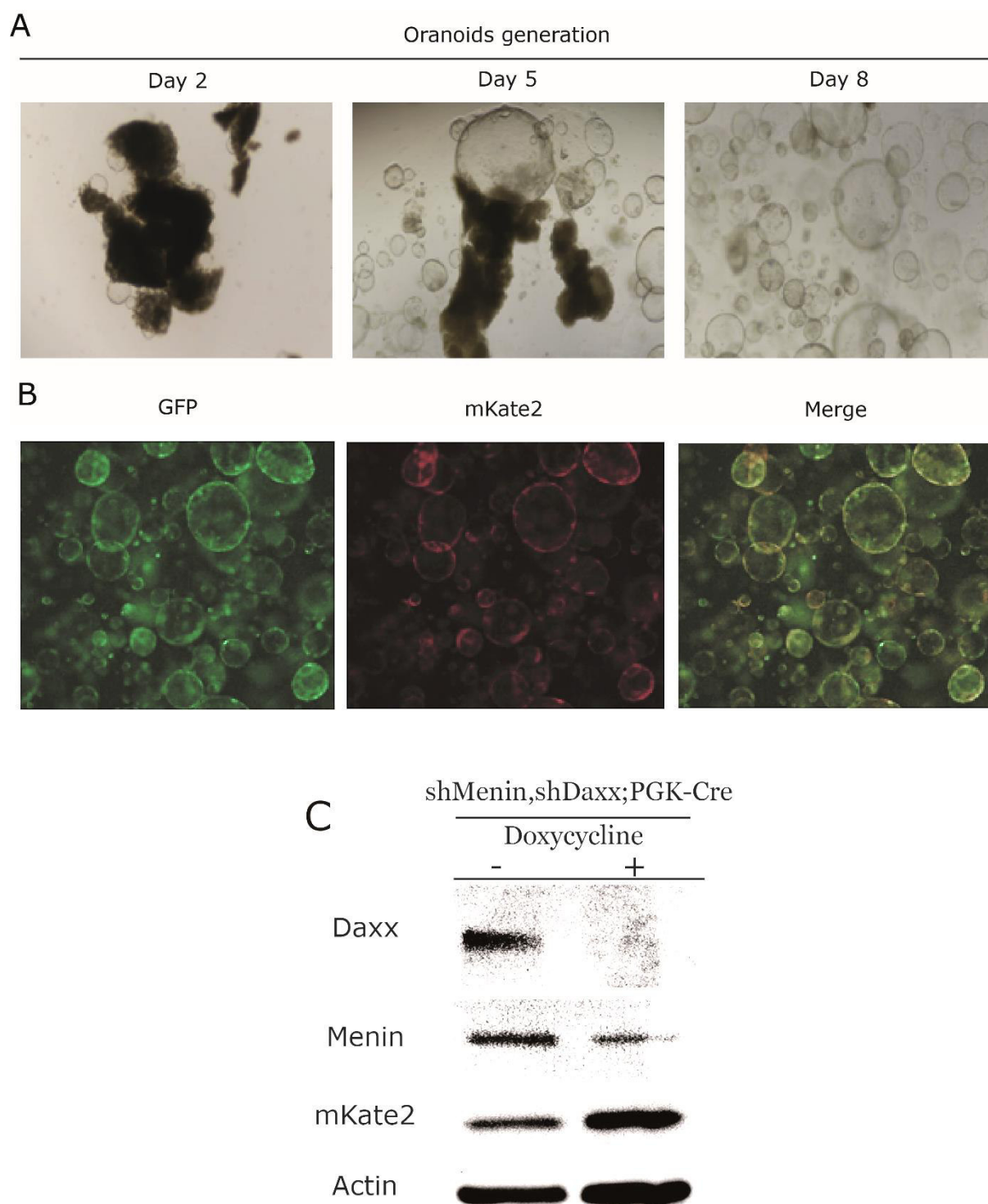


Figure 19 (A) Representative images of a pancreatic organoid culture growing at the indicated time points (2-5-8 days). (B) All organoids start to express GFP protein after co-transfection with GFP-PTA and PGK-Cre plasmids and express mKate2 as a sign of Cre recombinase activity.

4 Discussion

Genetic alterations frequently cooperate with the environment and other genetic mutations to promote cancer (Amanda R. Wasylshen¹, 2020). In the last few years, Next Generation Sequencing (NGS) technologies have opened up new scopes in nucleic acid analysis and developed cancer research enormously (Wilson, 2009). NGS data has accelerated the discovery of many cancer susceptibility genes (Wing Chan, 2020) and advanced our understanding of cancer genomes' mutations. Like all types of tumors, NGS data has identified novel biomarkers and a large number of mutated genes in pancreatic neuroendocrine tumors. NGS explored genomic data, which can support physicians in early diagnosis and advance personalized treatment (Mardis, 2012). For example, whole-genome and epigenetic analyses of 98 resected PanNETs demonstrated an increased number of mutations *in MEN1*, *DAXX* (in chromatin modeling genes), and genes involved in the mTOR pathway like *PTEN* (Aldo Scarpa, 2017), (Kebebew, 2020), (Scarpa, 2019). In order to use the NGS data to study PanNETs development and progression, there is a need to establish an *in vivo* system for pancreas-specific loss of *Menin*, *Pten*, and *Daxx*. It is also necessary to evaluate these genetic lesions' cooperation in the induction of pancreatic neuroendocrine tumor resemble human PanNETs. Our hypothesis to generate PanNETs mouse models replicating human PanNETs by combining different genetic lesions first became possible in 2017 when Aldo Scarpa et al. published their NGS data from PanNETs patients.

The use of Genetically Engineered Mouse Models in research is so crucial as they play an essential role in understanding different types of human disease development and progression, especially human cancers (Jessica C. Walrath, 2010), (Noorani, 2019), (Kelly Kersten 1. K., 2017). In cancer research studies use

of traditional transgenic mouse models have been very important, but GEMM-ESC based models have revolutionized the cancer mouse models (Ursa Lamprecht Tratar, 2018). The inducible and reversible RNAi-based transgenic mouse models can engineer a broad range of mutations found in cancers at a specific time point and in particular tissues and provide solutions to previously unsolvable problems (Ross A Dickins, 2007).

Inducible and reversible shRNA expression within the microRNA backbone is a potent way to reduce target protein expression (Julia E. Prier, 2019). This system's utility was demonstrated in the Saborowski et al. study to incorporate RNAi technology into the GEMM-ESC model to make an inducible and reversible gene knock-down model to study the role of Pten and c-Myc in pancreatic cancer progression (Michael Saborowski, 2014). The use of the RNAi system with ECS-based GEMM has several advantages. In the present study, we used D34 ESC with the "*CAGs-LSL-rtTA₃-IRES-mKate2*" alleles. mkate2 (far-red fluorescence) is a reporter for Cre-mediated recombination. In our targeting vector cTGM, the GFP reporter is linked to the shRNA and facilitates the detection of shRNA expression in cells (Michael Saborowski, 2014); thus, the dual fluorescent reporter system gave us more flexibility to analyze the resulted mice. Furthermore, temporal control of shRNA expression enables the degradation of targeted mRNA at different stages of pancreas development or reactivating it by stopping the Doxycyclin-enriched diet (NATSUKI MATSUSHITA, 2013).

Conditional knock-down solves the embryonic lethality problem in conventional transgenic mice (Jian Zhang, 2012). Conditional knock-down mice are generated by breeding chimeras to Cre recombinase controlled by a tissue-specific promoter (Hyeonhui Kim M. K.-K., 2018). It has been proved that homozygous conventional *Men1* knock-out mice die at embryonic day 10.5-13.5 (Judy S. Crabtree P. C.-B.-B., 2001), (Philippe Bertolino, 2003), but heterozygous mice

develop a broad range of tumors such as pancreatic, pituitary, thyroid, parathyroid, adrenal glands, testis, and ovaries tumors which make it less ideal for preclinical studies (Kay, 2006), (Brian Harding 1, 2009). Another example of conventional gene mutation is the mouse model of mutated *Daxx* that leads to apoptosis and embryonic lethality (E 9.5) (Jennifer S. Michaelson, 1999). Conventional homozygous deletion of *Pten* mice die at an embryonic day 9.5, but heterozygous mutation of *Pten* developed tumors in multiple organs such as the brain, lung, prostate, bladder, breast, colon, and endometrium (A Suzuki 1, 1998), (Eng, 2003), (Cristina Mirantes, 2013). In our study, to achieve tissue-specific knock-down, we used *Pdx1*-Cre mice. *Pdx1* expresses in pancreatic progenitor cells, and breeding chimeras to *Pdx1*-Cre mice lead to knock-down protein in pancreatic cells.

4.1 Transgenic shRNA technology for inducible and reversible pancreas-specific gene silencing

In the current study, we aimed to develop three RNA interference ESC base mouse models of tissue-specific, inducible, and reversible loss-of-function of Menin, Menin-Daxx, and Menin-Pten. This system can be used to knock-down any candidate gene product entirely or in any specific tissue. Instead of KC-RIK-ESCs and generating shRNA knock-down transgenic mice, CRISPR/CAS system can be used to inducible gene editing and validate the role of gene loss of function *in vivo* (Amrendra Mishra, 2020).

This system showed a substantial protein loss of function without any leaky expression of rtTA3 and TRE *in vitro* and a reduction in protein expression level in *Pdx1* expressing cells *in vivo*.

4.2 Development of Menin shRNA conditional knock-down mouse model

Multiple Endocrine Neoplasia 1 (*MEN1*) is a hereditary condition characterized by the presence of multiple tumors arising from neuroendocrine tissues such as pancreas, parathyroid glands, and anterior pituitary (Judy S. Crabtree 1. P.-B., 2003), (Pellegata, 2017). It has been reported that *MEN1* plays an important tumor suppressor role in the endocrine pancreas and also plays a functional role in the exocrine pancreas (Amanda R. Wasylishen, 2020), (Anna Karpathakis, 2013). *Men1* mouse model offers exclusive opportunities to study pancreatic neuroendocrine tumor development, progression, and test potential therapies (Judy S. Crabtree 1. P.-B., 2003). In this project, we aimed to explain the effect of the tissue-specific protein reduction of Menin by using the transgenic shRNA technology after pancreas development in pancreatic cells that express *Pdx1*. Although after birth, *Pdx1* expresses at high levels in the β cells, some δ cells, and at lower levels in exocrine cells (Peter O. Wiebe, 2007), lacks of Menin expression did not lead to tumorigenesis or any major remodeling of endocrine or exocrine tissues but mild phenotypic changes of endocrine cells in shMenin; *Pdx1* mice until 24 weeks of age. In our mouse model, blood glucose and serum insulin levels roled out any hormone production in mice until 24 weeks of age. It has been reported that the lack of *Men1* in *Pdx1*-Cre; *Men1*^{f/f} mice do not affect normal pancreas development but developing functional pancreatic neuroendocrine tumors (H.-C. Jennifer Shen, 2009). *MEN1* mutation in humans results mainly in nonfunctional pancreatic neuroendocrine tumors (Mark J C van Treijen, 2018). However, all pancreatic knock-out mouse models of *Men1* have developed functional PanNETs (Table 1).

4.3 Generation of RNA interference conditional double knock-down of Menin and Daxx

Genome sequencing data revealed that epigenetic regulators have a high percentage of genetic mutations in PanNETs (Hui Shen, 2013), (Jueng Soo You, 2012), (Scarpa, 2019). Based on Scarpa et al., *DAXX* has the highest mutation rate after *MEN1* in PanNETs patients, and the co-mutation of *DAXX* and *MEN1* happens in several patients. Since mouse models play a vital role in understanding the physiologically relevant functions of genes, and no conditional *Daxx* murine model was available at the time that we started our study, we decided to generate a mouse model to combine loss of Menin and Daxx in order to evaluate their corporation in the induction of PanNETs. By the time we were cross-breeding our chimeras, a paper was published by Amanda R. Wasylishen et al. about the role of *Daxx* loss of function and loss of *Daxx* in combination with *Men1* in the mouse pancreas (Amanda R. Wasylishen, 2020). They showed that loss of *Daxx* is well tolerated in pancreas development by using two different Cre tissue-specific promoters (*Pdx1*-Cre^{tg} and *Rip*-Cre^{tg}) to derive deletion of *Daxx* in the different pancreatic cell types.

Assessing the mice at the molecular level showed a significantly higher level of expression of *Bglap3* in *Daxx* deficient mice than in the control group. They also generated a mouse model combining loss of *Daxx* and *Men1* in the pancreas using *Pdx1*-Cre^{tg}. *Daxx*^{fl/fl}*Men1*^{fl/fl}*Pdx1*-Cre^{Tg} showed significant changes in exocrine tissue compare to their single knock-out models. They observed acinar atrophy, acinar to ductal metaplasia, ductal proliferation, cyst formation, chronic inflammation, and fibrosis in pancreas sections. They proposed that damage of homeostatic response resulted from a combination of *Men1* and *Daxx* loss in the pancreas. They also suggest that significant exocrine changes resulted from the loss of *Men1*

induce tissue stress, damage, and inflammation to ducts and block vessels (Amanda R. Wasylishen¹, 2020). In our model, combining *Menin* and *Daxx* deficiencies did not affect endocrine tissue; however, it did cause changes in the exocrine compartment at 12 weeks of age. At this age, they developed fibrosis, pancreas parenchyma atrophy, chronic pancreatitis, and acinar to ductal metaplasia in some samples. By the age of 24 weeks, we could detect fibrosis, parenchyma atrophy, and acinar to ductal metaplasia in 50% of the mice. Considering that we knocked down both proteins after pancreas development, double knock-down still remodeled exocrine tissue but not as severe as discussed in the mentioned mouse model.

4.4 Generation of a conditional shRNA knock-down mouse model for two well-known tumor suppressors *Menin* and *Pten*

PTEN is considered one of the most commonly altered genes in cancer (Shen, 2008), but its mutation frequency is not as high as *MEN1* in PanNETs patients. *PTEN* is a phosphatase that antagonizes the mTOR/PI3K/AKT pathway. It limits cell proliferation and cell survival and helps to prevent cancer. In 2017 we hypothesized that combining *Pten* deficiency with *Menin* can induce PanNETs and accelerate tumorigenesis. In 2019 when we were expanding cTRE GFP.sh*Menin*.sh*Pten*; CAGs-lox stop lox rtTA3 IRES mKate2 chimers, Chung Wong et al. published a paper based on whole-genome sequencing PanNETs data. They generated two well-differentiated PanNETs GEMMs using *Mip*-Cre (express in pancreatic β -cells) and *Rip*-Cre (express in pancreatic islets and hypothalamus) mice to derive deletion of *Men1* and *Pten* in the pancreas. *Men1*^{flox/flox}*Pten*^{flox/flox} *RIP*-Cre(MPR) developed well-differentiated PanNETs and pituitary neuroendocrine tumors with complete penetrance by 13 weeks of age. *Men1*^{flox/flox}*Pten*^{flox/flox} *MIP*-Cre (MPM) developed only well-differentiated PanNETs with complete penetrance by 17

weeks of age. MPR and MPM models developed well-differentiated PanNETs replicating human pancreatic neuroendocrine tumors.

In our mouse model, we knock-down Menin and Pten 5 days postnatally in pancreatic cells expressing *Pdx1*. shMenin,shPten; *Pdx1* mice pancreas did not undergo extensive remodeling of exocrine and endocrine compartments, but from 12 weeks of age, histopathological analysis of pancreatic tissues exhibited big lymph nodes, fibrosis, especially fibrosis around the ducts, inflammation, pancreatic parenchyma atrophy, loss of pancreatic parenchyma, and pancreatitis and at 18 weeks they exhibited dilated ducts, loss of acinar cells, fibrosis, chronic inflammation, ductal dysplasia, and parenchymal atrophy, and by the age of 24 weeks, we observed some spots with acinar to ductal metaplasia in all pancreas sections.

It has been shown that experimental outcomes depend on several variants. Cre recombinase activity timing and efficiency influence phenotype (Osipovich, 2013). For instance, using the same Cre tissue-specific promoters (*Pdx1*-Cre) to derive deletion of β -*catenin* in the different pancreatic cell types in three independent studies led to a noticeably different conclusion (Jessica Dessimoz 1, 2005), (L Charles Murtaugh 1, 2005), (Patrick W Heiser 1, 2006). Besides different Cre drivers, the time of gene loss of function induction also plays a role in experimental results. Complete deletion of a gene using *pdx-1* Cre mice prenatally leads to a loss of function in all pancreatic cells since *pdx-1* expression in pancreatic progenitor cells starts in embryonic day 8.5 (Reshmi Dassaye S. N., 2016). Moreover, mouse genetic background can confound experimental results (Doetschman, 2009). For example, it has been shown that C57BL/6J are diabetes-resistant strains while C57BLKS/J strain develops severe diabetes (Mao, 2006).

4.4 Outlook

Several methods are available for genome editing and generating genetically engineered animal models, and each one has its own advantages and disadvantages. What makes our model unique is the possibility of having temporal control of shRNA expression and checking the effect of protein reactivation at any desired time. The other advantage of this system is a dual fluorescence reporter that enables us to check the system functionality in resulting mice in a more flexible and faster way.

There are some possible explanations as to why our shRNA knock-down mouse models did not develop PanNETs. First of all, we activated shRNAs after pancreas development. In this case, *Pdx1* does not express in all pancreatic cells after pancreas development. Furthermore, it has a mosaic expression pattern. There might still be a possibility of PanNETs development if inducing shRNA expression starts prenatally before embryonic day

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Abbreviations

PanNETs- Pancreatic Neuroendocrine Tumors

PDAC- Pancreatic ductal adenocarcinoma

ESC- embryonic stem cells

rtTA3- reverse tetracycline transactivator

GEMM- Genetically engineered mouse model

RMCE- Recombination mediated cassette exchange

Dox- Doxycycline

Col1a1- collagen, type I, alpha 1

shRNA- short hairpin RNA

MEF- Mouse embryonic fibroblasts

FFPE- formalin-fixed paraffin-embedded

DMSO- Dimethyl sulfoxide HBS-

HEPES-buffered saline

FACS- Fluorescence assisted cell sorting

H&E- hematoxylin and eosin

IHC- Immunohistochemistry LB- Luria-Bertani

LIF- leukaemia inhibiting factor

PBS- Phosphate buffered saline

PCR- Polymerase Chain Reaction

EGFP- Enhanced green fluorescent protein

Declaration

Herewith, I confirm that I have written the present PhD thesis myself and independently, in compliance with “the policy of Hannover Medical School on the safeguarding of good scientific practice and procedural rules for dealing with scientific misconduct” and that I have not submitted it at any other university worldwide.

Herewith, I agree that MHH can check my thesis by plagiarism detection software as well as randomly check the primary data. I am aware that in case of suspicion, ombudsman proceedings according to § 9 of MHH ‘Guidelines of Hannover Medical School to guarantee good scientific practice and dealing with scientific fraud’ will be initiated. During such proceedings, the PhD process is paused.

Hannover, (June 2021)

Curriculum vitae

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CORE COMPETENCE SUMMARY

Highly-motivated and results-driven Ph.D. candidate in life science with profound knowledge and hands-on experience in cell and molecular biology, oncology, genetics, and genome editing. Highly developed cross-cultural communication skills combined with an ability to motivate others. A flexible and creative team member who thrives in a fast-paced international environment.

TECHNICAL SKILLS

- Strong molecular biology skills include DNA, RNA, protein handling, cloning, qPCR, quantification methods, and next-generation sequencing.
- Expert in cell biology techniques, including 2D and 3D (organoids) and stem cell culture.
- Highly experienced in mammalian genome editing using CRISPR/CAS and RNAi systems.

PROFESSIONAL SKILLS

- Strong time, project, and network management, problem-solving, and analytical skills.
- Adapt from one situation to another quickly.
- Willing to learning new technologies and new skills.
- Self-motivated and proactive.
- Ability to travel up to 75% both domestically and internationally.

PROFESSIONAL EXPERIENCES

10/2017 – Present

Ph.D. in Molecular Medicine

- Genome editing using CRISPR/CAS and RNAi system.
- Generation of ES cell-based GEMM using RNAi system to study pancreatic neuroendocrine tumor.
- Identification and functional characterization of several stroma modulators in cholangiocarcinoma.
- Generation of ES cell-based GEMM using CRISPR/CAS system to study the role of fusion proteins in induction of pancreatic ductal adenocarcinoma.
- Planning and executing scientific experiments related to three or more projects.
- Mentoring, teaching, and assisting junior researchers in executing scientific experiments, conducting analysis, and reporting results.

03/2014 – 11/2016

Product Manager and Field Application Specialist (Medical and laboratory equipment)

- Worked with sales specialists to meet/exceed territory sales targets and provided technical leadership during the sales process.
- Installed, implemented, and optimized medical equipment and laboratory tests and trained customers, acting as a primary liaison throughout the life cycle of solutions.
- Provided strategic and competitive customer feedback to Marketing and product development teams.
- Developed and maintained positive relationships with customers in assigned territory.

- 05/2011 – 05/2013 **Project Manager and Data Analysis** (Family business)
- Developed and implemented software to analyze non-destructive tests with the IT department's help to save time, minimize human errors, maximize results quality and customer satisfaction.

EDUCATION

- 10/2017 – PRESENT **Ph.D.:** Molecular Medicine
Medizinische Hochschule Hannover – Germany
- 02/2012 – 02/2014 **Master of Science:** Cell and Molecular Biology
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- 01/2009– 01/2011 **Associate of Arts:** Interior Design
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ENTREPRENURIAL INITIATIVE

- 01/2011 – 12/2016 **Interior designer:** Freelancer
- Delivered inspired and efficient interior designs for residential markets.
 - Exceeded milestones and specs for tens of satisfied clients.
 - Provided solutions to design problems and generated design alternatives.
 - Developed and managed various initiatives that support a consistent approach to further developing a robust workplace design approach.
 - Kept all projects under budget and delivered on time.

LANGUAGES

Farsi	Native
English	Fluent
German	Intermediate
Arabic	Basic

SOFTWARE SKILLS

Microsoft Office	Graphics
- Word	- Graphpad Prism
- Excel	- Adobe Illustrator
- PowerPoint	- Adobe Photoshop
- Outlook	

INTERESTS

Sports:	Running, TRX, fitness
Art:	Oil and pencil painting and mosaic art
Others:	Traveling, reading, and supporting disadvantaged children

Date:

Signature:

Acknowledgment

In the last four years and throughout the writing of my thesis, I have received a great deal of support and assistance.

I would like to thank my supervisor, PD Dr. Michael Saborowski, for his continuous support of my PhD and immense knowledge. His guidance helped me in all the time of research and writing of this thesis.

My appreciation extends to HBRS, Prof. DR. Reinhold Schmidt, Prof. Dr. Georg Behrens, Dr. Susanne Kruse, and Brigit Müller for their support all through the PhD.

I would like to thank Prof. Dr. Arndt Vogel, Dr. med. Anna Saborowski, for their insightful comments and encouragement.

My sincere thanks also go to my co-supervisors Pro. Dr. Sascha David and Prof. Dr. Florian Vondran for their support.

Special thanks to my fellow labmate Dr. Amrendra Mishra for his continuous support and help. I could not have imagined having more supportive colleagues than him.

A big shout-out to my wonderful colleagues and friends in K5 and J11, especially Barbara Seller, Steve Duong, Florian Hamberger, Franziska Wölfl, Dmitrij Ostroumov, Miriam Nassiri, and Eric Jende, for all the supports.

Above ground, I am indebted to my mother for all her unconditional support and love. This journey would not have been possible without the dedicated support of my mother.

Dedication

I dedicate this thesis to
my mother and all of my
friends, without whom
none of my success would
be possible