Accepted Preprint first posted on 25 July 2017 as Manuscript ERC-17-0249

Animal Models of MEN1

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Keywords: Multiple endocrine neoplasia type 1; Endocrine tumors; Menin; Animal models

Word count: 7806

Abstract (240 words)

Animal models of cancer have been instrumental in advancing our understanding of the biology of tumor initiation and progression, in studying gene function, and in performing preclinical studies aimed at testing novel therapies. Several animal models of the MEN1 syndrome have been generated in different organisms by introducing loss-of-function mutations in the orthologues of the human *MEN1* gene. In this review, we will discuss MEN1 and MEN1-like models in Drosophila, mice and rats. These model systems with their specific advantages and limitations have contributed to elucidate the function of Menin in tumorigenesis, which turned out to be remarkably conserved from flies to mammals, as well as the biology of the disease. Mouse models of MEN1 closely resemble the human disease in terms of tumor spectrum and associated hormonal changes, although individual tumor frequencies are variable. Rats affected by the MENX (MEN1-like) syndrome share some features with MEN1 patients albeit they bear a germline mutation in *Cdkn1b* (p27) and not in *Men1*. Both *Men1*-knockout mice and MENX rats have been exploited for therapy response studies testing novel drugs for efficacy against neuroendocrine tumors (NETs) and have provided promising leads for novel therapies. In addition to presenting well-established models of MEN1, we also discuss potential models which, if implemented, might broaden even further our knowledge of neuroendocrine tumorigenesis. In the future, patient-derived xenografts in zebrafish or mice might allow us to expand the tool-box currently available for preclinical studies of MEN1-associated tumors.

Introduction

Multiple endocrine neoplasia type 1 (MEN1) is a complex syndrome defined by the neoplastic transformation of at least two endocrine organs, most frequently parathyroid glands, pancreatic islets, anterior pituitary, endocrine pancreas. Less frequently patients present with adrenal cortical tumors, carcinoids, facial angiofibromas, collagenomas and lipomas (reviewed in Thakker 2014). In 1997, by linkage analysis and tumor deletion mapping the *MEN1* gene located on chromosome 11q13 was identified as the gene responsible for the MEN1 syndrome (Chandrasekharappa et al. 1997; Lemmens et al. 1997). The encoded 610-aa long protein named Menin is a tumor suppressor, and tumors of MEN1 patients usually show loss-of-heterozygosity (LOH), which leads to loss of Menin function. The protein plays a role in cell division, genome stability and transcriptional regulation (Thakker 2014). Menin binds to a MLL-containing complex with histone methyltransferase activity and recruits this complex to the promoters of the cyclin-dependent kinase (CDK) inhibitors *Cdkn1b* (p27) and *Cdkn2c* (p18), thereby activating their transcription (Karnik et al. 2005). The binding to JunD, a member of the Jun family of transcription factors, suppresses Menin's ability to activate transcription. Not surprisingly, missense mutations in *MEN1* that disrupt Menin's interaction with JunD or MLL were reported in MEN1 patients and correlated with loss of Menin's tumor-suppressor function (Huang et al. 2012) (Fig. 1A).

Menin is highly conserved among species, with a 97%, 97%, 67% and 45% sequence homology between human and mouse, rat, zebrafish or fly, respectively (Fig. 1B). Two important Menin's binding partners, JunD and MLL, are also relatively conserved through evolution. The sequence identity between human JUND and the mouse, rat, zebrafish or fly orthologue proteins is 79%,

79%, 61% and 25%, respectively, whereas the identity to human MLL in the above mentioned species is 91%, 88%, 50% and 17%, respectively (source: Esembl).

To date, over 1300 germline pathogenic mutations in the *MEN1* gene have been reported (Concolino et al. 2016). These alterations are spread over the whole coding sequence, including the promoter and other regulatory regions. Most of the reported alterations are frameshift or nonsense mutations leading to lack of Menin expression or to a truncated (non-functional) protein variant (Lemos and Thakker 2008). Most missense mutations (68%) and the most frequently reported human mutations occur within highly conserved regions between human, rat, mouse, zebrafish and Drosophila (Marini et al. 2009, http://www.umd.be/MEN1/) (Fig. 1B). No genotype-phenotype correlation has been found in MEN1, since individuals with the same *MEN1* mutation may have different clinical presentations.

To date, several animal models of MEN1, or having a MEN1-like phenotype, have been described. We here review the existing models but we also discuss model organisms that could potentially be useful to study MEN1-associated pathogenesis (Fig. 2).

1. Drosophila melanogaster: a model to study Menin's function

The fruit fly Drosophila melanogaster has a long history as model organism and it has helped elucidate the basic principles of inheritance before DNA was discovered to be the carrier of genetic information (Beller and Oliver 2006). Genetic screening in Drosophila revealed that several genes important in tumorigenesis are conserved between fly and man, including *Notch*, *Shh* (sonic hedgehog), *Wnt* (Wingless) and *Men1*. The advantages of this model organism are on the one hand extremely short life span and generation time, numerous progeny, low maintenance costs, and, on the other, well-established methods to modify its genome. Disadvantageous is of course their lower complexity, as well as the necessity to keep mutant fly

strains as living stocks since embryos cannot be frozen. Due to their short life span and limited cell divisions, flies do not spontaneously develop cancer. However, transgenic flies show hallmarks of cancer such as evasion from apoptosis, sustained proliferation, metastasis, survival, genomic instability, and metabolic reprogramming when cancer-associated genes are mutated (Tipping and Perrimon 2014).

The *Mnn1* gene is the Drosophila orthologue of the human *MEN1* gene and both genes share a similar genomic organization. *Mnn1* consists of two transcripts, one which is found only in embryos while the other is expressed in adult tissues and encodes a 763 aa long protein. In humans, six different *MEN1* transcripts were reported having a different 5' UTR, however only two with substantial differences in the coding sequence (Marini et al. 2009). *Mnn1* encodes a protein having 46% overall identity with human Menin (Guru et al. 2001; Marini et al. 2009) (Fig. 1). Although the overall identity is rather low, the N-terminal part, which harbors the binding sites to several Menin interaction partners, has higher homology. Moreover, sequences at the C-terminus that were shown to be important for Menin's nuclear localization are conserved between human and fly. Although initial studies could not demonstrate an interaction between fly Menin and human JunD in yeast two hybrid assays, this binding was then confirmed when the fly homologue of JunD was used (Cerrato et al. 2006).

Three transgenic Drosophila strains have been generated by introducing slightly different deletions in *Mnn1* that result in loss of menin expression (Busygina et al. 2004; Cerrato et al. 2006; Papaconstantinou et al. 2005). All flies having a homozygous *Mnn1* deletion were viable, suggesting that the Menin orthologue is dispensable for Drosophila development. However, deletion of *Mnn1* resulted in a 5-7% reduction in viability, which was identified by screening

6000 flies. Such progeny numbers cannot be obtained using rodent models, thereby making of Drosophila a useful model organism to study genetic alterations causing subtle changes in survival.

As stated above, *Mnn1*-deleted flies developed rather normally, yet when exposed to DNA damaging agents or ionizing radiation they displayed a higher sensitivity than wild-type flies (Busygina et al. 2004). The authors ascribed this phenotype to a defect in nucleotide excision repair in the transgenic flies, which resulted in loss of genomic integrity. Interestingly, *MEN1* expression is frequently lost in human melanomas due to epigenetic silencing and deletion of the gene in melanocyte cell lines impairs homologous recombination-directed DNA repair while concomitantly inducing the error prone mechanism of non-homologous end-joining (Fang et al. 2013). Thus, *Mnn1*-deleted flies share molecular mechanisms with MEN1-associated human cancers.

In another *Mnn1*-knockout strain, flies were found to be more sensitive to a variety of different stressors (Papaconstantinou et al. 2005). Mutant flies exposed to heat shock, hypoxia, hyperosmolarity and oxidative stress had a higher degree of developmental arrest and lethality when compared with wild-type flies. Mechanistically, it was shown that Menin activates the transcription of the heat shock protein genes *Hsp70* and *Hsp23*. This induction was abolished in the knockout flies, thereby impairing their response to stress. In a follow up study, the same authors showed that the lack of heat shock proteins induction caused by *Mnn1* deletion can be linked to genome maintenance (Papaconstantinou et al. 2010). These studies broadened our knowledge of the role of Menin in regulating stress response and genomic stability.

Collectively, these Drosophila models provided us with valuable information about the function of Menin as a regulator of transcription and DNA repair, and the potential implications of these characteristics for tumorigenesis. Although Drosophila as a model organism may be a bit old-fashioned, it can still be useful to elucidate the genetic events leading to secondary mutations or genomic instability, or to screen for drugs that might counteract the abnormal DNA repair due to loss of Menin.

2. Zebrafish as a potential model of MEN1

2.1 Transgenic fish strains to study NET-associated genes

While the zebrafish Danio rerio has been used as animal model of developmental disorders for over 50 years, only recently it became a focus in cancer research. The overall advantages of the model lie in the high fecundity (up to 200 progenies/week), the simple assessment of the transparent embryos that develop outside the mother, and the conservation of most of the vertebrate organs. Also the endocrine system is conserved between human and zebrafish, with relevant orthologues of neuro-hormones being present (Vitale et al. 2014). In addition, a very broad genetic tool-box allows easy manipulation of the zebrafish genome for large genetic screens or for specific site-directed mutagenesis (nicely reviewed in Gut et al. 2017). A few years ago a global initiative was set in motion with the aim to target every gene in the zebrafish genome and provide researchers with the resulting mutants (www.zfin.org/). Unfortunately, although 36284 mutant alleles have been generated so far, no *Men1* mutation has been described. Yet, a functional orthologue of human *MEN1* exists, named *Men1*, which encodes a 617 aa long protein with 67% identity and 80% similarity to human Menin (Manickam et al. 2000) (Fig. 1). Expression patterns of Menin in fish larvae correlated with those in murine

tissues, and the binding ability of fish Menin to mouse and human JunD was also conserved (Manickam et al. 2000).

The generation of transgenic strains with defective Menin could provide us in the future with a promising MEN1 model especially since zebrafish develop tumors that quite well resemble human cancers at histological and molecular levels (White et al. 2013). Although such strains are currently not yet available, several zebrafish mutants exist that develop tumors belonging to the MEN1 spectrum including parathyroid, pancreatic and pituitary tumors. These models may be useful to study specific characteristics associated to NETs (e.g. interaction with the tumor microenvironment, metastatic potential, angiogenesis).

Primary hyperparathyroidism (1°HPT) is the most common phenotypic manifestation of MEN1. It is defined by an excess of parathyroid hormone (PTH) which results in hypercalcemia and ultimately in bone thinning and formation of kidney stones (Giusti et al. 2012). Fish do not possess a typical parathyroid gland, yet studies from Okabe and Graham (2004) proved that the gills of fish are evolutionarily related structures that express calcium-sensing receptors and PTH. A transgenic zebrafish strain deleted for *cdc73* has been suggested as a model for parathyroid tumors, as the human homolog HRPT2/CDC73 is responsible for the hyperparathyroidism–jaw tumor syndrome (Bourque and Houvras 2011; Carpten et al. 2002).

Pancreatic NETs were observed in zebrafish overexpressing the human *MYCN* gene under control of the core-zymod-promoter (Yang et al. 2004). The few analyzed transgenic fish mostly expressed insulin in the tumors, with glucagon expression found in one case. The tumor morphology indicated a malignant phenotype. In MEN1 patients, insulinomas are also more

frequent than glucagonomas, however all these tumors are usually benign (Tonelli et al. 2012). Interestingly, although the overexpression of MYCN was ubiquitous, only tumors in the pancreas arose. This establishes a parallel with *MEN1*, which is also ubiquitously expressed but its defective function causes tissue selective tumorigenesis. The possibility to perform large genetic screens is among the strengths of the zebrafish model and this could be applied to search for genes associated with tissue-specificity of pancreatic cancer.

Extensive work has been done to analyze the temporal and spatial development of the zebrafish pituitary gland, which shares with the organ in higher vertebrates the organization into intermediate and anterior lobe (Pogoda and Hammerschmidt 2009). At the 96h embryonic stage, the pituitary gland is already fully developed, thereby facilitating studies focusing on alterations that affect pituitary development. Observing anomalies during development is further simplified by the transparency of the embryos and by several well established imaging techniques (Ignatius and Langenau 2011). Mutation of the ubiquitin specific peptidase 39 (usp39), a protein involved in RNA splicing, promotes the expansion of anterior pituitary cells (hyperplasia), making of this transgenic zebrafish a potential model for these lesions (Rios et al. 2011). Liu and coworkers generated a transgenic zebrafish overexpressing the pituitary tumor transforming gene (*Pttg*) under the control of the proopiomelanocortin (POMC) promoter (Liu et al. 2011). These animals (Tg: Pomc-Pttg) develop corticotroph adenomas associated with decreased glucocorticoid sensitivity, oversecretion of the corticotroph hormone (ACTH) and subsequent metabolic disturbances similar to the hypercortisolism seen in Cushing's disease patients (Lacroix et al. 2015). Although ACTH-secreting tumors represent a minority (4%) of the pituitary adenomas occurring in MEN1 patients (Uraki et al. 2017), this zebrafish model might be a valuable tool to identify molecular pathways associated to pituitary tumorigenesis, or to

screen for potential anti-tumor drugs. Indeed, zebrafish embryos can be maintained in cell culture dishes, thus simplifying large-scale screens for therapeutic agents in a cost-effective way. In the study of Liu et al. (2011), R-roscovitine, a CDK inhibitor, was found to suppress corticotroph expansion in the transgenic zebrafish embryos. The effect of this drug was also validated in a mouse model of ACTH-secreting pituitary tumors, and R-roscovitine was therefore proposed as a potential therapeutic option for Cushing's disease (Liu et al. 2011).

2.2 Patient-derived xenografts (PDXs) in zebrafish

Another important use of zebrafish in cancer research involves xenotransplantation studies. A hallmark of tumors is the ability to engraft after transplantation into an appropriate recipient animal. While xenografts in mice are still the gold standard, xenografting in zebrafish is increasing in popularity and examples of transplanting human cancer cell lines (Lara et al. 2011; Stoletov et al. 2007), patient-derived cancer cells (Gaudenzi et al. 2016) or tissues (Marques et al. 2009) have been reported. Tumor cells can be engrafted in zebrafish embryos, juveniles or adults, and working protocols have been established that outline the advantages and disadvantages of each approach. The problem posed by the fact that for optimal growth human cells and zebrafish require the temperature of 37°C or 28°C, respectively, can be overcome by using 31°C for embryos and 35°C for adult zebrafish (Haldi et al. 2006).

From the early embryonic stages up to one month after birth, zebrafish do not possess a completely developed immune system, so that immune suppression to engraft xenotypic tissues is not necessary (Tobia et al. 2011). Furthermore, in the first 3-4 days of life the embryos do not need an established blood circulation system as the oxygen can perfuse through the tissues (Pelster and Burggren 1996). Thus, transplanted cells can survive until they

228 are able to induce neovascularization. Monitoring angiogenesis in zebrafish is simplified by the
229 availability of several transgenic strains where blood vessels are fluorescently labelled (e.g.
230 Tg(fli1-eGFP), Tg(flk1:mCherry), Tg(vegfr2:g-rGFP) (Lawson et al. 2002; Jin et al. 2005). In the
231 translucent embryo, the newly forming blood vessels can be measured in real time by confocal
232 fluorescence microscopy. In Fig. 3 is illustrated the example of a xenograft of a prolactin-
233 secreting pituitary adenomas in zebrafish embryos inducing the growth of the new vessels
234 (green-fluorescent labeled endothelial cells), which sprout towards the transplanted tumor
235 cells (chemically labeled in red).
236
237 Wurth and coworkers transplanted human pituitary adenoma stem cells (hPASCs) in zebrafish
238 and in NOD/SCID mice (Wurth et al. 2016). While there was no proliferation of hPASCs in
239 murine hosts up to 8 months after injection, these cells readily engrafted into zebrafish
240 embryos. In the latter system, neoangiogenesis towards the tumor mass could be detected 2-3
241 days after injection. Taking into account that 2-5x10^6 cells had to be injected into mice against
242 0.5x10^3 cells in zebrafish embryos, and that the time required for cell engraftment in the
243 embryos was a few days, exploiting this model organism for xenotransplantation experiments
244 may bring personalized cancer therapy within reach.
245
246 Xenograft studies in zebrafish suggest that the aggressiveness of human primary tumors
247 correlates with their ability to spread from the initial implantation site (Marques et al. 2009).
248 To conduct these studies, tumor cells are labeled with a fluorescent dye before implantation
249 and then invasion, migration and formation of micrometastases are followed in the translucent
250 embryo by laser scanning confocal live imaging. This approach has been tested also for NETs. In
251 a proof-of-concept study, using eight different human primary NET samples, Gaudenzi and

colleagues evaluated angiogenesis and cell migration in zebrafish xenotransplants (Gaudenzi et al. 2016). The tumors engrafted in 6 of 8 cases and tumor originating from metastases showed a higher migration capacity, thereby proving the general applicability of the concept. In MEN1 patients, the leading cause of death is the malignant potential of pancreatic endocrine tumors (Ito et al. 2013). Using this assay in future applications might allow us to assess pancreatic NETs for their propensity to metastasize, with important implications for predicting disease outcome and selecting appropriate therapeutic interventions.

3. Murine MEN1 models

3.1 Constitutive and conditional Men1 knockout mice

Not always murine models of cancer recapitulate the corresponding human disease. This is not the case for mouse strains with defective *Men1* function, which possess a remarkable phenotypic overlap with the human MEN1 syndrome. This despite the fact that most MEN1 mutations in patients are point mutations leading to truncated peptides, whereas the mouse models were generated by deleting entire exons of the *Men1* gene. Therefore, to mimic the human disease it is not necessary to have a specific genetic mutation as long as Menin's function is abolished.

We will here focus on the phenotypic differences among the various *Men1* knockout models in comparison to the human disease (see also Fig. 4).

Four different transgenic mouse lines were created by constitutive deletions of the *Men1* gene. In each model, different exons of *Men1* were targeted, resulting in loss of *Men1* transcription [e.g. deletion of exon 1-2, $Men1^{\Delta 1\text{-}2}$ (Harding et al. 2009), and deletion of exon 2, $Men1^{\Delta 2}$ (Loffler et al. 2007)] or in truncated *Men1* transcripts [e.g. deletion of exon 3, $Men1^{\Delta 3}$ (Bertolino et al. 2003a) or of exons 3-8, $Men1^{\Delta 3\text{-}8}$ (Crabtree et al. 2001)]. Regardless of the

targeting site, these mouse strains share a similar tumor spectrum, albeit the frequency of the individual tumor types differs among them. In all four transgenic lines, homozygous *Men1* knockout mice died at embryonic stages E10.5-E13.5. Therefore, studies were performed on heterozygous knockout animals. In all mouse lines, loss of the wild-type *Men1* allele was found in the tumors. This closely resembles the situation in MEN1 patients whose tumors usually show LOH (Valdes et al. 1998). In addition to these conventional knockout models, conditional mouse lines with tissue-specific deletion of *Men1* were generated and are here presented and compared with the constitutive models.

Parathyroid glands

In MEN1 patients, primary hyperparathyroidism is the most prevalent, and often the first, symptom with an incidence of 95-100%, and is usually due to hyperplasia or adenoma in the parathyroid glands (Giusti et al. 2012). In the constitutive knockout mouse lines this phenotype occurred with an incidence of only 17-42%. In $Men1^{\Delta 3\text{-}8/+}$ mice, although serum calcium levels were not elevated, the incidence of parathyroid adenomas was 12 fold higher than in wild-type animals. In one case, progression to parathyroid carcinoma was detected. In $Men1^{\Delta 3/+}$ mice, PTH levels were not significantly elevated, yet a few animals had enhanced secretion of the hormone (Bertolino et al. 2003a). Serum calcium levels were not assessed. In these mice, parathyroid adenomas were observed starting at 12 months (41% of mice) and by 19+ months they reached the frequency of 64%. Increased incidence of parathyroid tumors with age was also a characteristic of $Men1^{\Delta 2/+}$ mice but the rates were lower, reaching only 15% in 2-year-old mice (Loffler et al. 2007). In the $Men1^{\Delta 1\text{-}2/+}$ model, hypercalcemia and hypophosphatemia were observed, which were caused by overactivity of PTH, and not by increased levels of the

hormone (Harding et al. 2009). In conclusion, similar to MEN1 patients, all *Men1*-knockout lines show abnormalities in the parathyroid glands, albeit with variable frequencies.

In a conditional mouse model obtained by crossing *Men1*$^{\Delta 3\text{-}8\ flox/flox}$ mice with animals carrying Cre under control of the PTH promoter, up to 80 % of the homozygous mice developed hyperparathyroidism (Libutti et al. 2003). Elevated serum calcium levels were detected by 7 months of age, and an enlargement of the parathyroid glands was visible by 9 months. By 14 months, the size of the glands was 5-fold bigger in transgenic than in control animals. The specificity of the Cre-recombinase expression was very high, as no tissue other than the parathyroid glands was affected. Consequently, these mice are a suitable model to study primary hyperparathyroidism without the interference of other hormonal imbalances.

Gut and pancreas

Gastroentropancreatic (GEP) NETs are the second most common neoplasm in MEN1 patients. They can be subdivided into functioning tumors (=hormone secreting, frequency up to 40%) and non-functioning tumors (60-100%). Functioning GEP-NETs are defined based on the hormone they secrete. Insulinomas, pancreatic tumors secreting insulin, occur in 21% of patients, glucagonomas in 3%, while somatostatinomas, VIPomas and GHRH-omas are quite rare (1% of cases) (Tonelli et al. 2012). In MEN1 patients, gastrin-producing tumors (gastrinomas) mainly occur in the duodenum wall with a frequency of 50%, but micro-gastrinomas have also been observed in the pancreas (Pritchard 2007). GEP-NETs in MEN1 patients present as multiple lesions and tend to metastasize.

In *Men1*$^{\Delta 3\text{-}8/+}$ mice, pancreatic islet tumors developed at high frequency and correlated with elevated serum insulin levels, suggesting that they are insulinomas (Crabtree et al. 2001). The *Men1*$^{\Delta 3/+}$ model showed islet cell hyperplasia in 65% of cases at the age of 8-12 months, but

also adenomas (5%) and carcinomas (9%), whereas gastrinomas occurred in 19% of the analyzed mice. All pancreatic NET subtypes could be detected, with the occasional simultaneous overexpression of two hormones, a feature also observed in microadenomas of MEN1 patients (Anlauf et al. 2006). In this model (i.e. *Men1*$^{\Delta 3/+}$) GEP-NETs had a higher incidence then parathyroid tumors (41%).

Also in the *Men1*$^{\Delta 2/+}$ model of Loffler et al. (2007) over 80% of mice harbored pancreatic lesions of different grades up to adenomas. No gender difference was found regarding the incidence of GEP-NETs. It should be noted that in this study over 130 mice were analyzed thereby reaching more statistical power when compared with reports where fewer animals were studied. Most of the adenomas were insulinomas, some were glucagonomas, but gastrin immunoreactivity was usually absent. In line with other models, pancreatic islet hyperplasia and pancreatic adenomas were found at high incidence in *Men1*$^{\Delta 1\text{-}2/+}$ mice. Given that gastrinomas in patients develop in stomach and duodenum, 36 knockout mice between the ages of 18-21 months were intensively screened for the presence of these extrapancreatic gastrinomas, but none were found.

Altogether, the whole-body knockout mouse lines recapitulate the high incidence of pancreatic NETs seen in MEN1 patients but not that of the gastrinomas, much more rare.

Due to the underrepresentation of these tumors in the various mouse models, (Veniaminova et al. 2012) addressed the question as to whether the deletion of *Men1* is sufficient to induce gastrinomas by specifically deleting Menin in antral and intestinal epithelium. To this aim, the authors crossed *Men1*$^{\Delta 3\text{-}8\ flox/flox}$ mice with Villin-Cre or leucine rich repeat containing G protein coupled receptor 5 (Lgr5)-Cre mice. The resulting conditional knockout mice developed hypergastrinemia, but again no gastrin-secreting tumors. It needs to be noted that the

recombination of the floxed sites was not complete, so that residual Menin expression was still present in the targeted tissues and may have prevented tumor formation.

Three studies addressed the effect of tissue-specific deletion of Menin in pancreatic β-cells by crossing four different rat insulin promoter (Rip)-Cre mouse lines with three different floxed *Men1* lines (Fig. 4). In order to easily distinguish these models, we will name them $Men1^{\Delta 3\text{-}8flox/flox}$ (Crabtree et al. 2003), $Men1^{\Delta 3flox/flox}$ (Bertolino et al. 2003b) and $Men1^{\Delta 2flox/flox}$ (Biondi et al. 2004) based on the exonic region excised after recombination. Several versions of the Rip were used in these studies, leading to the conclusion that the stronger the promoter, the earlier and more pronounced was the phenotype. In all studies, homozygous tissue-specific deletion of *Men1* resulted in larger islet sizes compared to the heterozygous-deleted mice, as well as hyperplasias and insulinomas. In conditional $Men1^{\Delta 3\text{-}8flox/flox}$/Rip-Cre mice, the size of the insulinomas correlated with the secretion of insulin, the level of blood glucose, and the survival rate (Crabtree et al. 2003). Tumor latency in the conventional heterozygous knockout mice was dependent on the complete loss of *Men1,* which represented the rate limiting step, whereas in the conditional floxed mice both alleles are lost upon recombination and thus the tumors develop earlier. Tumor progression was characterized by dedifferentiation, angiogenesis and multistage tumorigenesis (Bertolino et al. 2003b). Studies of $Men1^{\Delta 3flox/flox}$/*Rip-Cre* and $Men1^{\Delta 2flox/flox}$/*Rip-Cre* models revealed a poor tissue-specificity of RIP-mediated Cre expression, and consequently these mice developed pituitary tumors too, which were mainly prolactinomas. Different chromosomal rearrangements were found in pancreatic and pituitary tumors of $Men1^{\Delta 3flox/flox}$*/Rip-Cre* animals, with duplication of chromosome 11 and of chromosome 15, respectively, being the most frequent alterations. One possible explanation for these findings is that loss of *Men1* increases the susceptibility to a second mutagenesis hit in a tissue-specific manner.

To study the early events associated with *Men1* deletion in pancreatic islets two conditional and inducible mouse models were generated. Schnepp et al. (2006) crossed the *Men1*$^{\Delta 3\text{-}8flox/flox}$/Rip-Cre mice described above (Crabtree et al. 2003) with *Cre*-ER (estrogen receptor) transgenic mice expressing Cre under the control of a ubiquitously active ubiquitin carrier 9 (UBC9) promoter. In the resulting mice (named *Men1*$^{l/l}$;*Cre*-ER) the excision of *Men1* can be induced by tamoxifen in a controlled fashion. *Men1*$^{l/l}$;*Cre*-ER mice at 12 weeks of age were treated with tamoxifen and their pancreata were analyzed 7, 14 and 30 days later. Loss of *Men1* caused an increase in islet cell proliferation detectable already 7 days post-treatment, which resulted in islet enlargement and hyperplasia at the 14-day time point (Schnepp et al. 2006). One limitation of this study is that mice were analyzed before tumor development could take place. Moreover, the *Men1* gene was deleted not only in islet cells but also in the exocrine pancreas and in other mouse tissues due to the broad expression of the Cre-ER transgene. Subsequently, another conditional, inducible mouse line was established by crossing Men1$^{l/l}$ mice (see above) with mice expressing the tamoxifen-inducible *Cre*-ER driven by the rat insulin promoter (*RIP2-Cre*-ER) for a Cre expression restricted to pancreatic endocrine cells (Lines et al. 2017). The resulting *Men1*$^{l/l}$;*RIP2-Cre*-ER transgenic mice were treated with tamoxifen at 12 weeks of age and analyzed up to 5 months later (age 7,5-8,5 months) when pancreatic endocrine tumors (insulinomas) were present. Loss of menin in the islets was observed in transgenic mice at all ages, along with a rise in both proliferation of pancreatic β-cells and islet area (Lines et al. 2017a). These inducible mouse models further strengthen the hypothesis that increased cell proliferation is the first effect of Menin loss.

Pituitary gland

Pituitary tumors were a feature of all conventional knockout mice. Interestingly, the gender difference that is seen in men is recapitulated in mice: females have a higher prevalence of pituitary adenomas. In the *Men1*$^{\Delta 2/+}$ line, pituitary tumors were present in 78 % of the female mice *versus* 42.0 % of the males (Loffler et al. 2007) (Fig. 4). In these mice, both anterior pituitary microadenomas and macroadenomas were observed, which were highly vascularized and showed signs of necrosis. Mass effects due to the significant increase in pituitary size were seen. Prolactin-positive and nonfunctioning tumors were found in these mice, but no ACTH-secreting adenomas. Pituitary adenomas were more frequent in females also in *Men1*$^{\Delta 3\text{-}8/+}$ mice, and all analyzed tumors were prolactinomas (Crabtree et al. 2001). In the *Men1*$^{\Delta 1\text{-}2/+}$ line (Harding et al. 2009) the incidence of pituitary adenomas was only 31.4%, and all tumor subtypes were seen (i.e. prolactin-, GH- and ACTH-secreting adenomas, as well as nonfunctioning ones). Considering pituitary adenomas, the various constitutive knockout mice are a relatively faithful model of MEN1, although differences in tumor incidence and subtypes were observed between mice and men.

Adrenal gland

All conventional *Men1*-deficient models developed tumors in the adrenal gland with frequencies ranging from 10% to 43%. Mainly adrenocortical tumors (20-43%) but also bilateral pheochromocytomas (7%) were found in *Men1*$^{\Delta 3\text{-}8/+}$ mice (Crabtree et al. 2001). *Men1*$^{\Delta 1\text{-}2/+}$ animals developed cortical hydroxysteroid hydroxylase-positive adenomas at low frequency (7%) and pheochromocytoma was detected in one case (Harding et al. 2009). Surprisingly, only male knockout mice developed adrenocortical adenomas. While gender differences in pituitary adenomas are a feature of MEN1, no differences in adrenal tumors development between genders have been reported in patients (Goudet et al. 2011).

Other tissues

MEN1 patients suffer from a variety of skin lesions that occur in 33%-84% of the cases. The presence of multiple collagenomas and angiofibromas is a good indicator of the MEN1 syndrome (Ashgarian et al. 2004). Cutaneous tumors were not observed in any of the above described mouse models. In contrast, all constitutive knockout mice developed tumors in the gonads. Leydig tumors of the testis were found in 22% to 88% of the male mice and sex-cord stromal tumors in the ovary were found in *Men1*$^{\Delta 3\text{-}8/+}$, *Men1*$^{\Delta 3/+}$ and *Men1*$^{\Delta 1\text{-}2/+}$ females with incidences ranging from 8% to 40%. These tumors do not belong to the MEN1 tumor spectrum. Detailed studies revealed that the wild-type *Men1* allele was not lost in the Leydig cell tumors of the heterozygous knockout mice (Loffler et al. 2007) and thus molecular events other than Menin loss-of-function might account for the tumorigenesis in these cells.

Non–small-cell lung cancer (NSCLC) was observed in *Men1*$^{\Delta 3\text{-}8/+}$ mice with an incidence varying from 22% (Crabtree et al. 2001) to 42 % (Pei et al. 2007) depending on the study. Lung tumors are rarely seen in MEN1 patients (8% of cases). The different frequency of NSCLC occurring in the same transgenic model might be due to the background strain of the mice, as Pei and colleagues backcrossed *Men1*$^{\Delta 3\text{-}8/+}$ animals to the C57/BL6 background.

For the sake of completeness, it needs to be mentioned that *Men1*$^{\Delta 3}$ mice older than 19 months of age develop mammary carcinomas at low frequency (8.3%) (Bertolino et al. 2003a), as well as carcinomas of the prostate in 12.8% of cases (Seigne et al. 2010). These neoplasms do not belong to the tumor spectrum of the MEN1 syndrome.

It should be taken into account that for the models described above, mouse lines were usually a mixture of different background strains. This hampers the comparison of the different murine MEN1 models, due to strain specific sensitivity to tumor development (Brayton 2006).

3.2 Preclinical studies with MEN1 mouse models

As stated above, the *Men1* knockout models recapitulate several of the key phenotypic features of the human MEN1 syndrome. Hence it is reasonable to exploit them for preclinical studies aimed at identifying novel effective therapies against NETs, and a few such studies have indeed been conducted so far. We here discuss examples evaluating different therapeutic approaches.

Since Menin-dependent tumorigenesis starts with the loss of both functional *Men1* alleles, Walls and colleagues (Walls et al. 2012) explored the feasibility of a *Men1* gene replacement therapy *in vivo* by using a recombinant replication-deficient adenoviral vector containing the mouse *Men1* gene under the control of a cytomegalovirus promoter (Men1.rAd5). The virus was injected into the pituitary adenomas of $Men1^{\Delta 1\text{-}2/+}$ female mice (Walls et al. 2012). Although, the proliferation rates of pituitary tumors decreased, no changes in tumor mass or apoptosis were detected 4 weeks after injection of the recombinant adenoviruses.

Promising results were obtained by treating $Men1^{\Delta 3\text{-}8/+}$ mice with the monoclonal antibody mAB-G6-31 directed against vascular endothelial growth factor A (VEGF-A), the best characterized pro-angiogenic factor (Korsisaari et al. 2008). Pituitary tumors are highly vascularized and thus by inhibiting angiogenesis tumor progression should be suppressed. Indeed, Korsisaari et al. (2008) could nicely demonstrate that both tumor size and vascularization were reduced by the drug both *in situ* in $Men1^{\Delta 3\text{-}8/+}$ mice and in syngeneic models of the mouse pituitary adenomas. Upon treatment with the anti-VEGFA antibody, mice bearing pituitary tumors showed a reduction in serum prolactin levels. In contrast, the insulin levels, elevated due to the insulinomas, were unaffected by the drug. Targeting angiogenesis with sunitinib, a multikinase inhibitor, reduced the proliferation rates of the insulinomas

458 developing in *Men*$^{\Delta 3\text{-}8flox/flox}$*/Pdx1-Cre* mice, which was not accompanied by changes in tumor
459 vascularization (Shen et al. 2009). Effects on insulin secretion were not assessed in this study.
460 Thus, both studies suggest that the treatment of MEN1-associated tumors with drugs targeting
461 angiogenesis might be effective at reducing tumor size.

462 NETs often express somatostatin receptors (SSTRs) on their cell membrane, and this has been
463 exploited for diagnostic imaging, radiotherapy and pharmacological treatment using stable
464 somatostatin analogues such as octreotide. Taking advantage of SSTR expression on pancreatic
465 NET cells, Smith et al. (2016) used an adeno-associated virus displaying octreotide on the
466 surface to deliver tumor necrosis factor (TNF) to the tumor cells in order to induce apoptosis.
467 These viral particles were tested in the conditional *Men*$^{\Delta 3\text{-}8flox/flox}$*/Pdx1-Cre* mice (Shen et al.
468 2009) *in vivo* and found to reduce tumor size, to lower tumor metabolism and insulin secretion
469 thereby leading to improved survival (Smith et al. 2016).

470 The efficacy of pasireotide (SOM230), a multi-ligand somatostatin analogue, against pancreatic
471 NETs was evaluated in the *Men1*$^{\Delta 3\text{-}8flox/flox}$*/Pdx1-Cre* mouse model (Quinn et al. 2012). This
472 agent reduced tumor volume of the insulinomas by activating apoptosis. Circulating insulin
473 levels decreased in the treated mice and, as a consequence, blood glucose reached more
474 physiological levels. Adjustment of the blood glucose levels had a positive effect on the survival
475 rates of the treated mice, but due to small group sizes no statistical analyses were performed.
476 In another study, treatment of *Men1*$^{\Delta 1\text{-}2/+}$ mice with pasireotide was found to reduce pancreatic
477 tumor volume and frequency, to suppress pancreatic islet cell proliferation and induce
478 apoptosis (Walls et al. 2016).

479 Chromatin remodeling via histone modifications has been shown to play an important role in
480 tumorigenesis and several drugs have been developed that target epigenetic pathways (Jones

et al. 2016). Menin interacts with histone methyltransferases in pancreatic β-cells thereby initiating specific transcriptional programs that promote cell proliferation (see above). Moreover, human sporadic and familial pancreatic NETs show mutations in chromatin remodeling genes such as *DAXX* and *ATRX* (Jiao et al. 2011). These findings provide the rationale for the evaluation of compounds targeting epigenetic regulatory proteins in these tumors. Recently, Line at al. (2017b) tested several epigenetic drugs for efficacy on pancreatic NET cells *in vitro*, and the most promising one, JQ1, was then evaluated *in vivo* in *Men1*$^{l/l}$;*RIP2-Cre*-ER mice. JQ1 is an inhibitor of the bromo and extra-terminal motif (BET) proteins that bind acetylated lysine residues. This agent was able to reduce proliferation and promote apoptosis in pancreatic NETs of the transgenic mice, suggesting that targeting epigenetic pathways might be an effective strategy for the treatment of these tumors.

Based on the observation that active β-catenin accumulates in pancreatic NETs of *Men1*-deficient mice, conditional *Men1*$^{\Delta 3flox/flox}$*/Rip-Cre* mice were treated with a small molecule antagonist of the T cell factor/β-catenin complex, i.e. PKF115-584 (Jiang et al. 2014). Mice at the age of 14 months were treated for 8 weeks with PKF115-584 and then their pancreatic tumors were assessed for proliferation, which was suppressed by the drug. In addition, PKF115-584 treatment improved hypoglycemia in these mice by reducing insulin secretion.

Altogether, these studies emphasize the potential of the various *Men1* knockout mouse models as translational platforms to identify effective therapies for MEN1 patients.

3.3 Patient-derived xenografts (PDXs) in mice

The variety of cell types, stages of progression and sequential mutational events contribute to the tumor heterogeneity typically seen in most human tumors, including NETs (Hessman et al. 2001). It has been suggested that one of the reasons behind drug failure in clinical testing is the

lack of complexity of the models used for preclinical testing. A possibility to circumvent the potential lack of predictive power of various lower model organisms could be the use of patient-derived xenografts (PDXs) in preclinical studies.

Transplantation of human tumor samples into appropriate immunocompromised murine hosts allows the propagation of the primary tumors while maintaining their histological and genetic characteristics (Cassidy et al. 2015). Protocols for successfully engrafting human tumor cells/tissues into mouse recipients have been established (Mattar et al. 2017). The engraftment rate is strongly dependent on the intrinsic characteristics of the primary tumor, with aggressive tumors having in general higher rates (Siolas and Hannon 2013). The slow proliferation rate of NETs has historically hampered the generation of tumor cell lines (Grozinsky-Glasberg et al. 2012) and poses a problem also for their xenotransplantation. Only a few studies so far have reported the engraftment of NETs but with rather low success rates. In a proof-of-concept study Powers et al. (2017) demonstrated for the first time the successful engraftment of pheochromocyomas and paragangliomas in the NSG mouse model. Histological analysis proved the conservation of tumor features in the PDXs, and BrdU labeling demonstrated the proliferation of tumor grafts in the host. The NSG mouse strain is more immunocompromised than the better-known NOD/SCID strain and the lack of thymoma formation allows the mice to age up to 1.5 years in appropriate housing conditions. Therefore, NSG mice represent the ideal strain to engraft slow-growing tumors (Shultz et al. 2005).

In another study, using fragments of 39 well differentiated grade 1 and 2 pancreatic NETs, only 1 tumor generated xenografts in 90 % of the host NSG mice over multiple passages (Krampitz et al. 2016). In a bigger tumor cohort, only 7 of 106 gastrointestinal NETs were successfully engrafted. Of these 7 xenografts, only one tumor could be passaged several times and maintained for two years in NSG mice. Remarkably, the tumor retained its morphology and

molecular characteristics over time (Yang et al. 2016). With this established PDX model the authors plan to carry out preclinical drug studies. Francois and colleagues have already used PDX models of pancreatic NETs in drug efficacy tests (Francois et al. 2015). Treating the PDX models with an inhibitor of fokal adhesion kinase (FAK), they could show a reduction in tumor progression over time. Interestingly, the tumor volume of the untreated control PDX doubled in two weeks, indicating a faster proliferation of the tumor cells when compared with the aforementioned studies.

MEN1 is a rare disease with 1 in 30.000 people being affected (Marini et al. 2009). Accessibility to patient material is therefore limited. This precious material could be preserved by subsequent passaging in nude mice to then conduct biomarker screening and drug testing using the same tissue. The finding that loss of Menin increases cell proliferation might come in handy for the engraftment of MEN1 patient-derived tumors that usually have no expression of the protein. Indeed, these tumors might grow and progress faster than usual NETs, thereby resulting in a higher engraftment rate. In a xenotransplantation study, A549 cells derived from a human lung carcinoma cell line were stably transfected with either control or Menin-overexpressing constructs. These cells were then injected into nude mice, and it was demonstrated that Menin levels negatively affect the engraftment and growth of the cells (Gao et al. 2009).

4. MENX rats as a MEN1-like model

The MENX multi-tumor syndrome was discovered by serendipity in a rat colony that spontaneously started to develop multiple NETs. Affected rats present with anterior pituitary adenomas, adrenal and extra-adrenal pheochromocytomas, thyroid C-cell hyperplasia, parathyroid hyperplasia and pancreatic islet cells hyperplasia (Fritz et al. 2002; Wiedemann and

Pellegata 2016). The MENX tumor spectrum shares features with both MEN1 and MEN2 human syndromes. However, in contrast to the human syndromes, MENX is inherited as a recessive trait and affected rats are homozygous for the underlying mutation. Rat tumors develop at high frequency (often 100%) with a progression from hyperplasia to neoplasia with time. Whereas wild-type rats live approximately 24-30 months, the average life span of MENX-affected rats is 10±2 months.

Following classical linkage studies (Piotrowska et al. 2004) and candidate gene analysis, a tandem duplication of 8 nucleotides in exon 2 of *Cdkn1b* (p27), which causes a frameshift, was identified as the causative mutation responsible for MENX (Pellegata et al. 2006). The mutant *Cdkn1b* allele encodes a very unstable p27, which is rapidly degraded *in vitro* (Molatore et al. 2010a) and virtually not detected in the mutant rat tissues *in vivo* (Pellegata et al. 2006). Therefore, we refer to it as a loss-of-function mutation. As mentioned earlier, the CDK inhibitor p27 was demonstrated to be a transcriptional target of Menin in pancreatic β cells (Karnik et al. 2005). Noteworthy, germline mutations in the human homologue, the *CDKN1B* gene, were discovered in patients with a MEN1-like phenotype (Pellegata et al. 2006). These findings identified a novel MEN syndrome, named MEN4, caused by alterations of p27 (Lee and Pellegata 2013).

Pituitary gland

MENX-affected rats develop multifocal tumours in the anterior pituitary (frequency 100%), which belong to the gonadotroph lineage and are histologically and ultrastructurally similar to human gonadotroph adenomas (Marinoni et al. 2013). In patients, gonadotroph adenomas are clinically nonfunctioning (named NFPAs). These tumors occur in about 5% of MEN1 patients. The lesions in affected rats start from 4 months of age as multiple neoplastic nodules and

progress to become large adenomas that efface the gland. Rat adenomas express the glycoprotein alpha-subunit (αGSU) at all stages of progression. Similar to NFPA patients, the expression of LHβ and FSHβ subunits is present in the early lesions but is then lost in the larger tumors, accordingly serum LHβ levels in the mutant rats decrease with tumor progression. Rat adenomas show mitotic activity and relatively high Ki67 labelling index (average 8% at >8 months) (Marinoni et al. 2013). In addition to morphology and hormone expression, rat and human pituitary adenomas also share common genetic signatures. Transcriptome analysis identified genes dysregulated genes in both species that are involved in tumorigenesis and may represent novel biomarkers for future clinical applications (Lee et al. 2015).

MENX rats are the only spontaneous model of NFPAs. Moreover, adenomas develop in all affected animals. Considering these two aspects, MENX rats are the ideal model to evaluate novel antitumor drugs for their efficacy against NFPAs. Studies were thus performed to test BEZ235, a dual PI3K/mTOR inhibitor, *in vitro* (on 3D cultures of rat primary pituitary adenoma cells) and *in vivo* in the rats. The results demonstrated that BEZ235 has anti-proliferative and pro-cell death activities against rat pituitary adenomas tumors both *in vitro* and *in vivo*. Diffusion weighted-magnetic resonance imaging (DW-MRI) was used to monitor treatment efficacy and emerged as a useful modality to assess early therapy response (Lee et al. 2015). These findings provided a rationale for the clinical investigation of PI3K/mTOR inhibition in NFPA patients.

Pancreas

MENX rats develop pancreatic islet hyperplasia (100%), which leads to an increase in islet mass already detectable 2 weeks after birth (Wiedemann et al. 2016a). The pancreatic islets consist of five types of cells each producing a specific hormone. In mutant rats, all five cell

597 populations are increased in number. The islet hyperplasia occasionally progresses to
598 insulinomas (Fig. 5). Following oral glucose stimulation test, mutant female rats showed
599 increased insulin output when compared with wild-type littermates, compatible with their
600 islet hyperplasia (Pellegata, unpublished).

601 *Adrenal gland*

602 Adrenal tumors belong to the spectrum of the MEN1 syndrome but usually arise in the cortex.
603 Tumors of the adrenal medulla (pheochromocytomas) occasionally occur in *Men1*-deficient
604 mice (see above). MENX rats develop adrenomedullary hyperplasia at 3-4 months of age, which
605 progresses to pheochromocytoma by 6-8 months (frequency 100%). The histology of these
606 lesions resembles that of human pheochromocytoma. The rat tumors show high mitotic counts
607 and elevated Ki67 labeling index (Miederer et al. 2011). Pheochromocytoma in patients
608 secretes an excess of catecholamines. To verify whether the same occurs in rats, urine
609 catecholamine levels were measured longitudinally in both MENX-affected and wild-type
610 animals by high performance liquid chromatography. Mutant rats at 8 months of age show
611 increased urinary concentrations of norepinephrine, normetanephrine, 3-methoxytyramine and
612 dopamine compared to wild-type age-matched rats, hence their tumors are noradrenergic
613 (Wiedemann et al., 2016b). This is consistent with the lack of expression of phenylethanolamine
614 N-methyltransferase (PNMT), the enzyme that converts noradrenaline to adrenaline, in these
615 tumors (Molatore et al. 2010b). In patients with pheochromocytoma, high catecholamine
616 secretion associates with an increase in blood pressure, which, if not controlled, can cause
617 severe symptoms. Non-invasive measurements performed on mutant and wild-type rats over
618 time showed that blood pressure increases in the MENX animal model together with tumor
619 progression, as in patients. Moreover, mutant rats show pathological changes in organs such as

heart and kidney, similar to those observed in the patients if blood pressure is not controlled (Wiedemann et al. 2016b). Rat and human pheochromocytomas also share gene expression signatures (Molatore et al., 2010b; Leinhauser et al., 2015). For the diagnosis and staging of pheochromocytoma functional imaging plays a crucial role. In addition to ^{131}I/^{123}I-metaiodobenzylguanidine (MIBG) scintigraphy, a gold standard procedure, a variety of tracers have been developed for the detection of pheochromocytoma using positron emission tomography (PET), including ^{18}F-fluorodopamine (DOPA), ^{11}C-hydroxyephedrine (HED) a norepinephrine analog, and ^{68}Ga-DOTATOC targeting somatostatin receptors. Noteworthy, also the rat tumors show uptake of these radiotracers (Miederer et al. 2011; Gartner et al. 2013). MENX rats were used to test a novel norepinephrine analog (e.g. LMI1195) for its ability to detect pheochromocytoma *in vivo* by PET imaging. High and specific accumulation of ^{18}F-LMI1195 in the adrenals of tumor-bearing mutant rats was seen over time. Its favorable biodistribution makes it a promising PET tracer for pheochromocytoma imaging (Gartner et al. 2013). Given that in MENX rats pheochromocytoma develops with complete penetrance and recapitulates several key features of the human tumors, therapy response studies were conducted to test the efficacy of BEZ235 (dual PI3K/mTOR inhibitor) in this model *in vivo*. The results showed that this agent holds promise for the treatment of pheochromocytoma (Lee et al. 2017).

Other organs

Parathyroid hyperplasia has been observed in MENX-affected rats. The incidence was 65% when considering macroscopically visible tumors at the time of death (Fritz et al. 2002). It is not known whether the blood levels of PTH are elevated in affected rats as a consequence of the

parathyroid hyperplasia. MENX rats also present with bilateral hyperplasia of the thyroid C-cells, a feature exclusively associated with MEN2.

Conclusive remarks

In cancer research, animal models are used to understand the underlying pathogenetic mechanisms and to develop strategies to diagnose and treat the corresponding human disease. Depending on the specific questions to be addressed, not necessarily model organisms with higher complexity represent the most appropriate option. For instance, high-throughput screening of new putative targets or antitumor agents might be more easily and cost-effectively carried out in Drosophila or zebrafish, whereas rodents or PDX models might be better suited for an in depth characterization of tumor biology. The available Menin-deficient transgenic Drosophila strains have shed light on the protein's function. The use of PDX models of NETs (in mice) is still in its infancy, as only a few studies have been so far performed and with variable success. However, this platform could allow to preserve and to propagate the rare tumors of MEN1 patients for further studies. Although tumors cannot be passaged in zebrafish xenografts, the implantation and characterization of primary patient samples are quite promising.

Interestingly, although mouse models of MEN1 show an impressive overlap of pathologically relevant features with the human syndrome, they have been underused for molecular and preclinical studies. Serum profiling of these mice or tumor transcriptome analysis could provide us with new therapeutic targets or biomarkers, whereas the evaluation of novel drugs or existing ones in off-label-use in these models could identify effective therapies for MEN1 patients. Moreover, these mouse models might be suitable to establish imaging modalities for diagnosis and therapy-response monitoring. Therefore, investing time and effort in further

characterizing the existing MEN1 models has the potential for a big return if we thereby create more reliable *in vivo* platforms for therapy assessment studies.

Declaration of interest

The authors declare that they have no conflict of interest.

Acknowledgements

We thank Dr. Germano Gaudenzi and Prof. Giovanni Vitale (University of Milan, Italy) for sharing with us unpublished data. The work of the authors is supported by the Deutsche Forschungsgemeinschaft SFB824, subproject B08, and by the Deutsche Krebshilfe (grant 70112383).

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958

Figure legends

Figure 1. Scheme of human Menin structure, its domains and mutations, and alignment of Menin across different organisms. (A) Scheme of the human *MEN1* gene, the Menin protein and location of the so far identified mutations according to the MEN1 database (http://www.umd.be/MEN1/). The domains of Menin mediating the interaction with MLL or JunD are reported. NLS, nuclear localization signal. Asterisks indicate the most frequent mutations. (B) Multi-species alignment of the Menin protein sequence. Protein sequences were obtained from UniProtKB for Homo sapiens (O00255), Mus musculus (O88559), Rattus norvegicus (Q9WVR8), Danio rerio (Q9IAA9), Drosophila melanogaster (Q9VM47). Asterisks indicate the position of the most frequent mutations, as highlighted in (A).

Figure 2. Animal models of MEN1 currently available and potential alternative models.

Figure 3. Xenografting human cancer cells into zebrafish embryos to follow angiogenesis and migration. (A-C) Example of xenografting human pituitary adenoma cells in zebrafish. Red stained primary cells isolated from a human prolactin-secreting pituitary adenoma were xenografted in 48 hours post fertilization Tg(fli1:EGFP)^{y1} zebrafish embryos, that express the enhanced green fluorescent protein (EGFP) in the vascular endothelium. In comparison to PBS-injected control embryos (A), 24 hours post injection tumor-grafted embryos (B and C) showed alterations in the pattern of sub-intestinal vessel (SIV) plexus and the presence of tumor induced-endothelial structures that sprouted from the SIV and reached the implant. The red channel was omitted in images B and B' to highlight the newly formed microvascular network.

B’ and C’ represent digital magnification of white box in B and C, respectively. All images are oriented so that rostral is to the left and dorsal is at the top.

Source: Picture kindly provided by Dr. Germano Gaudenzi and Prof. Giovanni Vitale (University of Milan, Italy)

Figure 4. Tumor spectrum of the MEN1 syndrome and phenotype of the *Men1* knockout mouse strains. The incidence of the various tumor types both in MEN1 patients and in the different mouse lines are reported.

Figure 5. Pancreatic islets in wild-type and MENX mutant rats. (A) Hematoxylin and eosin (HandE) staining of an area of the pancreas of a wild-type adult rat containing two islets. (B) HandE staining of an area of the pancreas of an adult MENX mutant rat. The endocrine tumor is composed mostly of insulin-expressing cells, as demonstrated by immunohistochemistry with an anti-insulin antibody (right figure). Size bars: panel A and two most right hand panels in B: 100µm; left hand panel in B: 200µm.

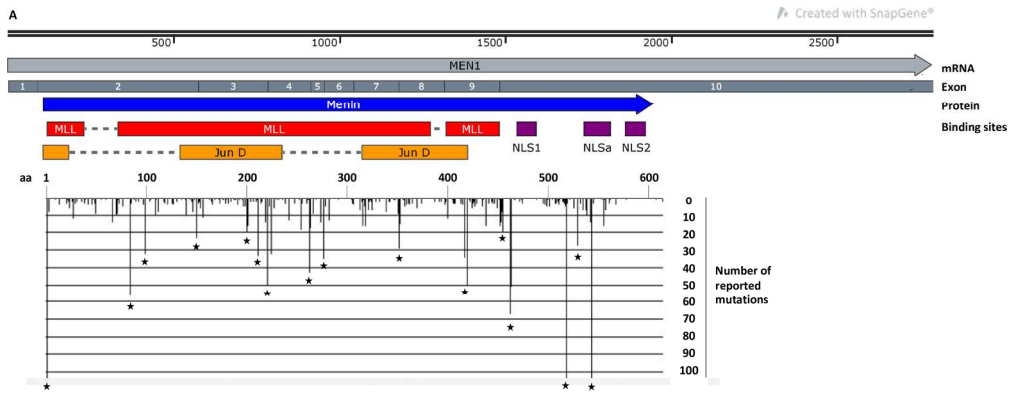

Figure 1A

190x73mm (300 x 300 DPI)

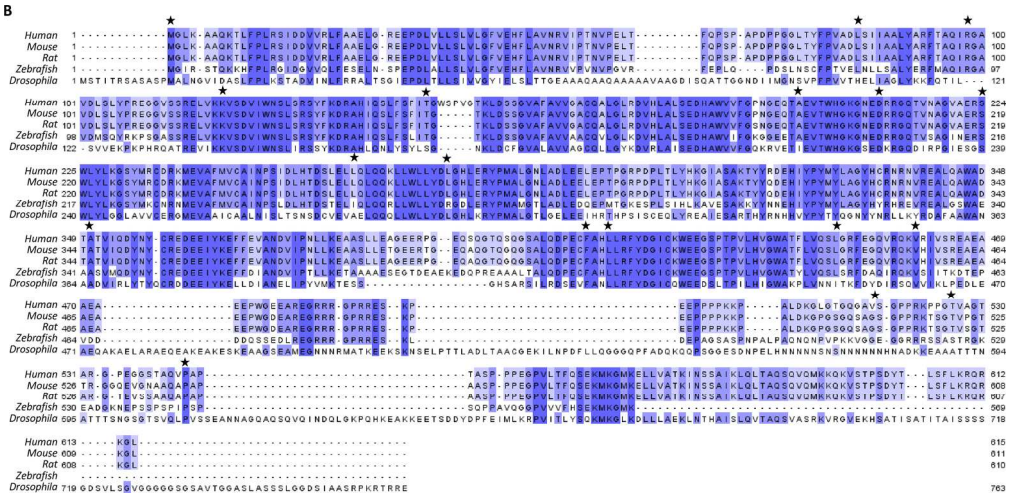

Figure 1B

190x92mm (300 x 300 DPI)




Genetically modified and spontaneous models	**Mnn1^{e200}** (Busygina, 2004) **Mnn1^{e173} and Mnn1^{e30}** (Papaconstantinou,2005) **Mnn1-** (Cerrato, 2006)	*Not described yet*	**MEN1 Δ3-8/+** (Crabtree,2001) **MEN1 1/T** (Bertolino,2003) **MEN+/-** (Loeffler,2007) **MEN+/-** (Harding,2009)	**MENX** *(Cdkn1b)*(Fritz,2002)
Xenograft models	no	yes	yes	no

222x71mm (300 x 300 DPI)

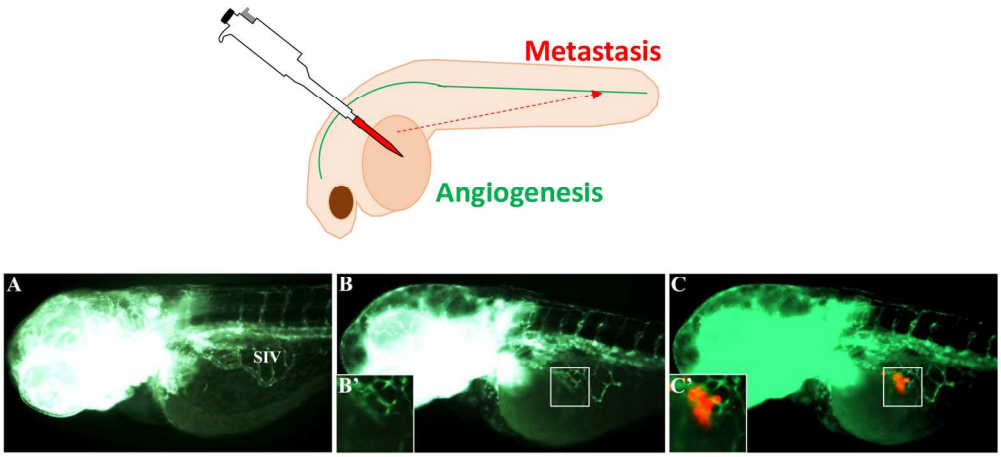

Figure 3

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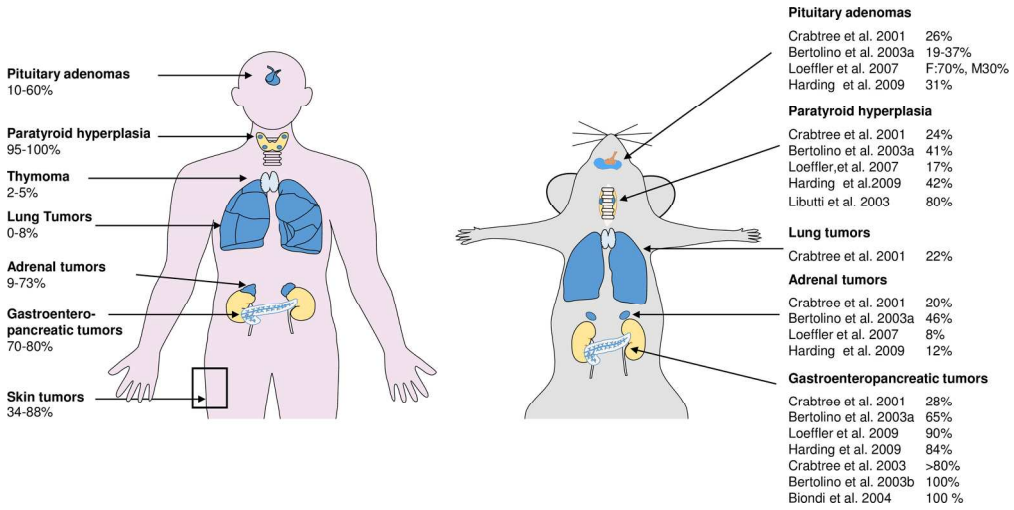

Figure 4

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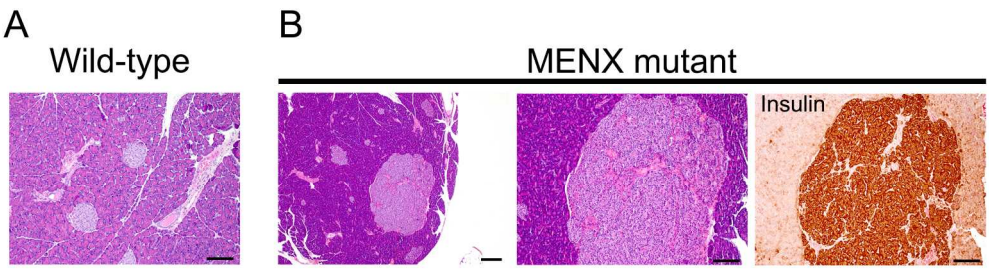

Figure 5

187x51mm (300 x 300 DPI)