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Genetic interaction of Pax3 mutation and canonical Wnt signaling modulates neural tube defects and neural crest abnormalities

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Summary

Mouse models provide opportunities to investigate genetic interactions that cause or modify the frequency of neural tube defects (NTDs). Mutation of the PAX3 transcription factor prevents neural tube closure, leading to cranial and spinal NTDs whose frequency is responsive to folate status. Canonical Wnt signalling is implicated both in regulation of *Pax3* expression and as a target of PAX3. This study investigated potential interactions of *Pax3* mutation and canonical Wnt signalling using conditional gain- and loss-of-function models of β-catenin. We found an additive effect of β-catenin gain of function and *Pax3* loss of function on NTDs and neural crest defects. β-catenin gain of function in the *Pax3* expression domain led to significantly increased frequency of cranial but not spinal NTDs in embryos that are heterozygous for *Pax3* mutation, while both cranial and spinal neural tube closure were exacerbated in *Pax3* homozygotes. Similarly, deficits of migrating neural crest cells were exacerbated by β-catenin gain of function, with almost complete ablation of spinal neural crest cells and derivatives in *Pax3* homozygous mutants. *Pax3* expression was not affected by β-catenin gain of function, while we confirmed that loss of function led to reduced *Pax3* transcription. In contrast to gain of function, β-catenin knockout in the *Pax3* expression domain lowered the frequency of cranial NTDs in *Pax3* null embryos. However, loss of function of β-catenin and *Pax3* resulted in spinal NTDs, suggesting differential regulation of cranial and spinal neural tube closure. In summary, β-catenin function modulates the frequency of PAX3-related NTDs in the mouse.

KEYWORDS
β-catenin, exencephaly, neural tube defects, Pax3, spina bifida, Wnt

1 | INTRODUCTION

Congenital anomalies (birth defects) affect up to 6% of infants worldwide, lead to approximately 300,000 deaths each year, and are the primary cause of infant mortality in developed countries (Finnell et al., 2021; Zaganjor et al., 2016). While significant progress has been made toward identification of the genetic basis of some disorders, there are large gaps in our knowledge, in part owing to multifactorial etiology,

genetic heterogeneity, and the potential influence of environmental factors. Hence, for common birth defects such as neural tube defects (NTDs), the genetic cause is not known in most individuals.

NTDs, including anencephaly and spina bifida, result from incomplete closure of the neural tube during embryonic development (Greene & Copp, 2014; Nikolopoulou, Galea, Rolo, Greene, & Copp, 2017). Failure of closure in the cranial region leads to exencephaly, the developmental precursor of anencephaly, while failed closure of the spinal neural tube leads to spina bifida. Mouse genetic models provide insight into the molecular requirements for neural tube closure, with NTDs arising in more than 200 different mutant and knockout strains (Harris & Juriloff, 2007, 2010; Nikolopoulou et al., 2017). Mouse models also provide opportunities to investigate the potential for multigenic causes of NTDs. Examples include strains in which there is a major risk allele with contributions from modifier genes, as in the *curly tail* strain in which NTDs result from a hypomorphic allele of *Grhl3*, with penetrance of NTDs influenced by contributions from other loci including *Lmnb1* (De Castro et al., 2012; Gustavsson et al., 2007; Sudiwala et al., 2016; Ting et al., 2003). Alternatively, a number of studies have identified additive interactions of heterozygous genetic mutations which individually do not result in NTDs. For example, the *Vangl2*^{Lp} mutation causes a tail flexion defect in *Vangl2*$^{Lp/+}$ mice but interacts with heterozygous mutations in genes such as *Celsr1* (encoding a component of the planar cell polarity signaling pathway) (Murdoch et al., 2014), as well as other genes including loss- and gain-of-function alleles of *Grhl3* (Caddy et al., 2010; De Castro et al., 2018).

In the current study, we investigated the potential for modulation of NTDs caused by mutation of *Pax3* by genetic alteration of canonical Wnt signaling. *Pax3* encodes a member of the paired- and homeodomain-containing family of PAX transcription factors that play roles in a variety of developmental contexts (Blake & Ziman, 2014). Key functions of PAX3 during embryogenesis are revealed by analysis of *splotch* mice, which carry mutations in *Pax3* and are named for the characteristic belly spot present in heterozygotes (Auerbach, 1954; Greene, Massa, & Copp, 2009). At least nine spontaneous and radiation-induced alleles of *splotch* have been identified, corresponding to a range of *Pax3* mutations and deletions and including the functionally null *Sp* and *Sp*^{2H} alleles (Epstein, Vekemans, & Gros, 1991; Epstein, Vogan, Trasler, & Gros, 1993). Several further knock-in alleles have been generated by gene targeting and recapitulate *splotch* phenotypes (Engleka et al., 2005; Mansouri, Pla, Larue, & Gruss, 2001; Zhou, Wang, Rogers, & Conway, 2008).

Pax3 is expressed in the dorsal neuroepithelium of the neural folds and closed neural tube, in populations of neural crest cells and in the developing somites (Goulding, Chalepakis, Deutsch, Erselius, & Gruss, 1991). Corresponding with its expression domain, homozygous mutation of *Pax3* in mouse embryos leads to multiple defects including cranial and spinal NTDs, muscular defects, and abnormalities in neural crest derivatives in the heart, gut innervation, and melanocytes (Auerbach, 1954; Conway, Henderson, Anderson, Kirby, & Copp, 1997; Conway, Henderson, & Copp, 1997; Lang et al., 2000; Lang et al., 2005).

Pax3-related abnormalities are assumed to result from dysregulated transcription owing to the role of PAX3 as a transcriptional activator, as a homodimer or heterodimer with other transcription factors, or as a transcriptional inhibitor with co-repressors (Boudjadi, Chatterjee, Sun, Vemu, & Barr, 2018). A large number of targets have been identified in different cell types, but the molecular mechanisms underlying *Pax3*-related NTDs have yet to be determined. Among PAX3 targets, *Wnt1* and *Wnt3a* appear to be regulated by *Pax3* during neurulation and in neural crest development (Conway et al., 2000; Fenby, Fotaki, & Mason, 2008; Monsoro-Burq, Wang, & Harland, 2005). Conversely, the presence of binding sites for Wnt signaling mediators, TCF/LEF, in intron 4 of *Pax3* as well as regulatory elements that confer indirect response to β-catenin in the proximal promoter suggests that *Pax3* may itself be regulated by canonical Wnt signaling (Degenhardt et al., 2010; Moore et al., 2013). This is supported by findings in neural crest and neural tube development (Taneyhill & Bronner-Fraser, 2005; Zhao et al., 2014).

Canonical Wnt signaling involves the repression of activity of the Axin-GSK3-APC-containing destruction complex, such that phosphorylation and ubiquitination of β-catenin is diminished, and β-catenin is free to translocate to the nucleus (Nusse & Clevers, 2017). Owing to the potential role of canonical Wnt signaling both upstream and downstream of *Pax3*, we tested whether the frequency of NTDs caused by PAX3 mutation is modified by gain- or loss-of-function of β-catenin. In the current study, we found that β-catenin gain of function exacerbates both cranial and spinal neural tube closure, leading to more frequent NTDs than with *Pax3* mutation alone. In contrast, cranial NTDs resulting from *Pax3* mutation are partially rescued by β-catenin ablation.

2 | RESULTS

2.1 | β-Catenin gain of function exacerbates the effect of *Pax3* mutation on neural tube closure

We explored potential effects of β-catenin gain of function on *Pax3*-related phenotypes using the *Ctnnb1*^{floxE3} allele, in which cre-mediated deletion of exon 3 (*Ctnnb1*$^{\Delta E3}$) leads to production of a stabilized β-catenin protein (Harada et al., 1999). Hence, we crossed mice of genotype *Pax3*$^{Sp2H/+}$; *Ctnnb1*$^{floxE3/+}$ with *Pax3*$^{cre/+}$ mice to generate litters containing embryos with combinations of *Pax3* and *Ctnnb1* alleles, for comparison of rates of NTDs. Litters include embryos of *Pax3*$^{Cre/+}$; *Ctnnb1*$^{\Delta E3/+}$ and *Pax3*$^{Sp2H/Cre}$; *Ctnnb1*$^{\Delta E3/+}$ genotype, which have β-catenin gain of function in the *Pax3* lineage in the dorsal epithelium and NCC derivatives. Within these litters, embryos of *Pax3*$^{Sp2H/Cre}$ genotype (either *Ctnnb1* wild type or gain of function) are functionally null for Pax3 as the cre knock-in ablates *Pax3* exon 1.

Cranial NTDs occurred in approximately 50% of *Pax3*$^{cre/Sp2H}$ embryos among litters collected at E9.5–10.5 (Figure 1a), a similar rate to that previously observed among *Pax3*$^{Sp2H/Sp2H}$ embryos (Burren et al., 2008). A low rate of cranial NTDs was also observed among

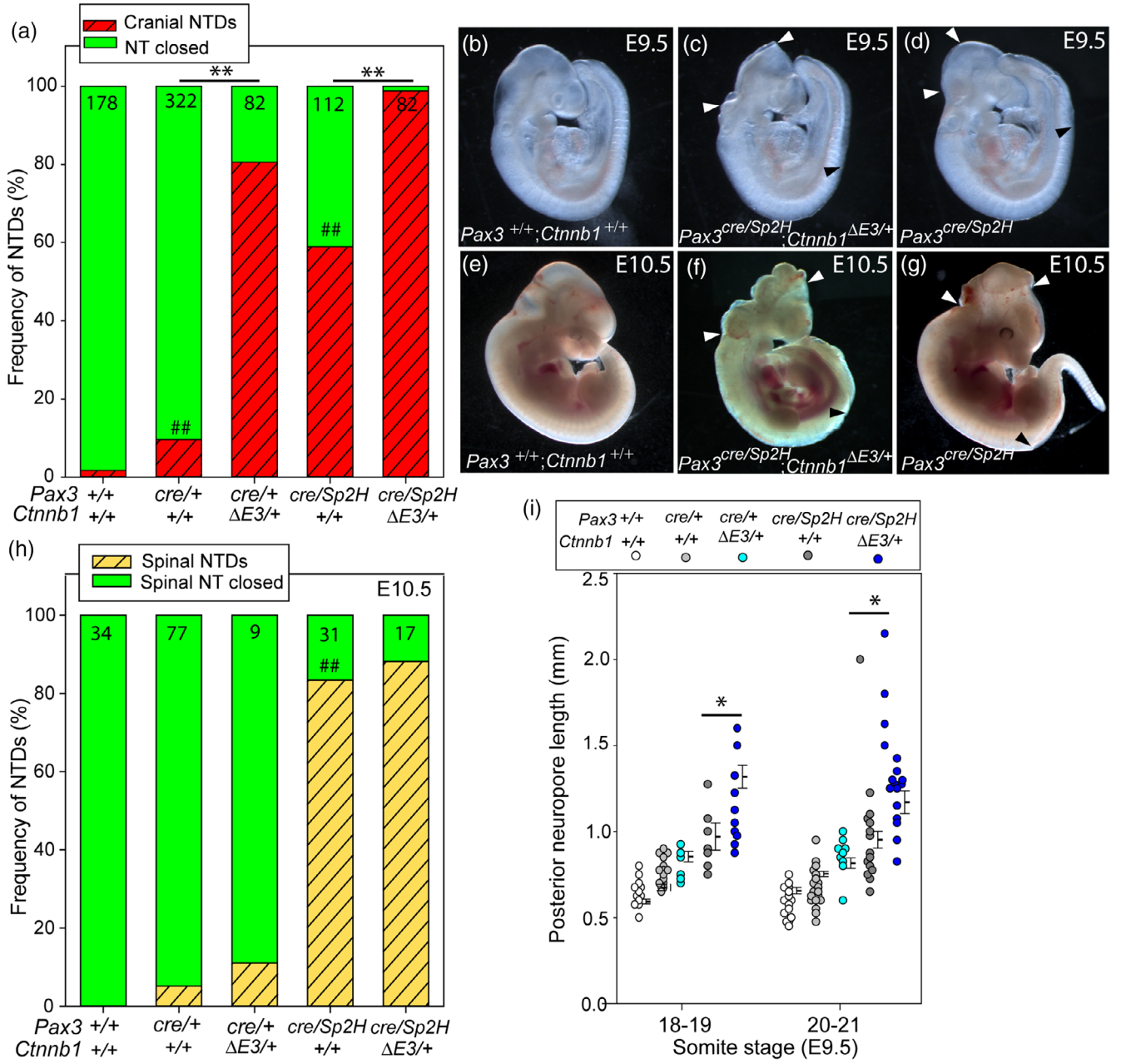

FIGURE 1 β-catenin gain of function exacerbates cranial NTDs and delays spinal neural tube closure in *Pax3* mutant embryos. (a) Embryos carrying combinations of functionally null alleles of Pax3 (Pax^{cre}, $Pax3^{Sp2H}$) and the $Ctnnb1^{floxE3}$ allele (recombined by $Pax3^{cre}$ to $Ctnnb1^{\Delta E3}$) were analyzed at E9.5 or E10.5 for the presence of cranial NTDs. Numbers of embryos per group are shown in each bar (the functionally equivalent $Ctnnb1^{floxE3}$ and $Ctnnb1^{+}$ alleles are combined). ** indicates significant difference between embryos of the same *Pax3* genotype with/without $Ctnnb1^{\Delta E3}$ ($p < .001$ Chi-Square). ## indicates significant effect of *Pax3* genotype among embryos that are wild type for *Ctnnb1* ($p < .001$ chi-square). (b–g) Compared with wild type in which the cranial NT is closed (b, e), and $Pax3^{cre/Sp2H}$ in which the cranial region is either closed (d) or has a moderately sized NTD (G), $Pax3^{cre/Sp2H}$; $Ctnnb1^{\Delta E3/+}$ embryos show severe cranial and spinal NTDs at E9.5 (c) and E10.5 (f). The region of open neural folds in the cranial region is indicated by white arrowheads and the rostral extent of the enlarged PNP is indicated by black arrowheads. (h) Embryos were scored for spinal NTDs (spina bifida) on the basis of failed PNP closure at E10.5 (30 or more somites). (i) Analysis at earlier stages (E9.5) revealed significant increase in anterior–posterior length of the PNP in $Pax3^{cre/Sp2H}$ embryos, suggesting exacerbation of spinal closure. * indicates significant difference between embryos of the same *Pax3* genotype with/without $Ctnnb1^{\Delta E3}$ ($p < .05$, ANOVA)

$Pax3^{cre/+}$ heterozygous embryos (with significant difference compared to wild-type). Recombination of the $Ctnnb1^{floxE3}$ allele in the *Pax3* expression domain led to a significant increase in the rate of cranial NTDs in $Pax3^{cre/+}$; $Ctnnb1^{\Delta E3/+}$ and $Pax3^{Sp2H/Cre}$; $Ctnnb1^{\Delta E3/+}$ embryos, compared with the equivalent *Pax3* genotype with wild-type *Ctnnb1* (Figure 1a). The overall appearance of cranial NTDs in $Pax3^{Sp2H/Cre}$; $Ctnnb1^{\Delta E3/+}$ embryos (Figure 1c, f) was typically more severe than in *Pax3* mutant *Ctnnb1* wild-type embryos (Figure 1d, g).

In embryos with β-catenin gain of function, the open cranial neural folds typically encompassed a more extensive region, including the entire hindbrain (Figure 1b–g), midbrain, and sometimes the forebrain.

Analysis of litters at E10.5 showed that spinal NTDs occurred with high frequency in Pax3 null embryos, irrespective of *Ctnnb1* genotype, and also occasionally in heterozygotes (Figure 1h). We observed a non-significant trend toward more frequent spinal NTDs with β-catenin gain of function, but the high frequency of spinal NTDs in $Pax3^{cre/Sp2H}$ embryos diminished sensitivity to detect exacerbation of these defects at E10.5, by which stage closure has either succeeded or failed (Figure 1h). We therefore evaluated spinal neurulation during the period of closure by measuring the length of open posterior neuropore (PNP) at E9.5, shortly after delay of closure (indicated by increased anterior–posterior length of the PNP) first becomes apparent in *Pax3* mutant compared with wild-type embryos (Sudiwala et al., 2019). Among stage-matched Pax3 null embryos, we observed significant enlargement of the PNP when the $Ctnnb1^{\Delta E3}$ allele was also present, indicating further delay of closure imposed by β-catenin gain of function (Figure 1i).

The high rate of cranial NTDs in $Pax3^{cre}$; $Ctnnb1^{\Delta E3/+}$ embryos suggests that there is an additive genetic interaction between PAX3 loss of function and β-catenin gain of function, in the dorsal neuroepithelium in which *Pax3* is expressed. To further investigate this hypothesis, we asked whether β-catenin activation is sufficient to induce NTDs in the absence of a co-occurrent *Pax3* null allele by using $Sox1^{cre}$ to recombine $Ctnnb1^{floxE3}$ throughout the cranial neuroepithelium. Among litters from a cross of $Pax3^{Sp2H/+}$; $Ctnnb1^{floxE3}$ mice with $Sox1^{cre/+}$, cranial NTDs arose among 25% (one out of four) of $Sox1^{cre/+}$; $Ctnnb1^{\Delta E3}$, and 20% (3 out of 15) $Sox1^{cre/+}$; $Ctnnb1^{+/+}$ embryos. The presence of a low frequency of exencephaly in $Sox1^{cre/+}$ embryos was surprising and may relate to the presence of the $Sox1^{cre}$ knock-in allele. Nevertheless, these findings suggest that β-catenin gain of function alone is not responsible for the high rate of NTDs in the $Pax3^{cre}$; $Ctnnb1^{\Delta E3}$ embryos. In contrast, the two embryos from this cross in which a *Pax3* mutant allele was present ($Pax3^{Sp2H/+}$; $Sox1^{cre/+}$; $Ctnnb1^{\Delta E3}$) both exhibited cranial NTDs (2/2; 100%), consistent with an additive effect of *Pax3* mutation with β-catenin gain of function.

2.1.1 | Canonical Wnt signaling appears unaffected by Pax3 mutation

We confirmed that canonical Wnt signaling was increased among embryos carrying the $Ctnnb1^{\Delta E3}$ allele by qRT-PCR (Figure 2a) and whole mount in situ hybridization for the target gene *Axin2* (Figure 2b–e). In contrast, in the absence of this allele we did not observe any effect of *Pax3* genotype on *Axin2* expression suggesting that there was not a pre-existing over-activation of canonical Wnt signaling in *Pax3* mutants that is exacerbated by β-catenin gain of function. Lack of an alteration in Wnt signaling in *Pax3* mutants was also consistent with findings obtained using the BAT-Gal reporter, in which *LacZ* is expressed under the control of LEF/TCF-regulatory elements (Maretto et al., 2003). After breeding the reporter into the $Pax3^{Sp2H}$ line, relative *LacZ* expression did not differ between $Pax3^{+/+}$ and $Pax3^{Sp2H/Sp2H}$ embryos at E9.5 (Figure S1a). Staining for β-galactosidase activity in BAT-Gal positive embryos at E9.5 confirmed that the domain of canonical Wnt signaling activity (Figure S1b) overlaps with the expression domain of *Pax3* (Sudiwala et al., 2019).

2.2 | β-Catenin loss of function lowers the frequency of cranial NTDs in *Pax3* null embryos but causes spinal NTDs

Having observed an effect of β-catenin gain of function on neural tube closure, we next tested whether the frequency of *Pax3*-related NTDs was altered by β-catenin loss of function, using a conditional allele in which cre-mediated recombination deletes exons 2–6 of *Ctnnb1*, creating a null allele, $Ctnnb1^{\Delta Ex2-6}$ (Brault et al., 2001). The frequency of NTDs was compared within litters from a cross of $Pax3^{Sp2H/+}/Ctnnb1^{floxEx2-6}$ with $Pax3^{cre/+}/Ctnnb1^{floxEx2-6}$. Cranial NTDs occurred at low frequency when *Ctnnb1* was deleted in the *Pax3* domain, generating $Pax3^{cre/+}$; $Ctnnb1^{\Delta Ex2-6/\Delta Ex2-6}$ (β-catenin loss of function in the Pax3 domain), but this did not differ significantly from embryos carrying the $Pax3^{cre/+}$ allele with $Ctnnb1^{+/\Delta Ex2-6}$ or $Ctnnb1^{+/+}$ genotype (Figure 3a). However, while *Pax3* null ($Pax3^{cre/Sp2H}$) embryos that were wild type or heterozygous for $Ctnnb1^{\Delta Ex2-6}$ showed cranial NTDs with an expected frequency of around 45%, this was significantly lower among $Pax3^{cre/Sp2H}$; $Ctnnb1^{\Delta Ex2-6/\Delta Ex2-6}$ embryos that were homozygous for the floxed loss of function β-catenin allele (Figure 3a; $p < .05$; *z*-test). Homozygous *Pax3* mutants displayed a high rate of spinal NTDs as expected, irrespective of *Ctnnb1* genotype. Similarly, β-catenin loss of function in the dorsal neuroepithelium of $Pax3^{cre/+}$; $Ctnnb1^{\Delta Ex2-6/\Delta Ex2-6}$ embryos also caused spinal NTDs with high frequency (Figure 3b), consistent with previous findings (Zhao et al., 2014).

Pax3 has been reported to be a target of Wnt signaling (Zhao et al., 2014). We therefore evaluated transcription from the *Pax3* locus by qRT-PCR using primers which amplify the wild-type and $Pax3^{cre}$ alleles. We found significant reduction of expression from the *Pax3* locus in *Ctnnb1* conditionally deleted embryos (Figure 4a) and this was replicated using allele-specific primers, which amplify the wild-type and $Pax3^{Sp2H}$ alleles (Figure S2). We tested whether β-catenin conditional gain of function may have a reciprocal effect on *Pax3* expression. However, we did not find that presence of the $Ctnnb1^{\Delta E3}$ allele led to altered *Pax3* expression (Figure 4b).

2.3 | β-Catenin gain of function exacerbates the effect of *Pax3* mutation on neural crest cell migration

In addition to NTDs, PAX3 loss of function leads to defects in NCC-derived tissues including peripheral nervous system, cardiac outflow tract, melanocytes, and limb musculature (Goulding, Lumsden, &

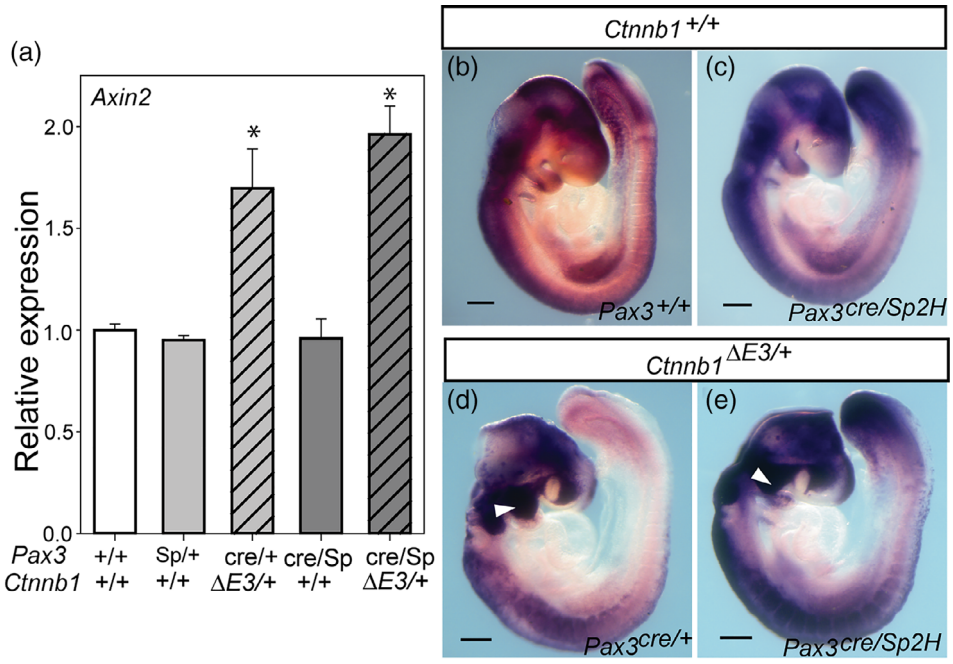

FIGURE 2 Upregulation of *Axin2* in embryos carrying the β-catenin gain of function allele. (a) Expression of *Axin2* was assessed by qRT-PCR using RNA extracted from whole embryos at E10 (22–24 somites). Expression did not differ between wild-type and *Pax3* mutant embryos in the absence of the $Ctnnb1^{\Delta E3}$ allele, whereas embryos in which $Ctnnb1^{\Delta E3}$ was expressed in the Pax3 domain showed significant upregulation of *Axin2* (* significantly different from embryos with $Ctnnb1^{+/+}$ genotype, $p < .05$ ANOVA; $n = 3–4$ per genotype). (b) Whole mount in situ hybridization at E9.5 showed more intense *Axin2* staining in embryos carrying the $Ctnnb1^{\Delta E3}$ allele and this was particularly evident in the pharyngeal arches (arrowhead in d). Scale bar represents 250 μm

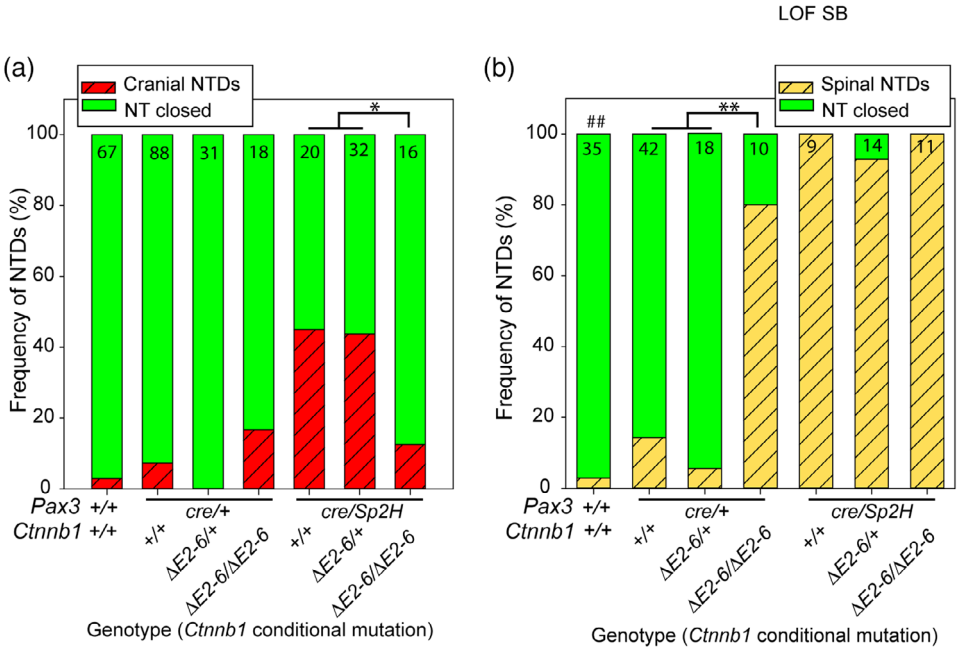

FIGURE 3 β-catenin loss of function has opposing effects on *Pax3*-related cranial and spinal NTDs. Experimental litters were generated by intercross of $Pax3^{Sp2H/+}$; $Ctnnb1^{floxE2-6/+}$ with $Pax3^{cre}$; $Ctnnb1^{floxE2-6/+}$ and analyzed at E9.5–10.5 for (a) cranial and (b) spinal NTDs. In $Ctnnb1^{+/+}$ embryos, NTDs occurred more frequently among $Pax3^{cre/Sp2H}$ than wild-type as expected. (a) Within $Pax3^{cre/+}$ genotype, the frequency of cranial NTDs did not differ significantly with *Ctnnb1* genotype, whereas (b) deletion of *Ctnnb1* in the *Pax3* domain led to a significant increase in spina bifida frequency (**, $p < .01$ *z*-test). (a) $Pax3^{cre/Sp2H}$; $Ctnnb1^{\Delta Ex2-6/\Delta Ex2-6}$ embryos exhibited a lower rate of cranial NTDs than $Pax3^{cre/Sp2H}$ embryos carrying the $Ctnnb1^{+/\Delta Ex2-6}$ or $^{+/+}$ alleles (*$p < .05$; *z*-test). Number of embryos per group is shown in each bar

Paquette, 1994; Greene et al., 2009). We therefore asked whether NCC specification and/or migration were exacerbated by β-catenin gain of function in *Pax3* mutant embryos, by analyzing expression of *ErbB3*, a marker of migrating NCC at E9.5–10.5 (Figure 5). *ErbB3* expression was diminished in $Pax3^{Sp2H/Cre}$ compared with $Pax3^{+/+}$ embryos when wild type for *Ctnnb1* (Figure 5a, f,k compared with d, i, n). The precursors of the dorsal root ganglia (DRG) did form in *Pax3* mutant embryos, but were abnormally small, and segmentation was visible (Figure 5d, i, n). The additional presence of the $Ctnnb1^{\Delta E3}$ allele in $Pax3^{Sp2H/Cre}$; $Ctnnb1^{\Delta E3/+}$ embryos resulted in lack of *ErbB3* staining in the trigeminal

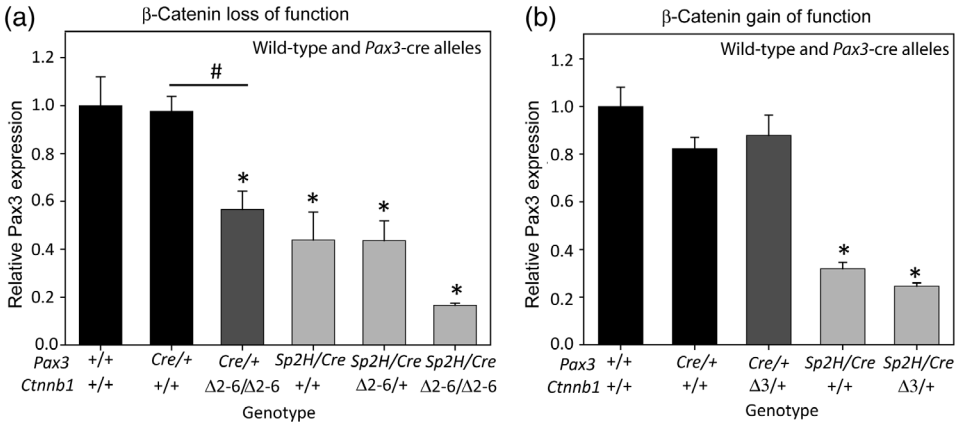

FIGURE 4 Expression from the *Pax3* locus is diminished by β-catenin loss of function but not stimulated by β-catenin gain of function. Expression of wild-type and $Pax3^{cre}$ alleles was compared between genotypes by qRT-PCR among stage-matched embryos (23–24 somite stage) at E10 ($n = 3$–4 embryos per group). (a) Conditional β-catenin loss of function ($Ctnnb1^{\Delta Ex2-6}$) led to a significant decrease in *Pax3* expression (#$p < .05$; ANOVA), whereas (b) conditional gain of function ($Ctnnb1^{\Delta Ex3}$) did not affect *Pax3* expression. Presence of the $Pax3^{Sp2H}$ allele (truncated transcript not detected by this primer pair) led to a significant reduction in *Pax3* transcript as expected (*indicates significant difference from $Pax3^{+/+}$; $Ctnnb1^{+/+}$, $p < .05$; one-way ANOVA)

ganglia, vagus nerve, and prospective DRGs by E10.5 (Figure 5e). Rudimentary and disorganized DRG primordia were evident in $Pax3^{+/Cre}$; $Ctnnb1^{\Delta E3/+}$ embryos at earlier stages (Figure 5c, h) but not by E10.5 (Figure 5m), while embryos of this genotype also showed loss of vagus nerve expression by E10.5 (Figure 5c, h, m).

Development of the NCC-derived peripheral nervous system was further evaluated by immunostaining for β-tubulin III (TuJ1) (Figure 6). Staining in the DRG of $Pax3^{Sp2H/Cre}$ embryos was less defined than in $Pax3^{+/+}$, corresponding with the previously reported diminished size of DRGs (Auerbach, 1954; Conway, Henderson, Anderson, et al., 1997; Conway, Henderson, & Copp, 1997). However, in $Pax3^{+/Cre}$; $Ctnnb1^{\Delta E3/+}$ and $Pax3^{Sp2H/Cre}$; $Ctnnb1^{\Delta E3/+}$ embryos the spinal DRGs showed disrupted patterning, segmentation was poorly defined and the vagus nerve appeared disrupted (Figure 6f compared with e and h compared with g). Hence, gain of function of β-catenin resulted in abnormalities of NCC derivatives, although the presence of *ErbB3* expression until E10 (24–25 somite stage) suggested that some migration did occur.

3 | DISCUSSION

Our findings reveal the potential for a multigenic cause of NTDs involving *Pax3* mutation and dysregulated canonical Wnt signaling. We find a significant deleterious effect of activated β-catenin function on neural tube closure when present in combination with heterozygous or homozygous *Pax3* mutation. This is sufficient to cause a high frequency of cranial NTDs, even though enhanced β-catenin function is present only in the dorsal neuroepithelial component of the neural folds corresponding to the domain recombined in embryos carrying the $Pax3^{cre}$ allele. Notably, conditional knock-out of β-catenin (using the same $Pax3^{cre}$ allele) led to partial rescue of cranial NTDs in *Pax3* null embryos.

Compared with cranial closure, there was a lesser effect of β-catenin gain of function on spinal closure, but further exacerbation of PNP closure defects did occur in homozygous *Pax3* mutants. As previously reported (Zhao et al., 2014), conditional knock-out of β-catenin in the *Pax3* domain is also sufficient to cause spinal NTDs in $Pax3^{cre}$; $Ctnnb1^{\Delta Ex2-6/\Delta Ex2-6}$ embryos. Hence, unlike in the cranial region, a genetic interaction of *Pax3* and either loss or gain of *Ctnnb1* can contribute to spinal NTDs, suggesting differential regulation of closure at cranial and spinal levels.

We did not find evidence that activation of β-catenin is sufficient to positively regulate *Pax3*, whereas reduced *Pax3* expression was found when canonical Wnt signaling was diminished, consistent with previous studies (Zhao et al., 2014). We also confirmed that conditional knock-out of *Ctnnb1* using $Pax3^{cre}$ causes spinal NTDs. These defects do not appear to be solely due to the loss of Wnt signaling, independent of the $Pax3^{cre}$ knock-in allele, as *Pax3* over-expression was found to rescue NTDs in an equivalent genotype (Zhao et al., 2014). Therefore, the reduction in *Pax3* expression that accompanies loss of *Ctnnb1* may contribute to spinal NTDs in double mutants, by further suppression of residual *Pax3* expression. In addition, there may be an additive effect of diminished Wnt signaling and heterozygosity for *Pax3* acting via distinct mechanisms. For example, although mice that are heterozygous for *Pax3* mutations do not develop NTDs, an additive effect with other NTD-predisposing mutations has also been found with genetic cross of the $Pax3^{Sp2H}$ allele into the *curly tail* (*ct/ct*) strain, which led to increased frequency of spina bifida compared with *ct/ct* alone (Estibeiro, Brook, & Copp, 1993).

PAX3-related NTDs have been found to be associated with diminished cell cycle progression, and premature neuronal differentiation, in the dorsal neuroepithelium corresponding to the *Pax3* expression domain (Sudiwala et al., 2019). Cranial NTDs in *Pax3* mutant (Sp^{2H}) embryos can be prevented by folic acid supplementation and exacerbated by maternal folate deficiency (Burren et al., 2008; Copp, Fleming, & Greene, 1998). Notably, rescue of cranial NTDs by supplemental

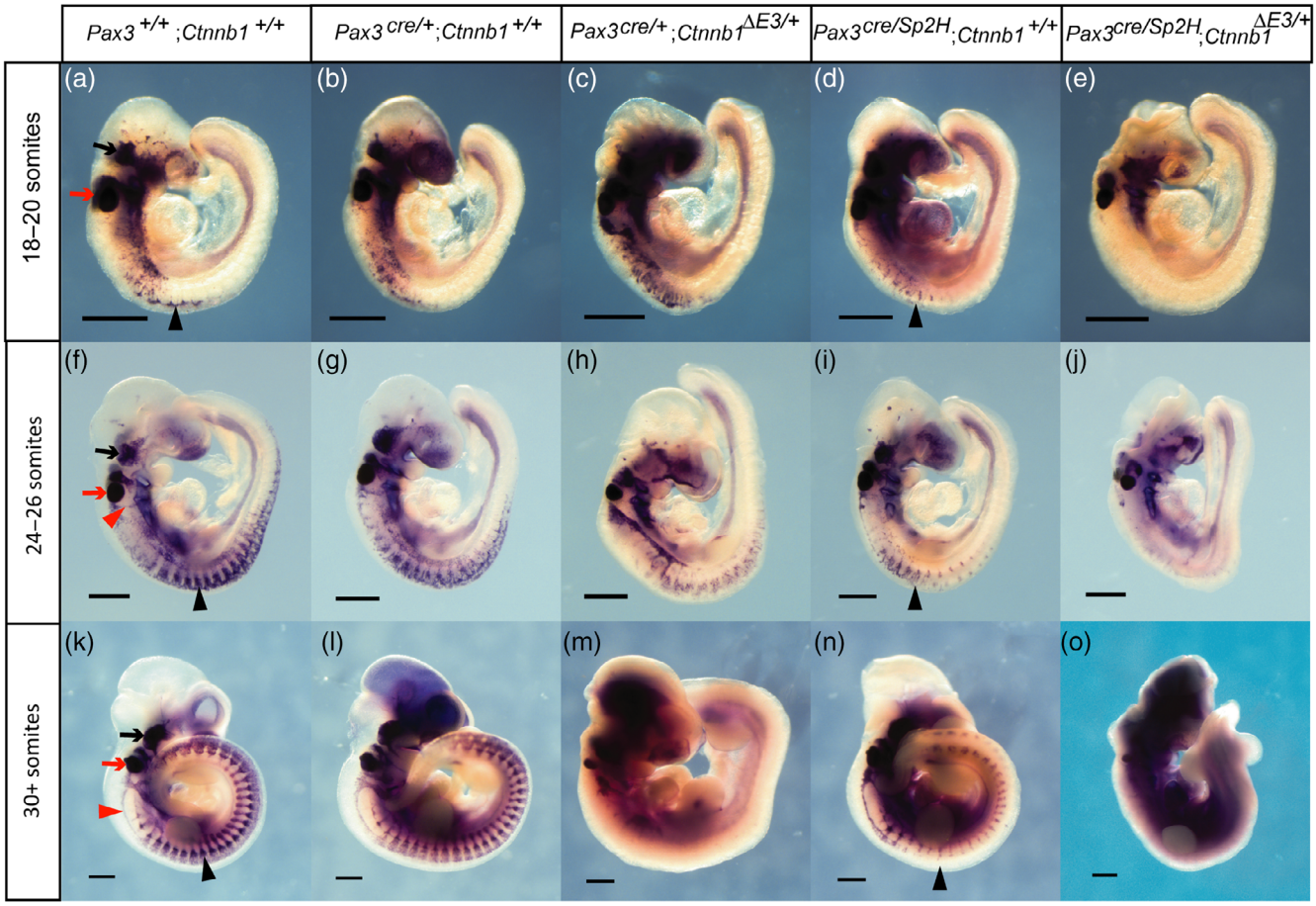

FIGURE 5 β-catenin gain of function disrupts neural crest development as visualized by expression of *ErbB3*. Whole mouse in situ hybridization for *ErbB3* at (a–e) E9.5 (18–20 somites), (f–j) E10 (24–26 somites), and (k–o) E10.5 (around 30 somites). In wild-type embryos, *ErbB3* is expressed in the DRG primordia (black arrowheads), trigeminal ganglia (black arrow), and otic vesicle (red arrow). The pattern is similar in *Pax3* heterozygotes (b, g, l) but staining is less intense in the DRGs of $Pax3^{Sp2H/cre}$ embryos (black arrowheads in d, i, n). Although *ErbB3* expression in the DRGs of $Pax3^{cre/+}$; $Ctnnb1^{\Delta E3/+}$ appeared comparable to wild-type at E9.5 (c), staining was less intense by E10 (h) and absent by E10.5 (m), by which stage expression in the vagus and trigeminal also appeared abnormal. $Pax3^{cre/Sp2H}$; $Ctnnb1^{\Delta E3/+}$ embryos (e, j, o) had even weaker *ErbB3* expression in the developing DRGs and this was absent by E10.5 (o), as was expression in the vagus and trigeminal ganglia. Scale bars represent 500 μm and 2–3 embryos per genotype were analyzed at each stage

folic acid was associated with correction of the proliferation defect, with treated embryos showing enhanced progression through S-phase (Sudiwala et al., 2019). These findings suggest that the causative mechanism for NTDs in *Pax3* mutant embryos involves diminished proliferation in the dorsal neuroepithelium. It will be of interest to determine whether the modulation of NTD frequency by canonical Wnt signaling is also mediated through effects on cellular proliferation.

4 | MATERIALS AND METHODS

4.1 | Mice

The $Pax3^{Sp2H}$ allele carries a 32 bp deletion in exon 5 (encoding the paired-type homeodomain), which generates a premature stop codon encoding a truncated and functionally null protein (Beechey & Searle, 1986). $Pax3^{cre}$ is a functionally null knock-in allele in which *Cre* replaces exon1 (Engleka et al., 2005). β-catenin gain of function was achieved using the $Ctnnb1^{floxE3}$ allele in which exon 3 is flanked by loxP sites. Recombination deletes exon 3, which encodes the GSKβ phosphorylation domain such that $Ctnnb1^{\Delta E3}$ is not targeted for ubiquitination and is stabilized (Harada et al., 1999). Conditional β-catenin loss of function was achieved using $Ctnnb1^{tm2Kem}$ ($Ctnnb1^{floxEx2-6}$), in which recombination of loxP sites flanking exons 2–6 leads to deletion of these exons and creation of a null allele (Brault et al., 2001). The $Sox1^{cre}$ allele carries an in-frame knock-in *cre* into exon 1 of *Sox1* (Takashima et al., 2007). The BAT-Gal reporter was previously described (Maretto et al., 2003). $Pax3^{cre}$, $Ctnnb1^{\Delta E3}$, $Ctnnb1^{flox2-6}$, and $Sox1^{cre}$ were all on a C57BL/6 background. The *splotch* ($Pax3^{Sp2H}$) mice have been maintained as a closed colony for more than 50 generations on a mixed background derived from CBA/Ca, 101 and C3H/He. Compound heterozygotes ($Pax3^{Sp2H/+}$; $Ctnnb1^{flox/+}$) used for experimental matings were the F1 generation (50% C57BL/6).

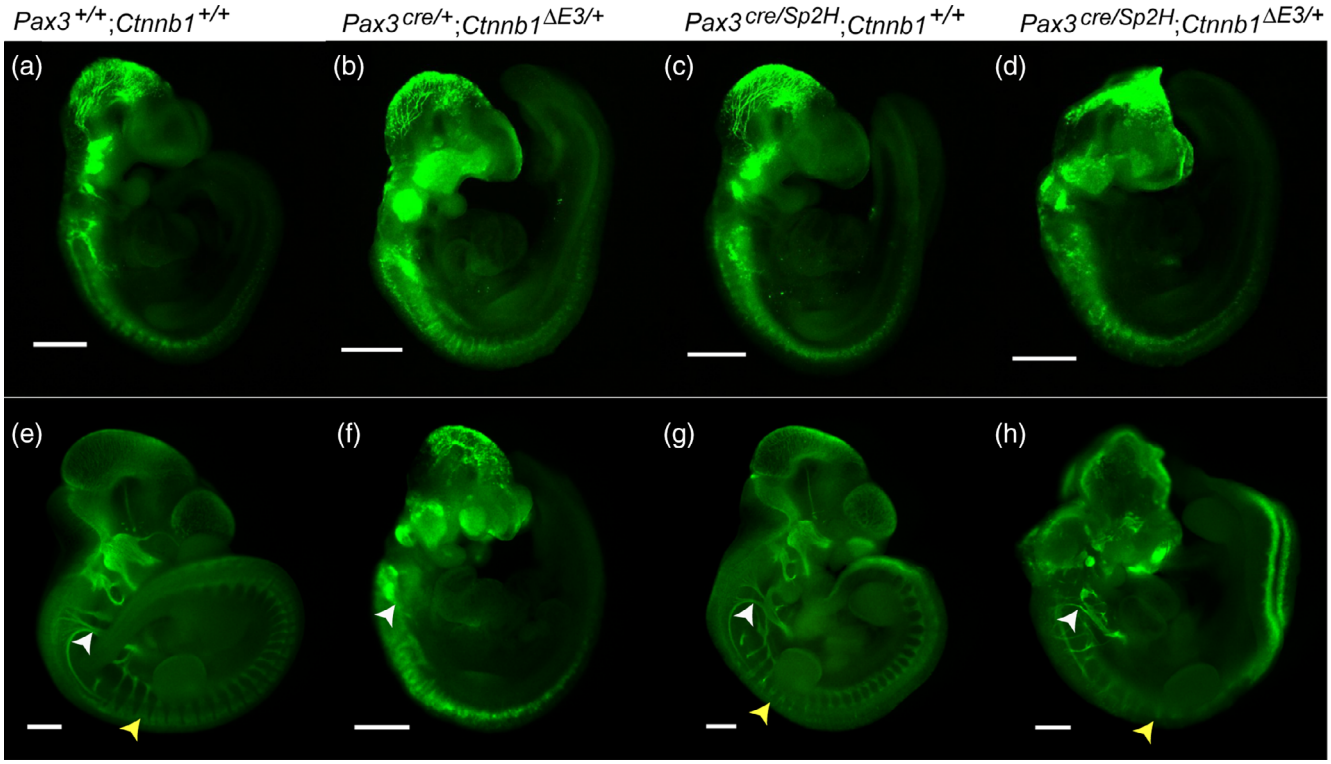

FIGURE 6 Neuronal differentiation is disrupted by β-catenin gain of function. Whole mount immunostaining for β-tubulin III (TuJ1) identifies differentiating neurons at (a–d) E10 (23–24 somites) and (e–h) E10.5 (approximately 30 somites). In wild-type embryos, neuronal differentiation is extensive in the cranial region at E10 and is progressing in a rostral to caudal direction in the spinal region (a). At E10.5 (e), the regular pattern of DRGs is evident in the spinal region (yellow arrowhead) and the vagus nerve is well defined (white arrowhead). In *Pax3*$^{cre/Sp2H}$; *Ctnnb1*$^{+/+}$ embryos (c, g), the vagus nerve is well defined and DRGs form but are smaller except in the caudal region where the neural folds remain open. In contrast, in the presence of *Ctnnb1*$^{\Delta E3/+}$, the pattern of cranial nerves appears disrupted and there is a failure of DRG segmentation, an effect that is more evident at E10.5 (f, h) that E10 (b, d)

Animal studies were carried out under regulations of the Animals (Scientific Procedures) Act 1986 of the UK Government, and in accordance with the guidance issued by the Medical Research Council, UK, in *Responsibility in the Use of Animals for Medical Research (July 1993)*.

4.2 | Collection of embryos and scoring of NTDs

Experimental litters were generated by timed matings. Litters were dissected from the uterus in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Cranial NTDs were scored by the presence of a persistently open neural tube in the cranial region of embryos with 18 or more somites. Spinal NTDs were scored by the presence of an open PNP in embryos with 30 or more somites. Embryos were rinsed in PBS and either frozen at −80, prior to RNA extraction for RT-PCR or fixed in 4% paraformaldehyde (PFA), dehydrated in a methanol series and stored at −20°C prior to in situ hybridization. Embryos were genotyped by PCR of yolk sac genomic DNA.

4.3 | Whole mount in situ hybridization and immunostaining

Whole-mount in situ hybridization (De Castro et al., 2018) was performed using sense and anti-sense digoxygenin-labeled riboprobes for *Axin2* (Andoniadou et al., 2007) and *Erbb3* (Henderson et al., 2001) were generated using a digoxygenin RNA-labeling kit (Roche) and purified on Chroma spin columns (Clontech).

4.4 | Real-time quantitative RT-PCR (qRT-PCR)

RNA was extracted using Trizol reagent (Invitrogen) and used for first strand cDNA synthesis using VILO Superscript cDNA synthesis kit (Invitrogen). qRT-PCR was performed using Mesa Blue qPCR Master Mix Plus for SYBR assay on an ABI 7500 Real-Time PCR machine (Applied Biosystems) with β-actin used as a housekeeping gene to normalize expression (De Castro et al., 2012; De Castro et al., 2018). Primers for amplification of *Axin2* were 5′AAGCCTGGCTCCA GAAGATCACAA and 5′TTTGAGCCTTCAGCATCCTCCTGT. Primers

for amplification of *Pax3* were designed to: (a) exons 5–6, 5′ GGCTTTCGAGAGAACCCACT and 5′ AGGTCTCCGACAGCTGG TAT (to evaluate expression of the wild-type and $Pax3^{cre}$ alleles) and (b) exons 1–2, 5′ GTGCTCGCTTTTTCGTCTCG and 5′ CAGAGGC CTGCCGTTGATAA (to evaluate expression of the wild-type and $Pax3^{Sp2H}$ alleles).

Statistical analysis was performed using Sigmastat version 3.5 (Systat Software). Multiple groups in qRT-PCR experiments were compared by One Way ANOVA with Holm-Sidak post-hoc test.

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CONFLICT OF INTEREST

The authors declare no competing or financial interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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