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**Loss of APC function in mesenchymal cells surrounding the Müllerian duct
leads to myometrial defects in adult mice**

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Running title: Loss of Apc leads to myometrial defects

Abstract

The WNT signal transduction pathway plays a rate limiting role in early development of many different organs. To study the functional consequences of constitutive activation of the canonical WNT pathway in the developing uterus, we generated a novel mouse model where loss of the tumor suppressor gene *Apc* was induced. A mouse model was generated and evaluated where *Amhr2*^{Cre/+} driven loss of *Apc* exon 15 was induced. The *Apc* recombination was detected mainly in the myometrial layer of the adult uterus. A significant loss of muscle fibers in myometrium was apparent, though with very few muscle cells earmarked by nuclear β -catenin. The finding was confirmed in the *Pgr*^{Cre/+}; *Apc*^{15lox/15lox} mouse model. Loss of APC function in mesenchymal cells surrounding the fetal Müllerian ducts results in severe defects in the myometrial layers of the uterus in adult mice, suggesting that the WNT signaling pathway plays important roles in maintaining myometrial integrity.

Key words: APC, WNT, myometrium, endometrium

1. Introduction

Shortly after the onset of testis differentiation during mammalian embryonic life, the first testicular hormone to be produced is anti-Müllerian hormone (AMH), secreted by the Sertoli cells, soon followed by production of testosterone by the Leydig cells. Fetal testosterone production stabilizes the Wolffian ducts, which give rise to the epididymides and vasa deferens. AMH binds to its receptors on the mesenchymal cells surrounding the Müllerian ducts, which induces apoptosis and results in Müllerian duct regression. Because embryonic female gonads do not produce any testosterone or AMH, Wolffian ducts will regress and Müllerian ducts are stabilized to form the upper part of the vagina, the uterus and the oviducts (Biason-Lauber, 2010). For the current investigations, we developed and analysed a novel mouse model to study the role of canonical Wnt/ β -catenin signalling in embryonic development and homeostasis of the uterus.

In canonical Wnt/ β -catenin signalling, a central role is played by the “destruction complex” consisting of three scaffolding proteins, namely AXIN1, AXIN2, and APC (adenomatous polyposis coli), two kinases, glycogen synthase kinase-3 β (GSK3 β) and casein-kinase 1 α (CK1 α), and β -catenin (CTNNB1). In the absence of extracellular Wnt ligands, formation of the destruction complex marks β -catenin by Ser-Thr phosphorylation, thus promoting its proteolytic degradation through ubiquitination. In the presence of Wnt signals however, formation of the destruction complex is inhibited and β -catenin is able to accumulate in the cytoplasm and eventually translocate to the nucleus where, in association with members of the TCF/LEF family of transcription factors, it activates transcription of a broad spectrum of downstream target genes (<http://www.stanford.edu/~rnusse/pathways/targets.html>). Defects in several members of the Wnt signalling cascade, i.e. loss of function

mutations at the *APC* gene, result in the accumulation of intracellular and nuclear β -catenin, thus leading to constitutive deregulation of target genes (Behrens et al., 1996; Fodde et al., 1994; Molenaar et al., 1996; Morin et al., 1997; Sparks et al., 1998). During the early phases of development of the female reproductive tract, Wnt ligands such as Wnt4, Wnt5a and Wnt7a play an important role (Mericskay et al., 2004; Miller and Sassoon, 1998; Vainio et al., 1999). *Wnt4* knock-out female mice develop normal Wolffian ducts but lack Müllerian ducts (Vainio et al., 1999). *Wnt5a* knock-out female mice are characterized by defects in the posterior outgrowth of the developing Müllerian ducts and in forming endometrial glands (Mericskay et al., 2004). Loss of Wnt7a expression on its turn, leads to incomplete demarcation between the vagina and the uterus and between the uterus and the oviduct. Furthermore, *Wnt7a* knock-out female mice do not form uterine glands and show myometrial aberrations (Miller and Sassoon, 1998).

More recently, two models have been developed to evaluate the consequences of conditional Wnt/ β -catenin activation in the uterus (Jeong et al., 2009; Tanwar et al., 2009). Jeong et al. (2009) investigated the effects of conditional activation of Wnt signaling in the uterus by *PgrCre* driven activation of β -catenin expression (*Pgr*^{Cre/+}; *Ctnnb1*^{f(Ex3)/+}) and showed profound endometrial hyperplasia, which did not further progress to endometrial cancer (Jeong et al., 2009). Tanwar et al. (2009) employed the *Amhr2* (Amh type 2 receptor) promoter to drive Cre expression (*Amhr2*^{Cre/+}) and activate β -catenin expression in mesenchymal cells surrounding the developing Müllerian ducts. This approach resulted in myometrial hyperplasia, adenomyosis, and mesenchymal tumors similar to leiomyomas and endometrial stromal sarcomas (Tanwar et al., 2009). Furthermore, hyperplasia of endometrial glands was occasionally observed suggesting that mesenchymal

activation of Wnt/ β -catenin signalling plays a role in the early events which may lead to endometrial carcinogenesis.

In approximately 40% of human endometrial cancers, nuclear β -catenin staining is observed (Saegusa and Okayasu, 2001; Scholten et al., 2003), and in over 50% of these cases loss of APC expression is involved (either LOH or promoter methylation, (Moreno-Bueno et al., 2002)). In order to further clarify the role of Wnt/ β -catenin signalling, and in particular loss of Apc expression in the uterus, we bred *Amhr2*^{Cre/+} (Jamin et al., 2002) mice with *Apc*^{15lox/15lox} mice (Robanus-Maandag et al., 2010) to conditionally inactivate the endogenous *Apc* gene from early embryonal life onwards in mesenchymal cells surrounding the Müllerian duct.

2. Materials and Methods

2.1. Animals and genotyping

All experiments conducted with mice were approved by our local animal ethics committee (DEC permit number EUR 1076) and were in accordance to international guidelines and regulations. *Amhr2-LacZ* animals (Arango et al., 2008; Arango et al., 1999) and *Amhr2Cre* animals (Jamin et al., 2002) were obtained from Dr RR Behringer. *Amhr2Cre* mice were bred with Rosa26 reporter mice (Soriano, 1999) to review Cre expression, and were bred with *Apc*^{15lox} mice (Miclea et al., 2009; Robanus-Maandag et al., 2010) to obtain *Apc*^{15flox/15flox} mice ("flox" means that the sequence between two loxP sites has been deleted by Cre). *PgrCre* animals (Soyal et al., 2005) were also bred with *Apc*^{15lox} mice to obtain *Apc*^{15flox/15flox} mice. All animals were maintained in a C57Bl/6J background. Genotyping was performed by routine PCR, on tail and uterus DNA, using the following primers: detection of the 380 bp *Apc*^{15lox} allele: TAGGCACTGGACATAAGGGC and GTAAGTGTCAAGAATCAATGG; detection of the 450 bp *Apc*^{15flox} allele: AACTTCTGAGTATGATGGAGG and GTAAGTGTCAAGAATCAATGG; detection of the 219 bp Cre allele: GGACATGTTTCAGGGATCGCCAGGC and CGACGATGAAGCATGTTTAGCTG; detection of the 500 bp Rosa26-LacZ^{lox} and 250 bp Rosa26-LacZ^{flox} allele: AAAGTCGCTCTGAGTTGTTAT, GCGAAGAGTTTGTCTCAACC and GGAGCGGGAGAAATGGATATG. For all animals, the stage of the estrous cycle was determined by a vaginal smear just before sacrifice.

2.2. β -Galactosidase staining

The β -galactosidase staining was performed as described before (Dannenberg et al., 2004) with the adaptation that here Bluo-Gal (Invitrogen, Breda, The Netherlands)

was used instead of X-gal. Fixed uteri were stained with Bluo-Gal overnight, embedded in paraffin, sectioned (5 μ m) and counter-stained with eosin.

2.3. Laser microdissection

Paraffin-embedded tissues were sectioned (10 μ m), adhered to uncoated microscope slides and stained with haematoxylin and eosin. Laser microdissection was performed by PALM laser microdissection (Carl Zeiss MicroImaging GmbH, Standort Göttingen, Germany). Approximately 1 mm² of myometrium, luminal epithelium and stromal tissue combined with glandular epithelium were collected, respectively. DNA was extracted from these tissues compartments, using a phenol-chloroform method. The genotypes of different uterine layers were assessed by PCR as described above.

2.4. Immunohistochemistry

Immunohistochemistry was performed essentially as described before (Klaassens et al., 2006). The antibodies employed are: mouse monoclonal anti-SMA (DAKO, Heverlee, Belgium) (1:150), rat monoclonal anti-cytokeratine 8 (CK8, 1:5000) (DSHB, Iowa, USA), rabbit polyclonal anti-ER α (Millipore, Billerica, USA) (1:2000) and rabbit monoclonal anti- β -catenin (Epitomics, Burlingame, USA) (1:800).

2.5. Histological view and measurement

Immunohistochemical staining for smooth muscle actin (SMA), which stains myometrium, was applied to distinguish between myometrium and endometrium. CK8 was used to specifically stain epithelial cells. Nanozoomer Digital Pathology (NDP) (Hamamatsu Photonics Deutschland GmbH, Herrsching am Ammersee, Germany) was used to scan the slides and this program also allowed for uterine area

measurements. The number of glands was measured per square millimeter of endometrium. Of each evaluated uterus, every 2mm one section was measured (7 sections per uterus), and in total thirteen control and ten experimental animals were assessed.

2.6. Statistical methods

Analyses were performed using Graphpad Prism 5 (GraphPad Software, Inc., San Diego, CA). Unpaired, two-tailed t-test was used to analyze the differences between groups. P values lower than 0.05 were considered statistically significant.

3. Results

3.1. The *Amhr2* promoter is mainly active in the myometrium

First we established the pattern of expression driven by the promoter of *Amhr2* in the uterus by employing previously developed *Amhr2*-LacZ reporter mice (Arango et al., 2008). As shown in Figure 1A, LacZ expression was limited to the myometrial layer of the uterus. This result was confirmed by breeding *Amhr2*^{Cre/+} transgenic animals with the Rosa26 reporter mice (R26R) carrying a loxP-STOP-loxP-lacZ reporter (Soriano, 1999). In the presence of active Cre, somatic deletion of the STOP cassette is achieved, thus leading to β -galactosidase expression from the *LacZ* gene. Figure 1B shows β -galactosidase staining of both myometrial layers (inner circular and outer longitudinal muscles) in compound *Amhr2*^{Cre/+};R26R animals. Notably, staining was not continuous throughout the myometrium as exemplified by patchy β -galactosidase expression within the outer myometrial layer, which is in accordance with a previous publication by Deutscher and Yao (Deutscher and Hung-Chang Yao, 2007). Staining for β -galactosidase also indicated that the *Amhr2* promoter is not active in stromal and epithelial cells of the endometrium.

Next, *Amhr2*^{Cre} animals were bred with *Apc*^{15lox/15lox} animals, a model developed by our laboratory (Robanus-Maandag et al., 2010) to conditionally inactivate Apc function by removing exon 15 of the endogenous gene. Deletion of both *Apc* alleles results in the constitutive activation of Wnt/ β -catenin signaling. To substantiate that Cre expression in the compound *Amhr2*^{Cre/+}; *Apc*^{15lox/15lox} animals was limited to the myometrial layer, and to verify that recombination of the *Apc* gene was indeed taking effect, laser capture microdissection (LCM) of different layers of the uterus was performed. As illustrated in Figure 1C and 1D, only a small percentage of cells from the stromal/glandular region showed *Apc* exon 15 deletion,

whereas no somatic recombination was detected in the luminal epithelial region. In contrast, *Apc* deletion was evident in cells from in the myometrium.

Somatic *Apc* deletion is predicted to result in the constitutive activation of the canonical Wnt pathway and, possibly, in the nuclear accumulation of β -catenin (Gaspar and Fodde, 2004; Robanus-Maandag et al., 2010). However, IHC analysis of β -catenin expression in the uterus of *Amhr2*^{Cre/+};*Apc*^{15lox/15lox} animals revealed that the majority of myometrial cells are marked by cytoplasmatic β -catenin staining with only few cells showing nuclear staining (Fig. 1E). This result is in agreement with previous studies from our and other laboratories showing that loss of *Apc* function is necessary but often insufficient for nuclear β -catenin accumulation (Fodde and Brabletz, 2007; Fodde and Tomlinson, 2010).

3.2. Loss of APC function results in myometrial defects

Histological assessment of uteri from adult compound *Amhr2*^{Cre/+};*Apc*^{15lox/15lox} mice revealed severe defects in the myometrium (an overview of the analyzed mice is presented in Table 1). To investigate the nature of these defects in more detail, smooth muscle actin (SMA) IHC staining was employed to specifically mark the myometrium. As shown in Figure 2, in contrast to control animals (Fig. 2A), all *Amhr2*^{Cre/+};*Apc*^{15lox/15lox} animals showed regions within the myometrium where the muscle layer appeared disorganized or even discontinuous (Fig. 2B). Out of the 19 *Amhr2*^{Cre/+};*Apc*^{15lox/15lox} mice here examined, 16 showed tissue architectural changes of the myometrium without interruption of the myometrium layer, as exemplified in Figure 2B (top panel). In the other 3 of 19 animals the same architectural changes in the myometrium appeared in association with a discontinuous myometrium layer, as exemplified in panels 2 and 3 of Figure 2B. Pregnancies in *Amhr2*^{Cre/+};*Apc*^{15lox/15lox}

animals only resulted in life born puppies in 2/4 cases. On the other 2/4 cases the mother died during delivery. In order to investigate these delivery problems further, we tried to obtain permission with our local animal ethics committee, but it was not allowed.

To obtain additional evidence that loss of Apc function in myometrial cells of the uterus indeed results in muscular defects in the adult uterus, *Apc*^{15lox/15lox} animals were bred with *PgrCre* animals. In these animals nuclear β -catenin staining was readily detected in the myometrium and in epithelial cells of the endometrium (Fig. 3A). Furthermore, marked myometrial defects were detected in the majority of investigated animals (7/10) (Fig. 3B). Interestingly, omissions in the myometrial layer were invaded by stromal and glandular cells from the endometrium reminiscent for a human disorder described as endometriosis interna or adenomyosis (Bergeron et al., 2006).

In order to assess the underlying basis for the effects of the induced Apc mutation, embryonal mice were sacrificed and the Müllerian ducts were stained for apoptosis (caspase-3). Time of sacrifice (E12) was chosen based on our own data in *Amhr2*^{Cre/+}; R26R-LacZ animals showing clear Cre activity around that day. No differences, however, were detected between controls and *Amhr2*^{Cre/+}; *Apc*^{15lox/15lox} littermates (data not shown).

3.3. Endometrial defects in *Amhr2*^{Cre/+}; *Apc*^{15lox/15lox} mice

Although recombination of *Apc*^{15lox} alleles in the endometrium could hardly be detected in the *Amhr2*^{Cre/+}; *Apc*^{15lox/15lox} animals, phenotypic expression of gene deletion was apparent from observed differences between targeted and control animals, such as a smaller endometrial area and less endometrial glands. To quantify

these differences, anti-SMA was used to stain myometrium, and anti-CK8 was used to stain endometrial glands (Fig. 4A and 4B). As indicated in Materials and Methods several areas were measured and gland numbers counted. Despite the myometrial defects described above, no significant differences could be detected in the myometrial area of *Amhr2*^{Cre/+};*Apc*^{15lox/15lox} animals when compared with control animals (Fig. 4C). However, significant differences were observed for the number of glands per mm² (Fig. 4D, $p = 0.0001$) and the area of the endometrium (Fig. 4E, $p = 0.0002$): *Amhr2*^{Cre/+};*Apc*^{15lox/15lox} mice show fewer glands and a reduction of the endometrial area.

4. Discussion

The functional role of canonical Wnt/ β -catenin signalling in uterine development and homeostasis is to date largely unclear. Recently, Jeong et al. (2009) investigated the effects of conditional activation of Wnt signaling in the uterus by *PgrCre* driven expression of oncogenic β -catenin (*Pgr^{Cre/+};Ctnnb1^{f(Ex3)/+}*) (Jeong et al., 2009). The progesterone receptor, however, is widely expressed throughout the uterus from week two after birth onwards, and accordingly a broad spectrum of defects was observed: the *PGR^{Cre/+};Ctnnb1^{f(Ex3)/+}* animals showed reduced offspring numbers resulting from a diminished decidual reaction, decreased uterine weight, increased ER α expression in the endometrium, enlarged endometrial glands, endometrial hyperplasia, and increased endometrial proliferation.

In the present study the aim was to investigate the consequences of Wnt/ β -catenin activation by *Apc* deletion in embryonal life for specific adult uterine tissues. In particular, the *Amhr2* promoter was employed to drive Cre expression to induce loss of *Apc* function (*Amhr2^{Cre/+};Apc^{15lox/15lox}*) in the myometrium but not in the endometrium. The main difference between the current approach and studies by others on the role of Wnt/ β -catenin signaling in the uterus (Arango et al., 2005) (Deutscher and Hung-Chang Yao, 2007; Jeong et al., 2009; Tanwar et al., 2009) is that here conditional knock-down of *Apc* is used (others use conditional inactivation or activation of β -catenin). The role of APC of course is different from the role of β -catenin: APC is involved in cell adhesion, migration, apoptosis and chromosomal segregation (Fodde, 2003). This is true, however, the main tumor-suppressing function of APC instigates from its role in the Wnt/ β -catenin signalling pathway.

Previous studies with the *Amhr2-LacZ* reporter mouse model showed that this promoter is active for a brief period from embryonic day 12.5 to 15.5 in mesenchymal

cells surrounding the Müllerian ducts (Arango et al., 2008; Jamin et al., 2002; Klattig et al., 2007). Notably, in a related reporter model, *Amhr2Cre*-driven recombination of a Rosa26 reporter (R26R) resulted in β -galactosidase staining of myometrial cells but not in endometrial stromal cells (Arango et al., 2008; Deutscher and Hung-Chang Yao, 2007). This observation was confirmed in the current study also by assessing somatic recombination of the *Apc*^{15lox} allele in laser-capture microdissected areas of the uterus of *Amhr2*^{Cre/+};*Apc*^{15lox/15lox} mice (Fig. 1).

A more detailed approach to the determination of Cre-mediate recombination efficiency is represented by β -catenin immunohistochemical staining. Nuclear β -catenin accumulation is often regarded as a hallmark of Wnt/ β -catenin signalling activation as the result of loss of Apc function. However, β -catenin IHC analysis of the myometrium of *Amhr2*^{Cre/+};*Apc*^{15lox/15lox} animals revealed a majority of cells with cytoplasmatic staining and only very few, sporadic cells with clear-cut nuclear staining. This is consistent with the observed patchy β -galactosidase staining (Fig. 1B). Furthermore, Miclea et al. (2009) and Robanus-Maandag et al. (2010) showed increased intracellular β -catenin staining upon somatic *Apc* deletion with only a minority of cells featuring nuclear accumulation (Miclea et al., 2009; Robanus-Maandag et al., 2010). Interestingly these authors did show a clear phenotype resulting from the intestinal Cre-mediated deletion of Apc: tumors in the large intestine (Robanus-Maandag et al., 2010). Accordingly, other reports have shown that loss of APC function is necessary but not always sufficient for nuclear β -catenin accumulation (Fodde and Brabletz, 2007; Fodde and Tomlinson, 2010). In contrast to this, *Pgr*^{Cre/+};*Apc*^{15lox/15lox} animals did show nuclear β -catenin staining indicating that recombination of Apc can indeed result in nuclear β -catenin staining. There is however a considerable difference between the two models: *Amhr2Cre* is activated

only from embryonal day 12.5 to 15.5 while *PgrCre* becomes active and stays active from approximately postnatal day 10 onwards. It is possible that the majority of mesenchymal cells surrounding the embryonal Mullerian duct (where loss of *Apc* function is established) selectively undergo apoptosis, while more mature myometrial cells in which *Apc* is knocked down, do not. In fact apoptosis as a result of activation of Wnt/ β -catenin signalling has previously been shown for NIH-3T3 fibroblasts (Kim et al., 2000). The latter would also be in agreement with the observed loss of myometrial muscle tissue in some of the animals upon *Apc* somatic deletion, described herein (Fig. 2 and 3). Reviewing Müllerian duct sections from embryonal day 14 *Amhr2*^{Cre/+};*Apc*^{15lox/15lox} animals, however, failed to show increased apoptosis as measured by caspase 3 staining (data not shown). Evaluation of the uterus of *Pgr*^{Cre/+};*Apc*^{15lox/15lox} animals indicated that it can not be excluded that loss of myometrial muscle fibers develops gradually over a prolonged period of time, or alternatively from pubertal life onwards.

The most prevalent phenotype observed among *Amhr2*^{Cre/+};*Apc*^{15lox/15lox} mice is undoubtedly represented by the loss and disorganisation of myometrial muscle fibres resulting in a defective myometrium. Myometrial defects were also observed by others by employing comparable models to either induce or inhibit Wnt/ β -catenin signalling *in utero*. Arango et al. (2005) used the *Amhr2Cre* model to induce β -catenin depletion and observed profound myometrial defects (Arango et al., 2005). In that study, β -catenin depletion in mesenchymal cells surrounding the Müllerian ducts resulted in the appearance of adipocytes replacing myometrial cells. Miller and Sassoon (1997) found that in *Wnt7a* defective animals, among many other disorders, the myometrial layer became largely disorganized (Miller and Sassoon, 1998). Tanwar et al. (2009) used a model which mimics more closely our approach in that

Amhr2Cre was employed to express an oncogenic variant of β -catenin thus inducing constitutive Wnt activation in the uterus (Tanwar et al., 2009). However, in that study the affected animals developed muscular hyperplasia already at 6 weeks of age, which is substantially different from the current findings in *Amhr2^{Cre/+};Apc^{15lox/15lox}* mice. Tanwar et al. (2009) also observed mesenchymal tumors with characteristics of human leiomyomas and endometrial stromal sarcomas (Tanwar et al., 2009). To investigate whether similar neoplastic lesion could be observed in the *Amhr2^{Cre/+};Apc^{15lox/15lox}* mice, we carefully reviewed all our mice but did not observe any leiomyomas. However, we did observe, in a single mouse, a region reminiscent of an endometrial stromal sarcoma-like lesion (Supplementary Fig. 1). This region was subsequently stained for SMA, ER α and β -catenin. It was observed that ER α and β -catenin expression were both enhanced in this region, which was in agreement with the observations done by Tanwar et al. (Tanwar et al., 2009).

Recently, we have extended our analysis on the consequences of loss of Apc function in the uterus by employing *PgrCre* mice (Fig. 3). Myometrial breakdown in *Pgr^{Cre/+};Apc^{15lox/15lox}* animals was to some extent comparable to myometrial defects in *Amhr2^{Cre/+};Apc^{15lox/15lox}* animals. However, *Pgr^{Cre/+};Apc^{15lox/15lox}* animals were also characterized by areas in the myometrium nearing some similarity to human adenomyosis, a condition characterized by the presence of endometrial tissue within the myometrium. Accordingly, Tanwar et al. (2009) occasionally also observed glands and stroma in the muscle of *Amhr2^{Cre/+};Ctnnb1^{f(Ex3)/+}* animals (Tanwar et al., 2009). Because in the *Amhr2^{Cre/+};Apc^{15lox/15lox}* model the integrity of the myometrium is mainly affected, this seems to indicate that the presence of endometrial tissue into the myometrial layer in the Wnt-activated mice may be the result of a passive process rather than signifying active invasion. Recent investigations by Mehasseb et

al (2010) also indicate that invasion of stromal cells from adenomyosis is augmented by the presence of myocytes from affected uteri (Mehasseb et al., 2010).

In conclusion, we report that *in vivo* inactivation of the endogenous mouse *Apc* gene in mesenchymal cells surrounding the Müllerian ducts results in significant myometrial defects in adult mice. Overall, these results further consolidate the notion that Wnt/ β -catenin signalling is important for uterine development and homeostasis.

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Legends

Figure 1 ***Amhr2* promoter activity and *Apc* recombination in different regions of the uterus.** **A:** *Amhr2-LacZ* mice were stained for β -galactosidase activity with Blue-Gal. **B:** *Amhr2*^{Cre/+};*R26R* animals were stained for β -galactosidase activity. **C:** Indicated different layers of the uterus that were isolated by microdissection for DNA isolation. (0), before dissection; (1), glands and stroma; (2), luminal epithelium; (3), myometrium. **D:** Representative gel-image from a PCR reaction to determine recombination of *Apc* using DNA isolated from an *Amhr2*^{Cre/+};*Apc*^{15lox/15lox} mouse: (1), stroma and glands; (2) luminal epithelium; (3) myometrium. *Apc*^{15lox} represents the non-recombined lox allele; *Apc*^{fllox} indicates recombination of *Apc*; Actin was used as a control for PCR. L/L represents DNA displaying both non-recombined *Apc*^{15Lox} alleles; F/L represents DNA displaying one non-recombined and one-recombined *Apc* allele. Primers used for the PCR are described in Materials and Methods. **E:** β -catenin staining of a representative *Amhr2*^{Cre/+};*Apc*^{15lox/15lox} mouse. The arrows indicate myometrial cells which show nuclear β -catenin staining. Luminal and glandular epithelial cells only show cytoplasmic and membrane β -catenin staining. Control animals never showed nuclear β -catenin staining.

Figure 2 ***Amhr2*^{Cre/+};*Apc*^{15lox/15lox} mice show myometrial defects.** The uterine muscle was stained with an antibody against Smooth Muscle Actin (SMA) **A:** three control animals at different stages of the estrous cycle: from top to bottom: proestrus, estrus and diestrus. The staining shows a cellular myometrium with closely-packed muscle fibers. **B:** Three *Amhr2*^{Cre/+};*Apc*^{15lox/15lox} mice, matched for age and stage of the cycle to the controls. The top figure shows an affected uterus where the outer part of the myometrium contains tightly packed fibers, but the inner part shows more

dispersed and thinner fibers. The middle and bottom figures show a complete trans-section of the uterus where the muscle wall is focally incomplete.

Figure 3 ***Pgr*^{Cre/+};*Apc*^{15lox/15lox} mice also display myometrial defects.** β -catenin (A) and SMA (B) staining of a representative *Pgr*^{Cre/+};*Apc*^{15lox/15lox} mouse. Details on the right indicate presence of glandular and stromal cells in the muscle layer. Gland (red arrow), stroma (black arrow) and myometrium (green arrow).

Figure 4 **Quantification of endometrial and myometrial defects in *Amhr2*^{Cre/+};*Apc*^{15lox/15lox} mice.** **A:** a cross section from a control uterus stained for smooth muscle actin and counterstained with hematoxylin. Indicated regions provide information on the area of the myometrium and endometrium. **B:** consecutive section was stained for cytokeratine 8 in order to measure the number of endometrial glands. **C:** calculation of the myometrial area in control and affected animals **D:** measurement of the number of glands per area of stroma in control and affected animals. **E:** calculation of the endometrial area in control and affected animals. The panels **C-E** were derived from 13 controls and 10 *Amhr2*^{Cre/+};*Apc*^{15lox/15lox} mice (7 sections per animal). Con = control animals; Cre-lox = *Amhr2*^{Cre/+};*Apc*^{15lox/15lox} animals. Data are presented as: Mean (line), 25th and 75th percentiles (box), and 5th and 95th percentiles (whiskers).

Supplementary Figure 1 **Endometrial stromal sarcoma-like lesion observed in one *Amhr2*^{Cre/+};*Apc*^{15lox/15lox} mouse.** **A and B:** Hematoxylin/eosine staining of the affected uterus showing the lesion (red circle indicates the aberrant region); the

square indicates the magnified region depicted in figure b **B**. Consecutive sections were stained for SMA (**C**), Estrogen Receptor alpha (**D**) and β -catenin (**E**).

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Figure 1*Amhr2-LacZ***A** *Amhr2^{Cre/+}; Rosa26-LacZ^{lox}***B** *Amhr2^{Cre/+}; Apc^{15lox/15lox}***C** **D** **E***Amhr2^{Cre/+}; Apc^{15lox/15lox}*

Figure 2

Figure 3*PGR*^{Cre/+};*Apc*^{15lox/15lox}**A** **B**

Figure 4

Table 1: Animals investigated

Genotype	N	Age (weeks)	Phenotype
<i>Wild type</i>	21	7-31	normal
<i>Apc</i> ^{15lox/+}	9	9-33	normal
<i>Apc</i> ^{15lox/15lox}	5	8-34	normal
<i>Amhr2</i> ^{cre/+}	3	7-34	normal
<i>Amhr2</i> ^{cre/+} ; <i>Apc</i> ^{15lox/15lox}	19	8-34	Impaired myometrial architecture in 19/19 animals. Discontinuation of the myometrial layer in 3/19 animals.

Supplementary Figure 1

Amhr2^{Cre/+}; *Apc*^{15lox/15lox}

> AMHR2^{Cre/+};Apc^{15lox/15lox} and Pgr^{Cre/+};Apc^{15lox/15lox} mouse models were used to make conditional *Apc* knockout. > Loss of *Apc* was induced in the adult mouse uterus.> Minor defects in endometrium. > severe defects in the uterine myometrial layers. > The WNT signaling pathway plays important roles in maintaining myometrial integrity.