β-CATENIN SIGNALING DOSAGE DICTATES TISSUE-SPECIFIC TUMOR PREDISPOSITION IN *APC*-DRIVEN CANCER

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ABSTRACT

Apc-driven tumor formation in patients and *Apc* mutant mouse models is generally attributed to increased levels of β-catenin signaling. We and others have proposed that a specific level of β-catenin signaling is required to successfully initiate tumor formation, and that each tissue selects for different dosages of signaling. This is illustrated by *APC* genotype-tumor phenotype correlations in cancer patients and by the different tumor phenotypes displayed by different *Apc* mutant mouse models. *Apc*1638N mice, associated with intermediate β-catenin signaling, characteristically develop intestinal tumors (<10) and extra-intestinal tumors including cysts and desmoids. *Apc*1572T mice associated with lower levels of β-catenin signaling, are free of intestinal tumors but instead develop mammary tumors. Although the concept of β-catenin signaling dosage and its impact on tumor growth among tissues is gaining acceptance, it has not been formally proven. Additionally, alternative explanations for *Apc*-driven tumor formation have been proposed. To obtain direct evidence for the dominant role of β-catenin dosage in tumor formation and tissue-specific tumor predisposition, we crossed *Apc*1638N mice with heterozygous β-catenin knockout mice, thereby reducing β-catenin levels. Whereas all *Apc*1638N;*Ctnnb1*$^{+/+}$ mice developed gastrointestinal tumors, none were present in the *Apc*1638N;*Ctnnb1*$^{-/+}$ mice. Incidence of other *Apc*1638N-associated lesions including desmoids and cysts was strongly reduced as well. Interestingly, *Apc*1638N;*Ctnnb1*$^{-/+}$ females showed an increased incidence of mammary tumors, normally rarely observed in *Apc*1638N mice, and histological composition of the tumors resembled that of *Apc*1572T-related tumors. Hereby, we provide in vivo genetic evidence confirming the dominant role of β-catenin dosage in tumor formation and in dictating tumor predisposition among tissues in *Apc*-driven cancer.

Keywords: β-catenin/APC/intestinal cancer/mammary tumor

INTRODUCTION

The Wnt/β-catenin signaling pathway represents one of the main regulatory mechanisms to retain tissue homeostasis in the adult organism by balancing self-renewal, differentiation and apoptosis in several adult stem cell niches (1). Underscoring the relevance of this pathway, many tumor types exhibit enhanced Wnt/β-catenin signaling that strongly contributes to tumor growth. In the Wnt/β-catenin signaling pathway, the adenomatous polyposis coli (APC) protein is a central component regulating the degradation and concomitantly the transcriptional activity of β-catenin in the nucleus. As depicted in Figure 1a, several motifs in the central domain of APC are responsible for regulating intracellular β-catenin levels. Four 15 amino acid repeats (AAR) bind β-catenin, whereas seven 20-AARs are involved in both binding and downregulation. Interspersed within those 20-AARs are three binding sites for Axin required for an optimal recruitment of APC into the destruction complex. Inactivation of APC perturbs the formation of the β-catenin degradation complex, leading to increased nuclear translocation and target gene expression, thereby affecting important cellular decisions and favoring a genetic program that initiates tumor formation. In case of colorectal cancer, a small subset of tumors acquires activating mutations in β-catenin itself, whereas most others result from inactivating biallelic *APC* mutations (2). The vast majority of these *APC* mutations result in truncated proteins that lack all Axin binding motifs while retaining between one and three 20-AARs. As a result, these truncated proteins still have residual activity in downregulating β-catenin signaling. Accordingly, an inverse correlation is observed between the number of retaining 20-AARs and the resulting level of β-catenin signaling, i.e. more repeats means a lower β-catenin signaling level to the nucleus. Based on these observations, we and others have proposed that *APC* mutations are selected to one another to reach an optimal level of enhanced β-catenin signaling, described as the 'just-right' signaling model (2-6). According to this model, levels beneath the optimal β-catenin signaling window will not provide cells with sufficient activation of target genes to

gain growth advantage and trigger tumor formation, whereas levels exceeding the optimal window will trigger apoptosis instead. As reviewed in Albuquerque *et al*, optimal β-catenin signaling dosages differ throughout the body, indicated by different *APC* genotypes that are observed in tumors on different locations (2). Both sporadic as well as familial forms of desmoid and duodenal tumors select for *APC* mutations retaining 2-3 20AARs associated with moderate β-catenin signaling activation. On the other hand, most colorectal tumors are associated with shorter truncating proteins resulting in higher levels of β-catenin signaling. Interestingly, correlations are observed even within the colorectal tract, where right-sided colon tumors generally retain more 20-AARs than left-sided ones (2, 7). Although in human breast cancer patients mutations in β-catenin or *APC* are rarely found, aberrant activation of β-catenin signaling is observed frequently (8).

Phenotypes of *Apc*-mutant mouse models strongly support *Apc* genotype-tumor phenotype correlations (Figure 1b). $Apc^{Min/+}$ mice have high levels of β-catenin signaling and develop intestinal tumors at high multiplicity (>100). Animals carrying the hypomorphic *Apc*1638N mutation, associated with intermediate β-catenin signaling, characteristically develop intestinal tumors at lower multiplicity (<10) and in parallel show a high susceptibility for extra-intestinal tumor types such as cutaneous cysts and desmoid tumors (9). The *Apc*1572T mouse model, associated with lower levels of β-catenin signaling, is free of intestinal tumors but instead develops mammary tumors with high penetrance, in addition to cysts and desmoids albeit with reduced numbers compared to $Apc^{1638N/+}$ mice (10). Taken together, this indicates that tissue-specific dosages of β-catenin signaling are selected to efficiently trigger tumorigenesis, where intestinal tumors are associated with higher levels of β-catenin signaling than cysts and desmoids, which in turn are associated with higher β-catenin signaling than mammary tumors (Figure 1c).

Although the concept of β-catenin signaling dosage and its impact on tumor growth among tissues is gaining acceptance, tissue-specific tumor predisposition has not been formally proven to be a direct consequence of β-catenin signaling dosage. Furthermore,

alternative explanations for *APC*-driven tumor formation have been proposed. APC is a large, multifunctional protein and in addition to downregulating β-catenin signaling it is implicated in various other cellular processes, as APC can affect chromosomal segregation, cytoskeletal organization and bind C-terminal binding protein (CtBP) (11-15). Here, we provide direct genetic evidence for the dominant role of β-catenin in tumor formation and establish the impact of β-catenin signaling dosage in dictating tissue-specific tumor predisposition. To this aim, we reduced the pool of available β-catenin in *Apc*$^{1638N/+}$ (*Apc*1638N) mice by heterozygous β-catenin (*Ctnnb1)* knockout. Consequently, gastrointestinal tumor formation was completely prevented while mammary tumor predisposition was enhanced, shifting the phenotype towards the *Apc*1572T-related tumor phenotype.

RESULTS AND DISCUSSION

The *Apc*1638N mouse model is a representative model to investigate intestinal cancer, where mice characteristically develop about 1-7 gastrointestinal tumors (16). In addition, these mice are highly susceptible for extra-intestinal tumor types including desmoids and cutaneous cysts (9). To reduce their dosage of β-catenin, we crossed *Apc*$^{1638N/+}$ mice with *Ctnnb1*$^{-/+}$ mice (17). First, non-tumorigenic intestinal tissues were characterized of both *Apc*$^{1638N/+}$*/Ctnnb1*$^{+/+}$ and *Apc*$^{1638N/+}$*/Ctnnb1*$^{-/+}$ mice. Histological evaluation, β-catenin protein staining and RNA expression of the β-catenin target gene *Axin2* showed no alterations between both groups in the non-tumorigenic intestinal tissues (data not shown). These results were in line with expectations, since these normal tissues only harbor the heterozygous germline *Apc*-mutation and intestinal tissue homeostasis of mice harboring the *Apc*1638N mutation is unaltered compared to that of wildtype mice (18). To verify reduced β-catenin signaling as a consequence of heterozygous *Ctnnb1* knockout, we used a more sensitive approach. For this, β-catenin reporter assays were performed to measure the intrinsic β-catenin signaling of mouse embryonic fibroblasts (MEFs) that we generated of embryos of the different genotypes. In the absence of *Apc* mutation, levels of β-catenin signaling were low and not significantly affected by heterozygous β-catenin knockout (Figure 2). MEFs carrying the *Apc*1638N allele showed enhanced β-catenin signaling, and here, heterozygous β-catenin knockout clearly resulted in reduced β-catenin signaling. This verifies that heterozygous β-catenin knockout indeed substantially reduces β-catenin signaling in *Apc*1638N mice.

Subsequently, tumor phenotypes of 8-months aged *Apc*1638N*/Ctnnb1*$^{+/+}$ and *Apc*1638N*/Ctnnb1*$^{-/+}$ mice were examined. Strikingly, whereas all 19 *Apc*1638N*/Ctnnb1*$^{+/+}$ mice developed gastrointestinal tumors as characteristic for *Apc*1638N mice, none of the 21 *Apc*1638N*/Ctnnb1*$^{-/+}$ mice developed any gastrointestinal tumor (Figure 3a). This provides direct evidence for the absolute requirement of a sufficiently enhanced β-catenin level for

intestinal tumorigenesis. The complete absence of intestinal tumors in mice with heterozygous β-catenin knockout precluded us to compare β-catenin and associated signaling characteristics in intestinal tumors between groups following second hit *Apc* mutation. *Apc*1638N-associated extra-intestinal lesions were still observed in *Apc*1638N/*Ctnnb1*$^{-/+}$ mice with characteristic gender-specific distribution, although with a clearly reduced incidence (Figure 3b,c). Desmoid numbers were reduced from 8.6 ± 3.0 to 0.2 ± 0.4 in females and from 61.4 ± 14.4 to 19.1 ± 8.1 in males (Figure 3b). Cysts numbers were lowered from 5.6 ± 3.8 to 0.4 ± 0.6 in females and from 29.8 ± 19.5 to 2.4 ± 1.5 in males (Figure 3c). Thus, reducing β-catenin levels in *Apc*1638N mice prevented gastrointestinal tumor formation and significantly reduced the incidence of other lesions associated with the *Apc*1638N mouse model. Most strikingly, we observed that half of the *Apc*1638N/*Ctnnb1*$^{-/+}$ females developed mammary lesions, reflecting a strongly enhanced incidence of mammary lesions following heterozygous β-catenin knockout (Figure 3d). Normally, mammary lesions are rarely observed in *Apc*1638N mice but are characteristic of *Apc*1572T mice. Our findings nicely illustrate that by reducing β-catenin in *Apc*1638N mice, the tumor phenotype shifts towards an *Apc*1572T-related phenotype, which is associated with a relatively lower activation level of β-catenin signaling. This confirms that β-catenin signaling dosage by itself dictates tissue-specific tumor predisposition in *Apc*-mutant mice.

The mammary lesions we observed in *Apc*1638N/*Ctnnb1*$^{-/+}$ mice were relatively small, showing an average diameter of 2.6 ± 1.4 mm, compared to those generally observed in *Apc*1572T mice (10). In accordance with this relatively mild mammary tumor phenotype in our *Apc*1638N/*Ctnnb1*$^{-/+}$ mice, metastases were not observed. Microscopic characterization of the identified mammary lesions revealed a heterogeneous histology, displaying glandular and squamous regions, keratinizing components and inflammatory cells (Figure 4a). Immunohistochemical analyses further established the heterogeneity of the mammary tumors found in *Apc*1638N/*Ctnnb1*$^{-/+}$ mice. Hence, staining for cytokeratin-8 confirmed luminal epithelial differentiation, cytokeratin-14 indicated areas of squamous differentiation

and smooth muscle actin showed myoepithelial cell types (Figure 4b-d). This histological composition is virtually identical to that of the mammary lesions observed in *Apc*1572T mice (10). In the mammary lesions of the *Apc*1638N/*Ctnnb1*$^{-/+}$ mice, expression of β-catenin was observed in epithelial cells displaying membrane-bound and nuclear β-catenin (Figure 4e). Staining for Ki67 revealed moderate proliferation (Figure 4f). Thus, *Apc*1638N/*Ctnnb1*$^{-/+}$ mice develop heterogeneous mammary tumors that resemble those observed in *Apc*1572T mice histologically, although *Apc*1638N/*Ctnnb1*$^{-/+}$ tumors remain relatively small.

Our data show that by reducing β-catenin levels, the characteristic *Apc*1638N-related intestinal tumor phenotype shifts towards mammary tissues, where tumors typically develop in *Apc*1572T mice (10). Also, the reduced incidence of cysts and desmoids is in accordance with that observed in *Apc*1572T mice. The mammary tumors observed in *Apc*1638N/*Ctnnb1*$^{-/+}$ mice resembled those of *Apc*1572T mice histologically, although remaining smaller. We propose that in *Apc*1638N mice following loss of the wild type *Apc* allele required for tumor initiation, we reduced the β-catenin dosage by heterozygous β-catenin knockout to levels approaching those associated with *Apc*1572T mice, thereby enabling successful mammary tumorigenesis (9-10, 19). However, the exact β-catenin signaling level preferred to sustain fully penetrant mammary tumor growth and metastasis may not have been reached most optimally, explaining the smaller tumors observed in *Apc*1638N/*Ctnnb1*$^{-/+}$ mice compared to *Apc*1572T mice.

Uncovering this shift in tumor phenotype from the gastrointestinal tract towards mammary tissues following β-catenin dosage reduction provides direct in vivo evidence that β-catenin dosage by itself dictates tissue-specific tumor predisposition in the setting of *Apc*-driven cancer. This is in accordance with previously described *APC* genotype–tumor phenotype correlations and associated β-catenin signaling dosages among *Apc*-mutant mouse models and sporadic and familial cancer patients (2). Also, Buchert *et al* indicated specific β-catenin signaling thresholds being important for intestinal and hepatic

tumorigenesis (20). Comparably, tissue-specific biological output being determined by specific dosage has been reported for the proto-oncogene c-Myc as well, which is one of the main target genes of β-catenin signaling (21). Thus, our findings confirmed the dominant role of β-catenin signaling dosage in determining tumor phenotype among tissues. Moreover, our results and those of Buchert *et al*, show that for *Apc*-driven tumor formation in the gut, enhanced β-catenin signaling is absolutely required. Both studies showed that intestinal tumor formation can be prevented completely by reducing β-catenin signaling levels below a hypothetical threshold (20). These results contradict suggested alternative explanations for *Apc*-driven cancer and strongly argue against the model presented by Phelps *et al,* who recently proposed that *APC*-driven tumor formation is independent of β-catenin, but instead requires the transcriptional corepressor CtBP (10). CtBP has been shown to interact with APC at its 15-AARs thereby competing with β-catenin binding, and CtBP's levels appear to increase upon *Apc* loss in early adenomas (13-14). In their paper, Phelps and coworkers suggest that in contrast to CtBP, nuclear β-catenin cannot be detected following *Apc* loss alone using immunofluorescence, and suggested the additional activation of oncogenic KRAS to impose nuclear accumulation of β-catenin (13). As discussed by Fodde and Tomlinson, nuclear staining of β-catenin is a reliable indicator of active Wnt signaling, but its absence does not exclude the robust activation of β-catenin target genes (22). Using immunoperoxidase-based methods most investigators detect nuclear β-catenin accumulation in early adenomas, independent of KRAS mutation status (22-23). Moreover, whereas oncogenic *CTNNB1* mutations have been detected in a large number of tumor types and expression of oncogenic β-catenin leads to the development of numerous tumors in the mouse intestine, equivalent data indicating tumor-initiating capacity of CtBP do not exist. Furthermore, as CtBP binds the more N-terminal located 15-AARs of APC, it can not explain the selection of specific truncated APC proteins retaining between 1-3 20-AARs that is observed in tumors, whereas this is the case for β-catenin (2, 24). The same argument holds true for the C-terminal microtubular functions of APC, which are completely lost in all APC-

mutant proteins. Although loss of these C-terminal regions has been implicated in disturbed cell migration and chromosomal segregation (11-12), *Apc*-mutant mouse studies have shown that the C-terminal domains of Apc do not influence intestinal tumorigenesis. Hence, *Apc*1638T mice lacking the C-terminal regions of Apc but retaining an axin-binding repeat remain tumor-free (25), and the tumor phenotype of *Apc*1322T mice expressing a truncated Apc retaining only 1 20-AAR is not influenced by reintroduction of the C-terminal regions of Apc (26).

Although our findings provide genetic evidence for the dominant role of β-catenin signaling dosage in dictating tissue-specific predisposition for *Apc*-driven tumorigenesis, mechanisms underlying tissue preferences for specific levels of β-catenin signaling remain largely unknown. In the intestine, β-catenin signaling is one of the main regulatory pathways, however, it operates in concerted action with multiple other signaling routes. This complex interplay is poorly understood. Other tissues including the mammary gland have unique architectural organizations and other signaling pathways are likely to play a role into different degrees. Unravelling how the complexity of all those signalling pathways influences which β-catenin signaling dosage dictates tissue-specific tumor predisposition in *Apc*-driven tumorigenesis represents a challenge for future investigation.

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FIGURE LEGENDS

Figure 1. APC structure and associated β-catenin signaling dosage. (A) Structure of the Apc protein containing multiple regulatory domains. (B) Truncated Apc in different *Apc*-mutant mouse models. The number of remaining 20-AARs is inversely correlated with the β-catenin signaling dosage and associated with tumor development in different tissues. (C) Windows of β-catenin signaling dosages associated with specific tumor types in *Apc*-mutant mouse models. Whereas intestinal tumorigenesis requires high to moderate β-catenin signaling, cysts and desmoid development is associated with moderate β-catenin signaling and mammary tumors occur with the low level of β-catenin signaling as observed in *Apc*1572T mice. Figure modified from (2) with permissionof BBA Reviews on Cancer.

Figure 2. Heterozygous β-catenin knockout reduces β-catenin signaling. β-catenin reporter assay of MEFs of $Apc^{+/+}$ or $Apc^{+/1638N}$ genotype, each in combination with $Ctnnb1^{+/+}$ or $Ctnnb1^{-/+}$, showing their intrinsic β-catenin signaling. MEFs were isolated from embryos of embryonic day (E)13.5-15.5 and cultured and transfected as described previously (27). β-catenin reporter assay was performed in duplicate and twice as described previously (27). *p<0.05 Two individual cell lines were used per genotype.

Figure 3. Heterozygous β-catenin knockout prevents gastrointestinal tumor formation but predisposes for mammary tumors in *Apc*1638N mice. Compound heterozygous $Apc^{+/1638N}$; $Ctnnb1^{-/+}$ animals and corresponding single transgenic $Apc^{+/1638N}$ control mice (C57BL/6J) were examined for tumor formation at the age of 8 months. Number of (A) gastrointestinal tumors (B) desmoids and (C) cysts per mouse , distinguishing distribution in females (left) and males (right). ***p<0.001 (D) Number of females with/without mammary tumor development. *p<0.05

Figure 4. Characterization of *Apc*1638N/$Ctnnb1^{-/+}$ mammary tumors. Mammary tissues were fixed overnight in 4% PBS-buffered paraformaldehyde at 4°C, followed by routine paraffin embedding. Haematoxylin-Eosin (HE) staining reveals heterogeneous histology.

Immunohistochemical staining for Cytokeratin-8 (1:800, DSHB) showing luminal epithelial differentiation, cytokeratin-14 (1:10000, Covance) indicating squamous differentiation and smooth muscle actin (1:200, DAKO) showing myoepithelial cells. β-catenin staining (1:2000, Epitomics) revealing membrane-bound and nuclear localization and Ki67 (1:200, DAKO) indicating moderate proliferation.

Figure 1

A

B

Structure of Apc gene product	Apc mouse model	β-catenin signal following LOH	Tumor burden in GI tract	Extra-intestinal Tumors
	+/Δ716	+++	>100	?
	+/Min	+++	>100	occasional cyst or desmoid
(1-2%)	+/1638N	++	5-6	multiple cysts and desmoids
	+/1572T	+	0	mammary tumors some cysts and desmoids
	+/1638T	-	0	none

C

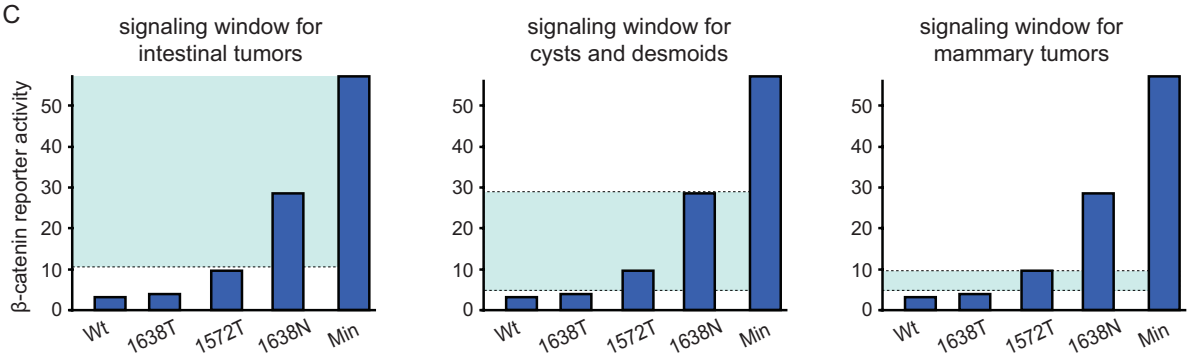

Figure 2

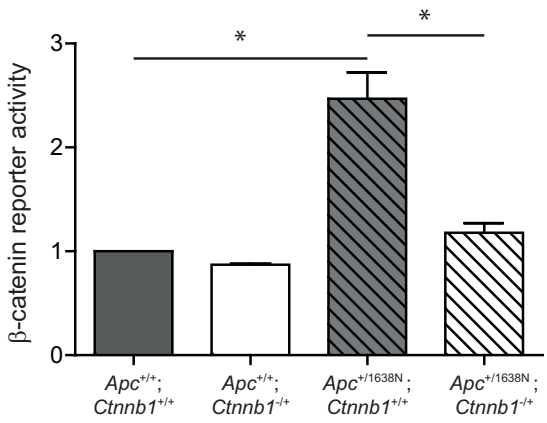
β-catenin reporter activity
3
2
1
0
*
*
Apc+/+; Ctnnb1+/+
Apc+/+; Ctnnb1-/+
Apc+/1638N ; Ctnnb1+/+
Apc+/1638N ; Ctnnb1-/+

Figure 3

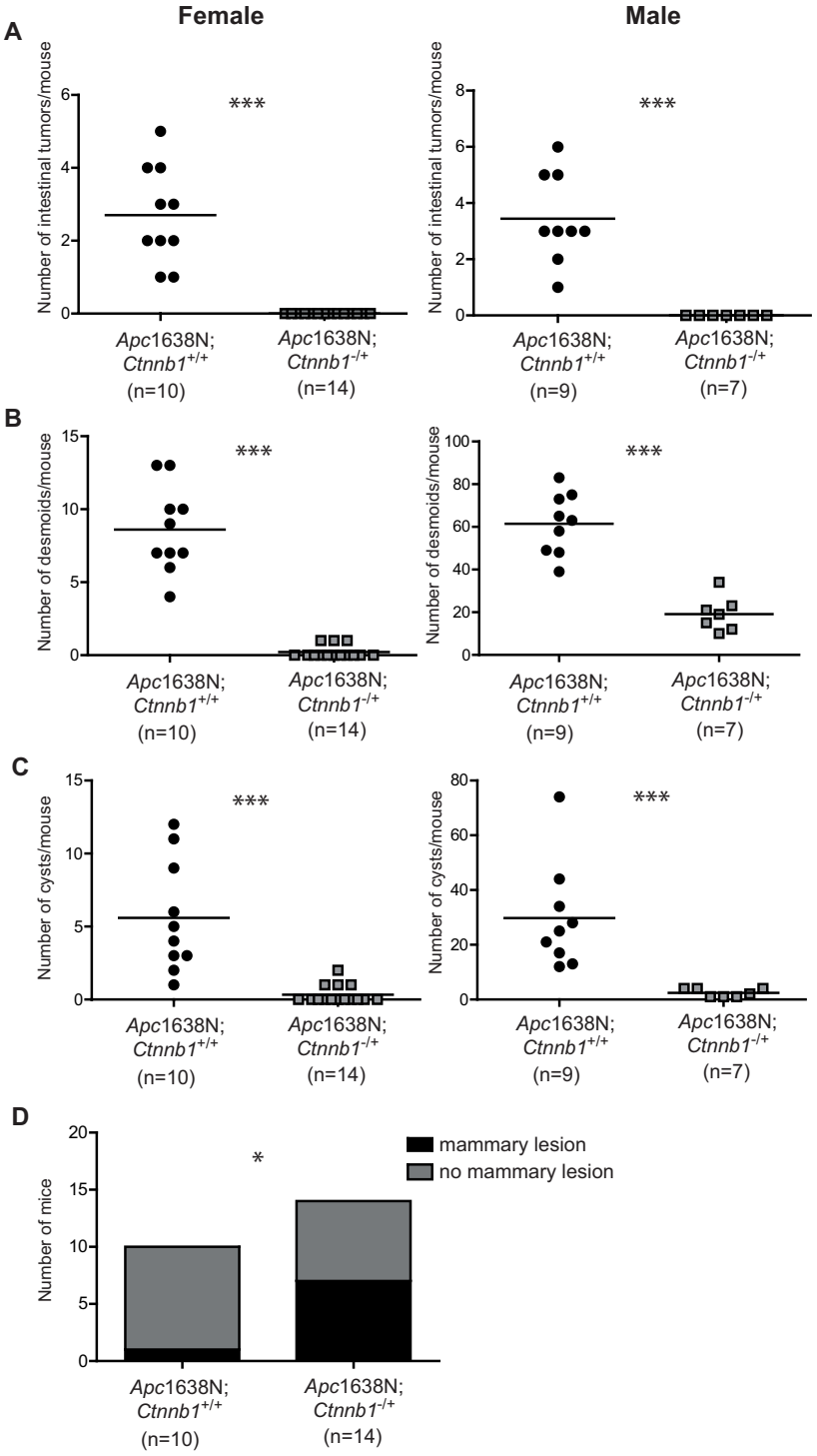
A
Female
Male
Number of intestinal tumors/mouse
***
Apc1638N; Ctnnb1+/+ (n=10)
Apc1638N; Ctnnb1-/+ (n=14)
Apc1638N; Ctnnb1+/+ (n=9)
Apc1638N; Ctnnb1-/+ (n=7)
B
Number of desmoids/mouse
C
Number of cysts/mouse
D
Number of mice
*
mammary lesion
no mammary lesion

Figure 4