EMBRYONIC LETHALITY IN MICE EXPRESSING CONDITIONALLY STABILIZED CTNNB1 UNDER CONTROL OF VIL-CRE

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by

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TABLE OF CONTENTS

ABSTRACT

Embryonic Lethality in Mice Expressing Conditionally Stabilized *Ctnnb1* Under Control of Vil-cre

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Beta-catenin (CTNNB1) is a key downstream effector of the WNT signaling pathway, a pathway involved in embryonic development and tumorigenesis. To further study the role of CTNNB1 in gut tumorigenesis, mice homozygous for a conditional stabilizing mutation of Ctnnb1(a floxed allele) were crossed with mice hemizygous for a Cre recombinase under a gut-specific promoter (Villin-Cre). However, contrary to expectations of Mendelian inheritance, no double heterozygous mice ($Ctnnb1^{flox}$, Villin-Cre) were observed at birth, suggesting embryonic lethality. Since the WNT signaling pathway is involved in embryonic development, and previous studies have confirmed the expression of Villin in the visceral yolk, this study primarily focused on potential placental defects that may have caused the lethality. After assessment of the embryonic lethality which occurred between embryonic stage 11.5 and 13.5, a Rosa26reporter was crossed with Villin-Cre and the resulting embryos and placentae were stained to localize the location of beta galactosidase activity. We found that Villin-Cre is expressed in the yolk sac epithelium, indicating potential defects in the hematopoiesis and/or cellular function caused by the Ctnnb1 mutation. We are currently assessing gene expression in

yolk sac epitheliums by RNAseq to provide insights into the molecular changes caused by ectopic WNT signaling due to stabilization of CTNNB1.

CHAPTER I

INTRODUCTION

β-catenin is an important gene which plays a crucial role in both tumorigenesis and embryonic development. The CTNNB1 protein is special in that it is a moonlighting protein, a protein that is able to perform two very different functions. CTNNB1's functions depend on its location. In the cytoplasm, CTNNB1 is involved in the proper formation of adherens junctions between cells (1). When CTNNB1 translocates in the nuclei it acts as a transcription factor of the WNT signaling system where it is involved in early development pathways (2). The *CTNNB1* gene has garnered a lot of clinical interest because of its involvement in cancer as a proto oncogene. It has been estimated that 10% of all tissue samples taken from cancerous tissue have mutations in the *CTNNB1* gene (3). To further understand the role CTNNB1 plays in tumorigenesis in the gut, we crossed mice that were homozygous for a stabilizing mutation of *CTNNB1* with mice that were hemizygous for a Cre recombinase under the gut specific promoter, Villin. However, none of the double heterozygous mice survived to birth pointing to an embryonic lethality. In this study we aim at elucidating underlying mechanisms of the embryonic lethality.

To this purpose, we generated a mouse model by crossing mice carrying the stabilizing mutation for Ctnnb1 with mice that were hemizygous for a Cre recombinase under Villin. The concentration of CTNNB1 is regulated by ubiquitination and is a substrate of the E3 ubiquitin ligase TrCP1(β-TrCP) (4). However, before β-TrCP can bind β-catenin, the protein must first be phosphorylated by two Glycogen synthase kinases, GSK3a and GSK3β (4). The phosphorylation sites for GSK3β are encoded by exon 3 in *CTNNB1.* When exon 3 is removed

CTNNB1 can no longer be targeted for ubiquitination and is stabilized. We cross these mice with mice that have a Cre recombinase under Villin so that the floxed exon 3 can be removed and localized to the gut epithelium.

However, contrary to Mendellian calculations (Figure 1) none of the mice that survived to birth were double heterozygous. Previous studies had shown that Ctnnb1 plays a role in embryonic development and that Villin is expressed in the yolk sac, so this study primarily focuses on the potential placental defects that may have caused the lethality. By counting the surviving embryos at different stages, it was determined that embryonic lethality occurred between embryonic stages 11.5 and 13.5 (Figure 3), the location of the protein expression was localized by crossing a Rosa26reporter with Villin-Cre. X-gal staining for β galactosidase expression of the collected placentas showed that the protein was expressed in the yolk sac epithelium (Figure 4), pointing towards complications in cellular function or in the hematopoietic pathway (5, 6).

CHAPTER II

METHODS

All figures and tables are found in the appendix.

Cre Recombinase to Excise Exon 3 from *CTNNB1*

B6.129X1-*Ctnnb1*^{tm1Mmt}, aka *Ctnnb1*^{flox} mice that have floxed alleles flagging exon 3 of *CTNNB1* were crossed with B6N.Cg-Tg(Vil1-cre)20Syr/J, known as *Villin-Cre* mice that are hemizygous for a Cre recombinase under a gut-specific promoter. The β-catenin protein is normally degraded by the ubiquitination protein β-TrCP (4) in which exon 3 of *CTNNB1* is involved in the encoding of the serine-threonine phosphorylation sites for GSK3β. GSK3β is one of the glycogen synthase kinases that must first phosphorylate the β-catenin protein before β-TrCP can ubiquitinate it (4). The Cre recombinase in *Villin-Cre* mice performs the recombination of the floxed exon 3 and the Villin localized it to the gut epithelium as described in Figure 2.

Mice and Collection of Embryos

All mice are maintained on a C57BL/6J genetic background. Genotypes were determined using PCR and confirmed with differential exon analysis. Embryos were collected based on time after coitus(day of mouse plug = 0.5, day post coitus dpc) (7). Embryos from the CTNNB1, VILLIN CRE cross were collected from a range of 10.5 to 13.5 dpc (Figure 3) to determine embryo resorption timeline. *Rosa26* reporters (B6.129S4-Gt(ROSA)26Sor^{tm1Sor}/J) was crossed with *Villin-Cre* to determine the location of Villin Cre expression in the placenta and yolk sac membrane by X-gal staining to determine beta galactosidase activity(Figure 4). The results in Figure 4 led to another CTNNB1, VILLIN CRE cross, from which embryos were collected at

11.5 and 12.5 dpc. Yolk sac membranes were collected and sent for HE staining and and for RNAseq analysis.

Clean DNA Extraction Protocol

The Promega Maxwell DNA Extraction/Purification Kit Catalog Number - AS1030 was used to extract and purify DNA for this study. Approximately 50 mg of tissue was obtained from collected samples and was heated to 56 ˚C with 180 µL of ATL Tissue Lysis buffer and 20 µL of proteinase K until the tissue was lysed. The purification conducted by following the protocols for Standard Elution Volume (SEV) mode. The embryos were genotyped after DNA extraction using the CTNNB1 - flox, CTNNB1 - wild type, and Cre PCRs.

Mucous Plug Check

After a mouse is impregnated, a mucous plus will appear on or in its vaginal opening. This plug only lasts for approximately one day but it is a reliable method of approximating the time of fertilization. This is important to determine the embryonic age of the mice. The day when the plug was found was assumed to be dpc 0.5. After dissection, the embryos were compared with literature to double check their embryonic age.

Determination of LacZ activity in Placentas

Villin-cre, R226R placentas were stained using the X-Gal staining protocol as described by Burn to determine the location of β-galactosidase activity in mouse placentas (8). The results are described in expression as seen in Figure 4.

RNA Sequencing

RNA sequencing was performed by the phenotyping core at Texas A&M University and was used to determine gene expression in mutant and control samples of yolk sac membranes. The RNA sequencing data was analyzed with the help members of the Threadgill Lab.

Hematoxylin & Eosin Staining (HE staining)

HE staining is currently being conducted on yolk sac membranes extracted from mutant and control samples. The protocol was followed as described by Mintz. This was used to characterize and analyze alterations cell morphology.

CHAPTER III

RESULTS

All figures and tables are found in the appendix.

Excision of Exon 3 from CTNNB1 under Control of Villin-Cre Causes Embryonic Lethality

In order to stabilize β-catenin in a gut specific manner, mice with a floxed exon 3 were crossed with mice hemizygous for Villin-Cre, a Cre recombinase under control of Villin, a gut specific promoter. The successful stabilization of β-catenin using this method has been described in previous studies (4). The expected ratio and genotypes of the offspring from the cross can be seen in Figure 1 and the gut specific manner in which Villin-Cre expresses the growth of function mutation, however none of the double heterozygous offspring survived to term.

Embryonic Death due to the Mutation Occurs on DPC 12.5

Embryo samples were collected and analyzed at a range of different dpcs(from 10.5 to 13.5) and were visually analyzed for aberrant development and reabsorption. Figure 3 shows drastic differences in development by dpc 12.5. By dpc 12.5, embryos displayed hemorrhaging and visible size differences. By dpc 13.5 most double heterozygous mice were seen in various stages of reabsorption.

β-galactosidase Activity in the Yolk Sac Epithelium

In order to narrow down the possible causes of the embryonic lethality, a Rosa26reporter was crossed with mice hemizygous for Villin-Cre. Dpc 12.5 embryonic death pointed to placental defects. Placentas were collected and were observed under microscope after X-gal staining to

view β-galactosidase Activity. The results of that experiment can be seen in Figure 4, which shows that Villin-cre is expressed in the yolk sac epithelium. Previous studies have shown that β-catenin signalling in the yolk sac epithelium is involved in the induction and maintenance of embryonic hematopoiesis (5, 6).

Tissue Samples Collected from Embryos were Genotyped to Select for RNAseq and HE Staining

Following localization of Villin expression in the yolk sac membrane, tissues were collected from embryos at dpc 11.5 and 12.5. These embryos were produced from the cross described in Figure 1. Table 1 shows the expected and confirmed genotypes of the 10 samples from which yolk sac membranes were collected and sent for RNAseq and HE Staining.

CHAPTER IV

CONCLUSION

This study reports on the current progress in elucidating the embryonic lethality resulting from stabilization of the β-catenin protein under the control of the gut specific promoter Villin. The protein was stabilized by excising exon 3 using a Cre recombinase, which is essential for the normal ubiquitination of β-catenin.

Upon confirming that the embryonic resorption was occurring at dpc 12.5 (Figure 3), the study was focused on placental defects. Previous studies have attributed placental defects with embryonic resorption at dpc 12.5, and the *Villin* gene is gut specific and models with gut developmental issues have been shown to survive significantly farther than dpc 12.5 (9). This previous research confirmed that embryonic gut development issues were unlikely to be the cause of the lethality. To further narrow down the possible causes of this lethality, a Rosa26reporter was crossed with Villin-Cre mice. Placentas collected from the embryos of this cross from a range of dpc 10.5 to 13.5 were collected and stained to locate β-galactosidase activity. Upon analysis of these placentas we realized that Villin is being expressed in the yolk sac membrane (Figure 4).

After localizing the expression in the yolk sac membrane, samples of mutant and control membranes were collected and sent for RNA sequencing and HE staining. The data from RNA sequencing will be used for analysis of gene expression. Previous studies have shown the abnormal endocytosis of the yolk sac endoderm caused embryonic death by dpc 12.5 in mice

(10). The study suggests that a knock out of *Pit-1* causes issues in the yolk sac visceral endoderm (10). Other studies have related the regulation of *Pit-1* to the functino of β-catenin(lorin e olson). β-catenin signalling has also been implicated in the proper hematopoiesis in vertebrate embryos. The formation of ventral blood islands in *Xenopus* has been connected to the canonical signalling of Wnt4 (5), and other studies have stated that Wnt signalling is a necessary mechanism to regulate hematopoietic mergence (6).

The data collected from RNAseq will help elucidate gene expression, and analysis of the differential levels of gene expression in mutant and wild type yolk sac membranes will aid in further narrowing down the possible mechanisms through which this mutation causes embryonic death.

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APPENDIX

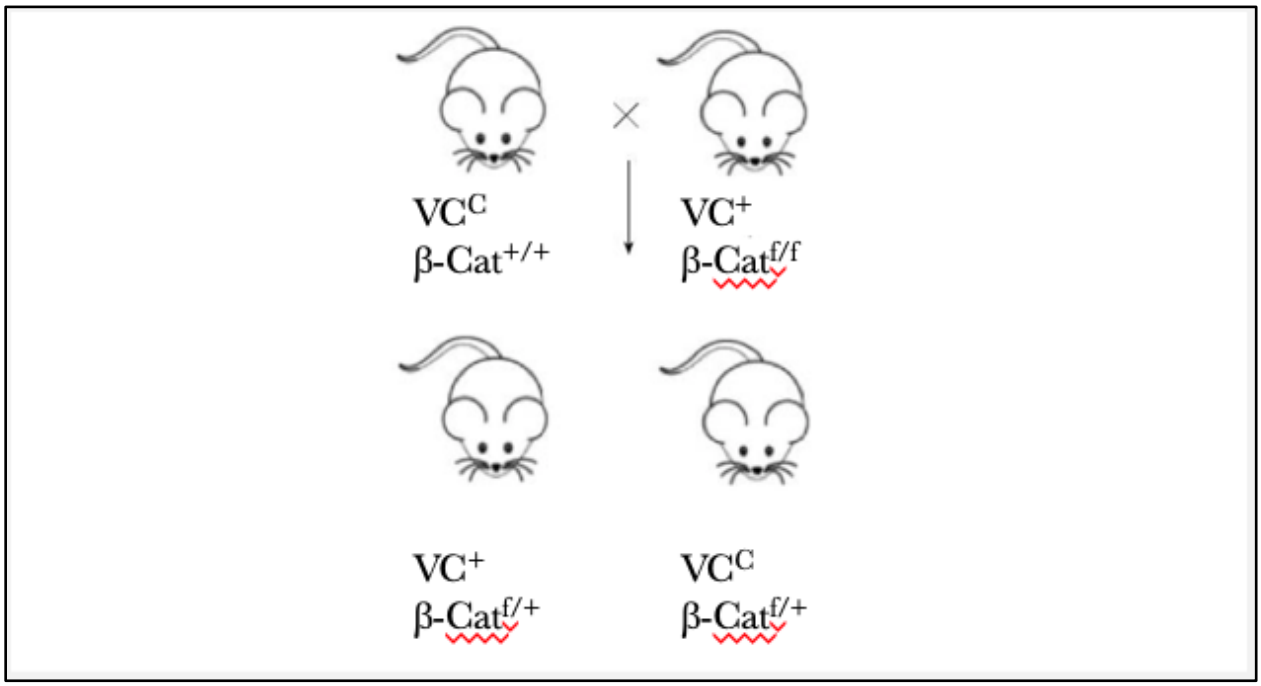

Figure 1. Visual description of mouse genotypes of control and mutant mice.

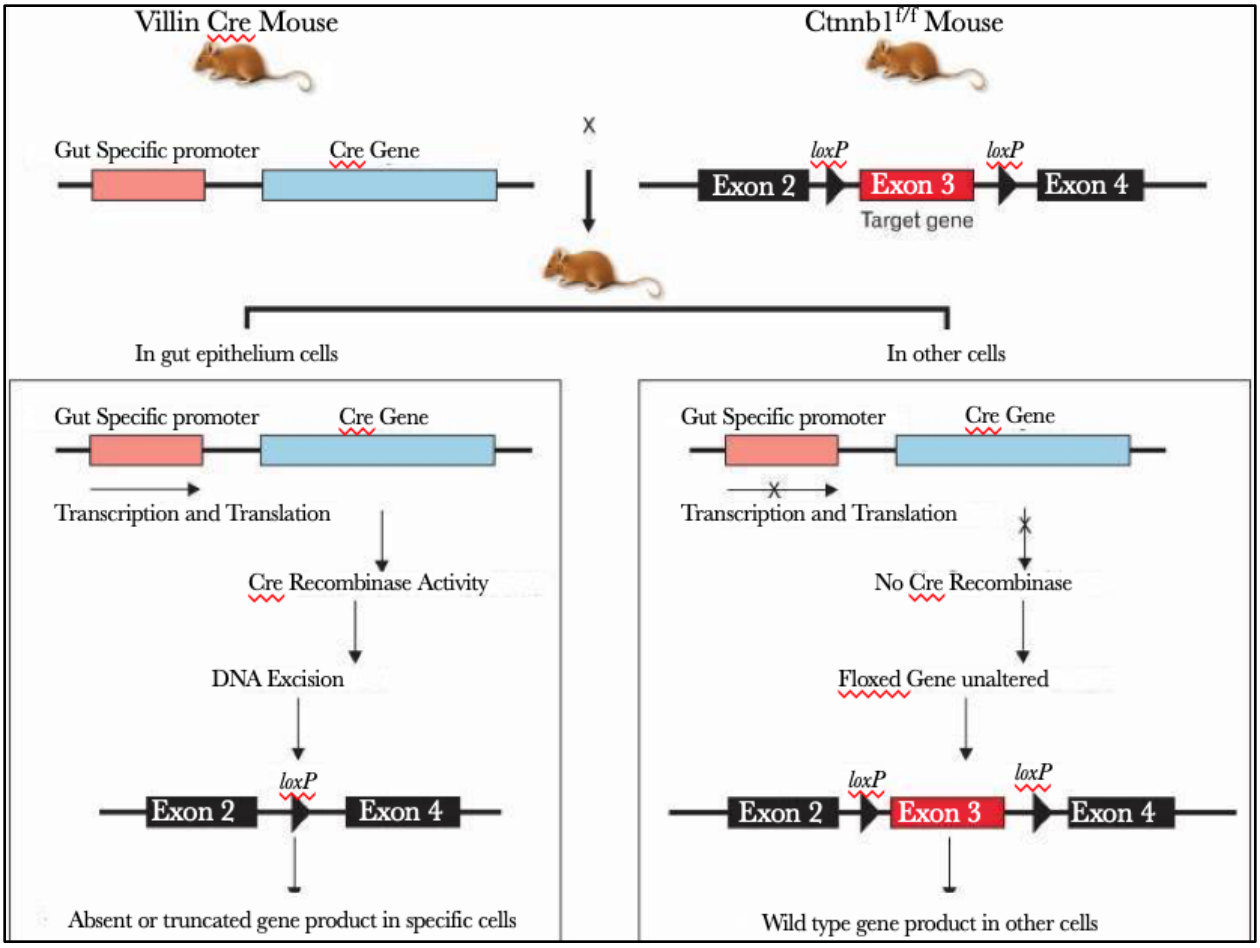

Figure 2. Cre Recombinase used to conditionally remove Exon 3 from Ctnnb1.

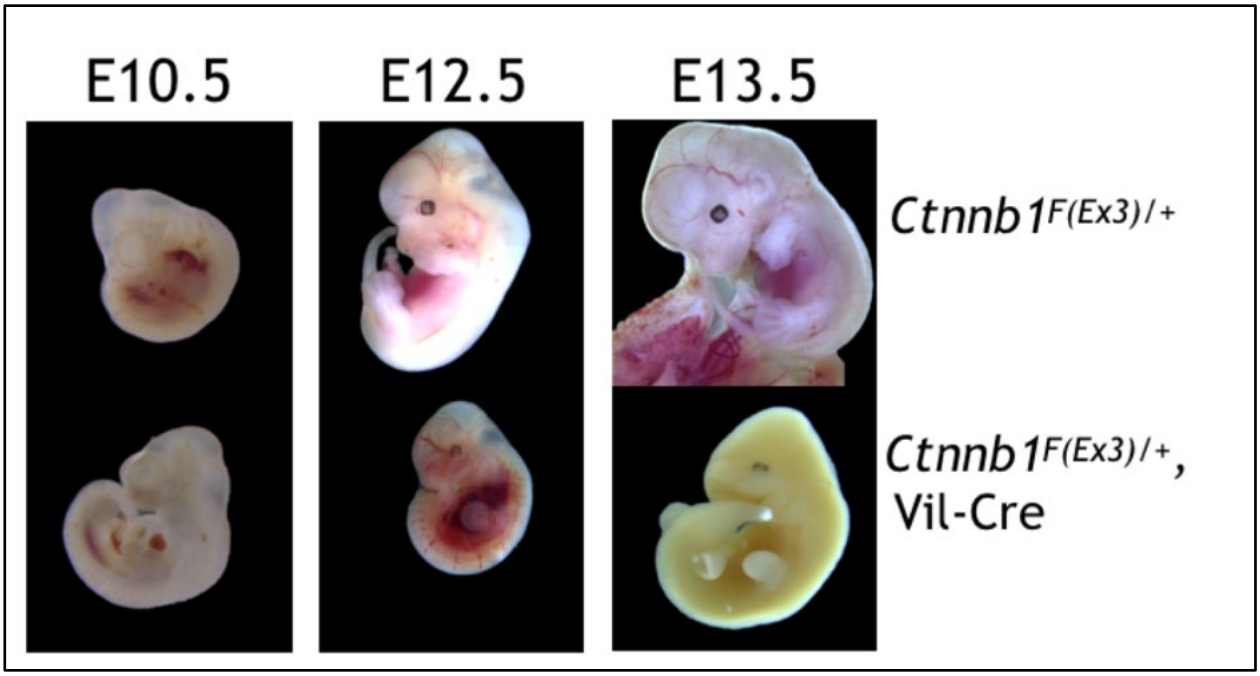

Figure 3. Ctnnb1$^{F(EX3)/+}$, Vil-Cre embryos show delayed development by dpc 12.5. By dpc 13.5 most Ctnnb1$^{F(EX3)/+}$, Vil-Cre embryos are in various states of resorption.

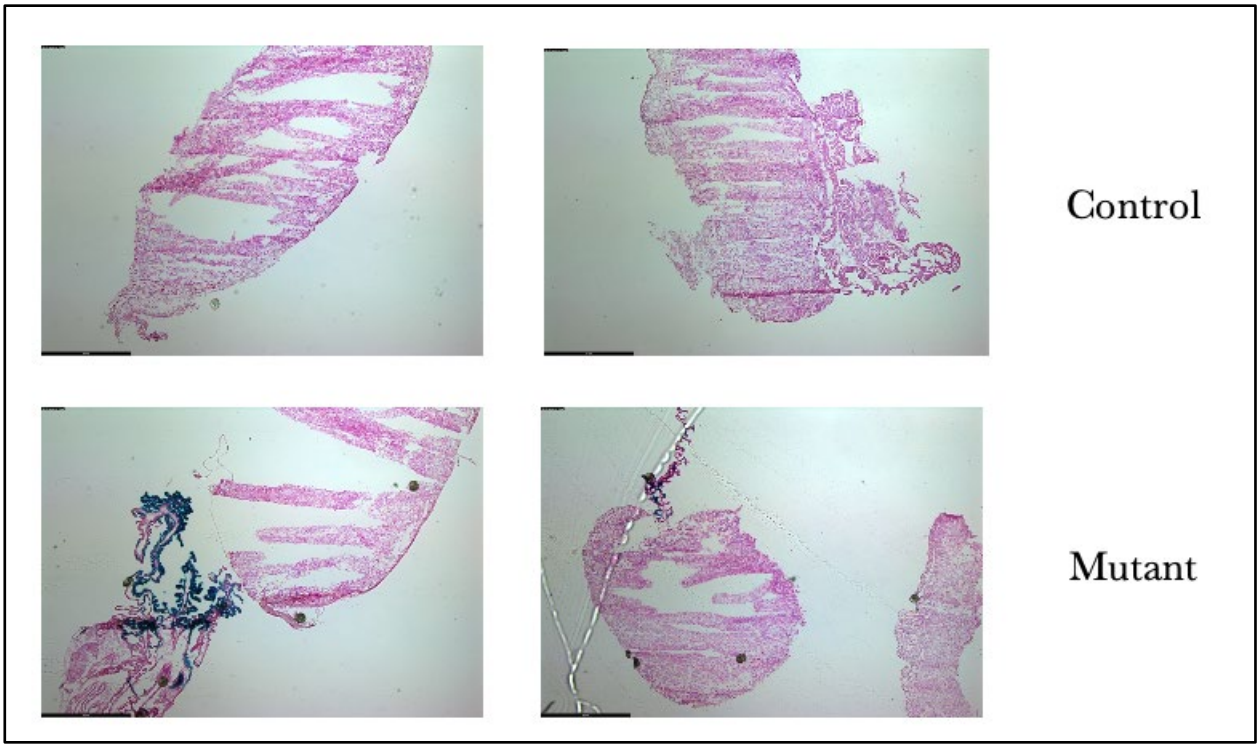

Figure 4. X-gal staining of mouse embryos. The top 2 figures show control samples with no β-galactosidase activity, whereas the two bottom samples show β-galactosidase activity in the yolk sac membranes in mutant placentas.