NEDD4 and NEDD4L regulate Wnt signalling and intestinal stem cell priming by degrading LGR5 receptor

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Abstract

The intestinal stem cell (ISC) marker LGR5 is a receptor for R-spondin (RSPO) that functions to potentiate Wnt signalling in the proliferating crypt. It has been recently shown that Wnt plays a priming role for ISC self-renewal by inducing RSPO receptor LGR5 expression. Despite its pivotal role in homeostasis, regeneration and cancer, little is known about the post-translational regulation of LGR5. Here, we show that the HECT-domain E3 ligases NEDD4 and NEDD4L are expressed in the crypt stem cell regions and regulate ISC priming by degrading LGR receptors. Loss of Nedd4 and Nedd4L enhances ISC proliferation, increases sensitivity to RSPO stimulation and accelerates tumour development in Apcmin mice with increased numbers of high-grade adenomas. Mechanistically, we find that both NEDD4 and NEDD4L negatively regulate Wnt/beta-catenin signalling by targeting LGR5 receptor and DVL2 for proteasomal and lysosomal degradation. Our findings unveil the previously unreported post-translational control of LGR receptors via NEDD4/NEDD4L to regulate ISC priming. Inactivation of NEDD4 and NEDD4L increases Wnt activation and ISC numbers, which subsequently enhances tumour predisposition and progression.

Keywords colorectal cancer; intestinal stem cell; Lgr5; NEDD4; Wnt

Introduction

The intestinal epithelium is constantly self-renewed through a small population of stem cells localised at the bottom of the crypts that continuously regenerate new epithelial cells (Cheng & Leblond, 1974). These ISC are maintained by a Wnt gradient at the intestinal crypts during homeostasis. Wnt signalling pathway plays central role in multiple cellular processes such as stem cell maintenance and cell fate decision (Clevers & Nusse, 2012). Perturbation of this pathway impairs tissue homeostasis, leading to many diseases including cancer and metabolic disorders (MacDonald et al, 2009; Clevers & Nusse, 2012; Novelladsdemunt et al, 2015). Wnt signalling controls the level of the key effector beta-catenin for signal transduction. This is regulated by the cytoplasmic beta-catenin destruction complex which consists of the adenomatous polyposis coli (APC), AXIN, glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1) (Aberle et al, 1997; Kitagawa et al, 1999; Liu et al, 2002). Wnt ligands initiate the signal transduction through binding to two types of cell-surface receptors: the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/LRP6) and the Frizzled (FZD) family of serpentine proteins (MacDonald et al, 2009; Clevers & Nusse, 2012). Apart from Wnt ligands, the four secreted R-spondin (RSPO) proteins have also been previously reported to enhance canonical Wnt signalling in the presence of Wnt ligands (Kazanskaya et al, 2004; Kim et al, 2005; Nam et al, 2006). Following the discovery of the seven-transmembrane receptor leucine-rich repeat containing G protein-coupled receptor 5 (LGR5) as ISC marker (Barker et al, 2007), several studies have further revealed the role of LGR5, and its homologues LGR4 and LGR6, as RSPO receptors for signal enhancement of low-dose Wnt (Carmon et al, 2011; Glinka et al, 2011; de Lau et al, 2011). LGR5 is a Wnt target gene that is enriched in ISCs and colon cancer. It marks adult stem cells in several tissues including the intestinal tract and the hair follicle (Barker et al, 2007; Jaks et al, 2008), while LGR5+ cells are also believed to be the cells-of-origin of intestinal cancer (Barker et al, 2009). Thus, precise control of LGR5 expression is crucial for stem cell homeostasis and tumour suppression. To date, little is known about the post-translational regulation of LGR5 protein stability.

Several E3 ligases have been previously shown to play important role in ISC maintenance and cancer progression. For instance, the RING finger ubiquitin ligases RNF43 and ZNRF3 have been reported as tumour suppressors and negative regulators of ISC by targeting FZD receptors (Carmon et al, 2011; Glinka et al, 2011; de Lau et al, 2011), while the E3 ligase Mule regulates ISC niche and cancer by...
targeting EpHB3 and β-catenin (Dominguez-Brauer et al., 2016, 2017). On the other hand, the role of Neuronal precursor cell developmentally downregulated protein 4 (NEDD4) and its homologue NEDD4L in cancer is controversial. They belong to the NEDD4 family of HECT-type E3 ubiquitin ligases (Rotin & Kumar, 2009). NEDD4 has been reported as a tumour suppressor (March et al., 2011; Zeng et al., 2014; Lu et al., 2016), while others have suggested that NEDD4 is a proto-oncogene by targeting the tumour suppressor PTEN for degradation (Kim et al., 2008; Eide et al., 2013). The latter observation was confounded by another study showing that NEDD4 is dispensable for ubiquitination of PTEN in mammalian central nervous system neurons (Hsia et al., 2014). Interestingly, NEDD4L has also been shown to inhibit Wnt signalling by targeting Dishevelled (Dvl) for proteasomal degradation (Ding et al., 2013).

Here, we investigate the role of NEDD4 and NEDD4L in the context of intestinal homeostasis and tumour development. Through comprehensive analysis of various mouse models, we show that deletion of Nedd4/Nedd4l in the intestinal epithelia increases ISCs and crypt proliferation during homeostasis. Loss of Nedd4/Nedd4l further promotes intestinal tumour progression to high-grade adenomas in Apcmin tumour model. We further demonstrate that both NEDD4 and NEDD4L inhibit Wnt signalling by targeting the RSPO receptor LGR5 for lysosomal and proteasomal degradation. Our data unveil the post-translational regulation of LGR5 by the NEDD4 homologues for ISC priming.

Results

Loss of Nedd4 and Nedd4l increases ISC numbers and crypt proliferation

We first examined the expression pattern of Nedd4 and Nedd4l in murine intestine. RNAscope in situ hybridisation (ISH) showed that Nedd4 was expressed predominantly at the crypt stem cell zone, while Nedd4l was homogenously distributed throughout the crypt–villus axis (Fig 1A). The result was confirmed by quantitative reverse transcription polymerase chain reaction (qRT–PCR) of crypt and villus fractions of intestinal crypts (Fig EV1A). Interestingly, expression of Nedd4 was significantly upregulated in Apcmin adenoma, while Nedd4l expression was unchanged (Fig 1B). This is consistent with the previous observation in human colorectal cancer tissues (Tanksley et al., 2013). The results suggest that Nedd4 expression could be regulated by Wnt signalling.

To investigate the functional role of Nedd4 and Nedd4l in intestinal homeostasis, we generated VillinCreERT2;Nedd4fl/fl (Nedd4 cKO) and VillinCreERT2;Nedd4lfl/fl (Nedd4l cKO) mice to induce gene deletion in intestinal epithelia upon tamoxifen administration. We further generated double-mutant VillinCreERT2;Nedd4fl/fl;Nedd4lfl/fl (DKO) mice to delete Nedd4 and Nedd4l simultaneously. Single- or double-mutant intestines were examined 50 days post-induction (dpi), which showed no significant changes in gross intestinal morphology or crypt proliferation (Fig EV1B). Interestingly, when we let the animals age for 1 year, significant increase in crypt proliferation was observed in the DKO intestine (Fig 1C, D and O) with elongated crypts (Fig 1E, F and P), indicating expansion of proliferative crypt compartment. RNAscope ISH analysis of the DKO intestine further showed increased expression of the ISC marker Olfm4 (Figs 1G, H and Q). Consistently, the number of Cyclin d1- and Sox9-positive cells was also increased in the DKO intestine (Fig 1I, L, R and S), suggesting that Wnt signalling is upregulated. qRT–PCR analysis further confirmed a significant increase in Wnt target genes and stem cell markers expression in the DKO intestine (Fig EV1C).

Nedd4/Nedd4l deficiency activates Wnt signalling and promotes growth advantage in intestinal organoids

To validate the stem cell expansion phenotype, we further examined the intestinal organoids derived from wild-type (WT), Nedd4 cKO, Nedd4l cKO and DKO animals at 7dpi. Loss of Nedd4 or Nedd4l was confirmed by qRT–PCR (Fig EV2A). Surprisingly, significant upregulation of Wnt target genes (Fig 2A) and stem cell markers (Fig 2B) was observed in all mutant organoids at short-term gene deletion (7dpi) as compared to 1 year in vitro. There was also a remarkable increase in proliferation (Fig 2C) and organoid formation efficiency in the mutant organoids (Figs 2D and EV2B), supporting the notion that Nedd4 or Nedd4l deletion increases numbers of ISCs. We speculate that the accelerated phenotypes in organoids may be attributed to the growth factor–enriched culture condition in vitro. To test whether the growth advantage of the mutant organoids is dependent on exogenous Wnt signal, we further challenged the organoids by reducing the amount of the Wnt agonist RSPO from the culture media, which is an essential component of the organoid media (Sato et al., 2009). Neither WT nor mutant organoids survived in the absence of RSPO. On the other hand, in the RSPO-low (1%) condition, most WT organoids died at day 3 while the mutant organoids were able to survive longer (~day 7–8) (Fig 2E and F). The results indicate that loss of Nedd4/Nedd4l results in organoid growth advantage upon RSPO depletion, while the survival of mutant organoids is still dependent on exogenous RSPO.

Loss of Nedd4 and Nedd4l exacerbates Apcmin intestinal tumour phenotype with increased tumour grade

Nedd4 has been previously reported to enhance growth of Apcmin intestinal tumours, which required deletion of Nedd4 in both intestinal epithelium and the surrounding tissues (Lu et al., 2016). The role of Nedd4 in colorectal cancer (CRC) remains uncharacterised. To clarify the role of Nedd4 and Nedd4l in intestinal tumorigenesis, we further crossed the mutant mice to Apcmin animals, a mouse model of colon cancer (Su et al., 1992), to obtain Apcmin Nedd4 cKO, Apcmin Nedd4l cKO, and the compound Apcmin DKO mice. While VillinCreERT2; Apcmin (designated Apcmin control) mice lived for approximately 5 months (151 days on average), Apcmin Nedd4l cKO and Apcmin DKO showed signs of sickness significantly earlier at around 4 months (125 and 126 days on average, respectively) (Figs 3A and EV3A). The survival rate of Apcmin Nedd4 cKO animals was also decreased but not significant (140 days on average) (Fig EV3A). Apcmin mice with single or double deletion of Nedd4 and Nedd4l exhibited an increase in tumour numbers in small intestine, while Apcmin DKO animals further displayed increased numbers of colonic tumours (Fig 3B and D). Histology
**Figure 1. Loss of Nedd4 and Nedd4l increases intestinal crypt proliferation.**

A, B Representative images of RNAscope ISH showing Nedd4 and Nedd4l expression in mouse small intestinal tissues derived from normal animal (A) and Apcmin tumours (B). High-magnification images are shown in the box. Scale bars, 50 µm (A), 100 µm (B).

C–N Histology and immunostaining of VillincreERT2 wild-type (WT) (C, E, G, I, K, M) and VillincreERT2-Nedd4fl/fl,Nedd4lfl/fl DKO (D, F, H, J, L, N) proximal intestine 1-year post-tamoxifen induction using the indicated markers (n = 3 per group). Scale bars, 50 µm.

O Quantitation of Edu+ proliferating cells per crypt from [C, D]. Each dot represents the average of at least 10 crypts per animal. Data are mean ± standard error. n = 3 per group.

P Quantitation of crypt length (µm) from (E, F). Each dot represents the average of at least 20 crypts per animal. Data are mean ± standard error. n = 3 per group.

Q–T Quantitation of Olfm4 (Q), Cyclin D1 (R), Sox9 (S) and lysozyme (T)-positive cells per crypt. Each dot represents the average of at least 20 crypts per animal. Data are mean ± standard error. n = 3 per group. Error bars represent ± SEM.

Data information: P values were determined using the unpaired two-sided t-test (*P < 0.05; **P < 0.01; ns, not significant).
analysis revealed that most adenomas were low-grade dysplasia, whereas Apc<sup>min</sup> Ned4 cKO and Apc<sup>min</sup> DKO further promoted high-grade dysplasia (Fig 4A–C). In contrast to the previous study (Lu et al., 2016), our data show that deletion of Ned4 and/or Ned4l in the intestinal epithelium alone is sufficient to accelerate adenoma development in Apc<sup>min</sup> animals. Of note, loss of Ned4l appears to be more effective in tumour progression than Ned4 ablation.

Since deletion of Ned4 and Ned4l increases ISC numbers under homeostasis, we further examined the ISC marker Lgr5...
expression in control and mutant adenomas. Consistently, loss of Nedd4 and/or Nedd4l resulted in an increase in Lgr5-expressing stem cells in the adenomas (Fig 4D–G). In addition, the number of lysozyme+ Paneth cells was also significantly increased in mutant adenomas as compared to the Apc min control (Figs 4H–K and EV3B). Next, we asked if the tumour-promoting role of Nedd4 and Nedd4l was associated with Wnt signalling. Immunohistochemistry analysis showed strong nuclear β-catenin staining in all control and mutant adenomas (Fig EV3C–F). On the other hand, the Wnt target gene Sox9 expression was significantly upregulated in all mutant adenomas when compared to control (Fig EV3G–J and O), suggesting that Wnt signalling is upregulated upon Nedd4 and Nedd4l loss. In addition, adenomas with single or double deletion of Nedd4 and Nedd4l further displayed increased proliferation as indicated by Edu+ cells (Figs 4L–O and EV3P), while apoptosis was not affected (Fig EV3K–N). Together, we conclude that loss of Nedd4 and Nedd4l in Apc min animals promotes intestinal tumour progression by enhancing Wnt activation with increased numbers of ISCs and Paneth cells.

The E3 ligases NEDD4 and NEDD4L negatively regulate Wnt signalling upstream of the β-catenin destruction complex

To study the Wnt regulatory role, we first deleted NEDD4 or NEDD4L via CRISPR targeting in HEK293T cells. Loss of NEDD4 or NEDD4L protein was confirmed by Western blot analysis (Fig EV4A). This resulted in significant increase in TCF-transcriptional (TOPFlash) activity in all the mutant cells in both Wnt3a and Wnt3a plus RSPO conditions (Figs 5A and EV4B). NEDD4L has been previously reported as Wnt negative regulator by targeting DVL for degradation (Ding et al, 2013). We asked if NEDD4 also contributed to DVL degradation. Interestingly, our data showed that overexpression of either NEDD4 or NEDD4L induced the degradation of DVL2 but not DVL1/3 (Figs 5B and EV4C). Immunoprecipitation (IP) analysis further showed that WT NEDD4, but not the catalytic inactive mutant C854S (NEDD4-CS), promoted DVL2 ubiquitination (Fig EV4D). These results indicate that both NEDD4 and NEDD4L play redundant roles in targeting DVL2 for proteasomal degradation.
Since our organoid data showed that Nedd4 and Nedd4l mutants exhibited growth advantage upon RSPO depletion, we asked if Nedd4/Nedd4l also play a role in the RSPO axis of the Wnt pathway. Ectopic expression of either NEDD4 or NEDD4L significantly suppressed the TOPFlash activity induced by Wnt3a and RSPO treatment (Fig 5C). To test whether this Wnt inhibitory role is DVL2-dependent, we further deleted DVL2 in HEK293T cells (Fig EV4E). Surprisingly, NEDD4 and NEDD4L were still able to suppress Wnt activity in the absence of DVL2 (Fig 5D), suggesting that there might be additional player for NEDD4/4L-mediated Wnt inhibition. To further dissect how NEDD4 and NEDD4L regulate Wnt signalling, we assessed their inhibitory effect using different cell lines carrying different mutations in the Wnt pathway. Similar to HEK293T cells, expression of NEDD4 and NEDD4L in the CRC cell line HCT116 strongly suppressed Wnt pathway activation (Fig 5E). On the other hand, ectopic expression of NEDD4 and NEDD4L in APC4 (HEK293T cells with APC truncation at 1,225a.a.) (Novellasdemunt et al., 2017) and DLD1 (APC-mutated CRC cells at 1,417a.a.) cells failed to inhibit Wnt signalling (Fig 5F and G). HCT116 cells have WT APC but heterozygous β-catenin mutation. Importantly, HCT116 cells have been reported to express Wnt ligands for autocrine Wnt signalling (Bafico et al., 2004).
These data suggest that NEDD4 and NEDD4L inhibit Wnt signalling upstream of the APC/β-catenin destruction complex, likely at the surface receptor level.

**NEDD4 and NEDD4L target LGR5 receptor for lysosomal and proteasomal degradation**

We speculate that NEDD4 and NEDD4L may inhibit Wnt signalling by targeting Wnt receptors for degradation. To test our hypothesis, we examined the effect of NEDD4/NEDD4L expression on the common canonical Wnt receptors FZD4 and FZD5, and the two RSPO receptors LGR4 and LGR5. Our data showed that over-expression of NEDD4 and NEDD4L WT constructs, but not their catalytic inactive mutants (NEDD4-CS and NEDD4L-CA), resulted in a marked reduction of LGR4 and LGR5 protein levels (Figs EV5A and 6A). In contrast, expression of NEDD4 and NEDD4L did not alter the protein levels of FZD4 or FZD5 Wnt receptors (Figs EV5B and 5C). Interestingly, two bands of LGR5-Flag proteins were noted in the immunoblot: the lower band for the immature unprocessed ER form and the upper band for the mature, glycosylated post-Golgi form (Fig 6A). The NEDD4/NEDD4L-mediated degradation appeared to happen predominantly in the upper mature form. To confirm these findings, we co-expressed HEK293T cells with NEDD4/NEDD4L and LGR5 that carried a SNAP-tag (SNAP-LGR5) in its extracellular domain. SNAP-LGR5 labelling with membrane-impermeable SNAP-Alexa488 showed its localisation at the cell surface (Fig 6B). Notably, expression of WT NEDD4 or NEDD4L strongly reduced this cell-surface pool of LGR5 signal, while the signal was largely unaffected in NEDD4-CS and NEDD4L-CA mutant-expressing cells (Figs 6B and EV5E). This is consistent with the immunoblot data that only mature form of LGR5 at the membrane level was being degraded by NEDD4 and NEDD4L, while the total LGR5 level (mostly cytoplasmic as immature form in the biosynthetic pathway such as ER) was unaffected. On the other hand, expression of NEDD4 and NEDD4L did not alter the surface levels of SNAP-FZD5 (Figs EV5D and 5E), which is in concordance with our earlier observation (Fig EV5C). Thus, our data support the notion that NEDD4 and NEDD4L selectively target LGR but not FZD receptors for degradation.

Next, we examined the effect of NEDD4 and NEDD4L on LGR5 protein turnover. Interestingly, LGR5 protein was highly unstable. Significant degradation of total LGR5 protein was detected as soon as 1 h after cycloheximide (Chx) treatment in control cells, which was stabilised in NEDD4 and NEDD4L CRISPR KO cells (Fig EV5F). This confirmed the increase in the endogenous Lgr5-HA protein level in the mouse organoids (Appendix Fig S1D). Western blot analysis confirmed the increase in the endogenous Lgr5-HA protein level in the DKO organoids when compared to WT upon RSPO3 administration (Fig 7A). Taken together, these results support the notion that the E3 ubiquitin ligases NEDD4 and NEDD4L target LGR5 for lysosomal degradation via their E3 ligase activities (Figs EV5G and 5H). To determine whether proteasomal degradation is also involved, we treated the cells with proteasomal inhibitor MG132 and repeated the IP experiment. Strikingly, expression of WT NEDD4, but not the mutant, resulted in robust accumulation of ubiquitinated form of LGR5 (Fig 6C). Similar results were observed using NEDD4L overexpression (Fig 6D), indicating that both NEDD4 and NEDD4L mediate proteasomal degradation of LGR5. In a reverse experiment, we examined LGR5 ubiquitination in the CRISPR-mediated NEDD4 and NEDD4L KO cells. Loss of NEDD4 or NEDD4L displayed a profound reduction in LGR5 ubiquitination, supporting the notion that LGR5 ubiquitination requires NEDD4/NEDD4L (Fig 6E). Together, our results indicate that NEDD4 and NEDD4L target LGR5 for both lysosomal and proteasomal degradation.

Next, we examined if NEDD4 and NEDD4L interact with their substrates. Co-IP analysis showed that NEDD4 and NEDD4L indeed bound to LGR4 and DVL2 at the endogenous level (Appendix Fig S1A). Consistently, endogenous LGR4 and DVL2 protein levels were stabilised in the NEDD4 or NEDD4L CRISPR KO cells (Appendix Fig S1B), indicating that they are the substrates for the NEDD4 E3 ligases. Unfortunately, we were not able to demonstrate LGR5 stabilisation at the endogenous level due to the lack of reliable antibodies. To validate that the NEDD4/NEDD4L-mediated Wnt regulation is dependent on LGR4/5, we further performed siRNA knockdown (KD) assays in the NEDD4 and NEDD4L CRISPR KO cells. Loss of LGR4/LGR5 abrogated the Wnt activation in the NEDD4/NEDD4L KO cells, indicating that the NEDD4/NEDD4L-mediated Wnt regulation is indeed dependent on LGR4/5 (Fig 6F and Appendix Fig S1C). To further study the endogenous Lgr5 protein level in the absence of reliable antibodies, we introduced HA tag to the endogenous Lgr5 locus (Lgr5-HA tag) in WT and DKO mouse organoids (Appendix Fig S1D). Western blot analysis confirmed the increase in the endogenous Lgr5-HA protein level in the DKO organoids when compared to WT (Appendix Fig S1E). Taken together, these results support the notion that the E3 ubiquitin ligases NEDD4 and NEDD4L target LGR4, LGR5 and DVL2 for degradation.

**Loss of Nedd4 and Nedd4l increases sensitivity to RSPO stimulation**

Given our observation that Nedd4 targets the RSPO receptor Lgr5 for degradation, we asked if deletion of Nedd4 and/or Nedd4l in vivo will synergise RSPO signalling. Tamoxifen was injected into WT and mutant animals 7 days prior RSPO3 injections for 3 days (Fig 7A). Injection of RSPO3 alone in WT animals resulted in increased crypt proliferation, while deletion of Nedd4 and/or Nedd4l further synergised the RSPO-induced hyperproliferation (Fig 7B and Appendix Fig S2A). Consistently, the population of Oflm4+ ISCs was further expanded in Nedd4l cKO and DKO intestine when compared to WT upon RSPO3 administration (Fig 7C and Appendix Fig S2B). The results indicate that deletion of Nedd4 or Nedd4l in vivo leads to hypersensitivity to RSPO stimulation. It is interesting to note that loss of Nedd4 and Nedd4l alone did not show ISC expansion phenotype until 1 year after deletion in vivo (Figs 1 and E1). In contrast, hyperproliferation of intestinal crypts and ISC expansion were observed as early as 10 dpi under RSPO stimulation, supporting the notion that Nedd4 and Nedd4l regulate RSPO-LGR axis of the Wnt pathway.
Figure 5. NEDD4 and NEDD4L negatively regulate Wnt signalling pathway.

A Relative TOPFlash reporter activities of HEK293T cells with the indicated CRISPR targeting. Cells were treated with Wnt3a-conditioned media.

B Western blot analysis of HEK293T cells transfected with Flag-DVL1, Flag-DVL2 or Flag-DVL3 with or without Myc-NEDD4 using the indicated antibodies.

C Relative TOPFlash reporter activity upon ectopic expression of the indicated plasmids in HEK293T cells treated with Wnt3a and RSPO.

D Relative TOPFlash reporter activities of HEK293T cells with CRISPR deletion of DVL2 upon ectopic expression of the indicated plasmids. Cells were treated with Wnt3a and RSPO.

E–G Relative TOPFlash reporter activities of HCT116 (E), APC4 (Novellasdemunt et al, 2017) (F) and DLD1 (G) cells transfected with the indicated plasmids.

Data information: Data represent mean ± standard error of at least three independent experiments. P-values were determined using the unpaired two-sided t-test (*P < 0.05; **P < 0.01; ***P < 0.001).
Figure 6.
Discussion

The bona fide ISC marker LGR5 is expressed exclusively at the intestinal crypt base under tight regulation, while LGR5+ ISCs are indispensable for regeneration and tumourigenesis (Metcalf et al., 2014; de Sousa e Melo et al., 2017). Transcriptional control of LGR5 by Wnt, ASCL2 and BMP has been reported in the past (van der Flier et al., 2009; Schuijers & Clevers, 2012; Qi et al., 2017), yet the regulation of LGR5 protein turnover is largely unknown. To our knowledge, this is the first study describing the post-translational modification of LGR5 receptors via the E3 ubiquitin ligases NEDD4 and NEDD4L.

Ubiquitin modification has been shown to drive cell-surface receptor internalisation and lysosomal degradation (Haglund & Dikic, 2012). For instance, RNF43 and ZNRF3 decrease Wnt signals by ubiquitinating FZD receptors (Hao et al., 2012; Koo et al., 2012), while deubiquitination has also been shown to play an important role in determining the surface level of FZD by recycling the receptor to the plasma membrane (Mukai et al., 2010). Indeed, NEDD4 has been previously shown to target a number of growth factor receptors for internalisation and degradation (Katz et al., 2002; Murdaca et al., 2004; Persaud et al., 2011; Huang et al., 2015). The current study provides compelling evidence that NEDD4 and NEDD4L function as Wnt negative regulators by targeting LGR5 receptor for lysosomal and proteasomal degradation (Fig 7D). We demonstrate that both NEDD4 and NEDD4L selectively degrade DVL2 but not DVL1/3. More importantly, we uncovered a new DVL-independent Wnt regulatory role of NEDD4 and NEDD4L by mediating the RSPO receptor LGR5 degradation. Our results further show that loss of Nedd4 and Nedd4l increases ISC numbers and sensitivity to RSPO stimulation, implicating that NEDD4/NEDD4L may contribute to the ISC priming (Yan et al., 2017b) by targeting LGR5 for degradation. RSPO potentiates Wnt signalling by forming a complex with LGR4/5 and RNF43/ZNRF3 to neutralise the RNF43/ZNRF3-mediated FZD degradation (de Lau et al., 2014). It will be interesting to further investigate the potential overlapping or distinct roles between RNF43/ZNRF3 and NEDD4/NEDD4L in Wnt activation and tumourigenesis. The selective regulatory role of NEDD4/NEDD4L to LGR over FZD receptors further supports two distinct negative regulatory mechanisms of Wnt and RSPO signalling: ZNRF3/RNF43 for targeting FZD receptors and NEDD4L4L for targeting LGR receptors.

The role of NEDD4 and NEDD4L in cancer progression has been controversial. NEDD4 has been reported as both tumour suppressor and oncosgene (Kim et al., 2008; March et al., 2011; Eide et al., 2013; Zeng et al., 2014; Lu et al., 2016), while the role of NEDD4L in cancer is largely unknown. Our current study provides the first comprehensive analysis of both Nedd4 and Nedd4l in intestinal tumourigenesis. Our data demonstrate that both Nedd4 and Nedd4l exacerbate Apcmin tumour phenotype, indicating that they are both tumour suppressors. Unlike the previous report showing that Nedd4-deficient tumour phenotype requires surrounding microenvironment (Lu et al., 2016), our results show that deletion of Nedd4/Nedd4l in the intestinal epithelium alone is sufficient to enhance Apcmin tumour growth with significant increase in ISC numbers.

Previous studies have identified several crypt-expressing Wnt inhibitors such as AXIN2, RNF43 and SH3BP4 that are involved in CRC development by targeting Wnt signalling pathway at different subcellular levels (Cancer Genome Atlas, 2012; Giannakis et al., 2014, Yan et al., 2017a; Antas et al., 2019). In this study, we uncover a new crypt-expressing Wnt regulator NEDD4 that targets two Wnt pathway components LGR4/5 and DVL2 to maintain ISC homeostasis and suppress intestinal tumourigenesis (Fig 7D). These findings highlight the complexity and importance of multi-level regulation of the pathway to fine-tune the Wnt signal strength at the crypt and to determine the cellular responses. It is interesting to note that deletion of Nedd4 and Nedd4l was able to exacerbate Apcmin phenotype despite the observation that Nedd4/Nedd4l inhibit Wnt signalling upstream of APC. The data imply that upregulation of Rspo-Lgr5 signalling upon Nedd4/Nedd4l loss increases tumour predisposition and progression in Apcmin animals by promoting Wnt activation and ISC self-renewal (Yan et al., 2017b). On the other hand, given that NEDD4/4L target both LGR receptors and DVL2 for Wnt regulation, the Wnt activation and stem cell increase in the Nedd4/Nedd4l-deficient intestine is likely caused by both Lgr5 and Dvl2 stabilisation. Despite the clear evidence showing the Lgr5-dependent role in the siRNA assay and the enhanced sensitive to RSPO stimulation in vivo, it is admittedly difficult to dissect whether the enhanced stem cell and tumour phenotypes in the mutant intestine are caused by Lgr5 or Dvl2 or both.

In conclusion, our work shows that NEDD4 and NEDD4L are crypt-expressing tumour suppressors by targeting the RSPO receptor LGR5 and DVL2 for degradation. Interestingly, inactivating mutations and deletions of NEDD4 and NEDD4L have also been reported in human CRCs (Cancer Genome Atlas, 2012) (Appendix Fig S2C), while low NEDD4L expression has been associated with poor prognosis (Tanksley et al., 2013). Indeed, expression of NEDD4L was profoundly downregulated in CRCs (Appendix Fig S2D),
Figure 7. Loss of Nedd4 and Nedd4l enhances ISC expansion upon RSPO stimulation.

A Experimental design of RSPO3 stimulation model.
B, C Representative images of EdU (B) and Olfm4 (C) staining in intestinal tissues obtained from the indicated genotypes. Scale bar, 50 µm.
D Model of proposed mechanism of NEDD4/NEDD4L-mediated regulation of Wnt pathway. NEDD4 and NEDD4L target two Wnt pathway components: (i) LGR4/5 receptor for lysosomal and proteasomal degradation, and (ii) DVL2 for proteasomal degradation.
suggesting that inhibition of the tumour suppressors NEDD4/ NEDD4L may be an alternative Wnt activating mechanism for some CRCs. Since NEDD4 is expressed in the crypt/stem cell region, it may imply a new negative feedback regulation of Wnt signalling pathway by degrading LGR5 and DVL2 to control the Wnt signal strength in the intestinal crypt.

Materials and Methods

Antibodies
β-catenin (610154, BD), caspase-3 (9661L, Cell Signalling), Cyclin D1 (2978S, Cell Signalling), DVL2 (1085) (sc-8026, Santa Cruz), GAPDH (sc-47724, Santa Cruz), Flag (F3165, SIGMA), HA (Y-11) (sc-7392, Santa Cruz), LGR4 (C-12) (sc-390630, Santa Cruz), Lysosome (A0099, Dako), c-Myc (9E10) (sc-40, Santa Cruz), NEDD4 (sc-7392, Santa Cruz), LGR4 (C-12) (sc-390630, Santa Cruz), Lysozyme (A0099, Dako), c-Myc (9E10) (sc-40, Santa Cruz), NEDD4L (4013S, Cell Signalling), SNAP (P9310S, NEB), Sox9 (AB5335, Millipore) and V5 (ab27671) were used in immunohistochemistry, immunoprecipitations or Western blot analysis.

Plasmids and other reagents
Full-length NEDD4 was a kindly gift from Igor V. Korobko (Kalini-chenko et al., 2012), and NEDD4L was a kindly gift from Olivier Staub (Oberfeld et al., 2011). Full-length human NEDD4, NEDD4L and LGR4 and LGR5 were cloned into XhoI site of pcDNA-Flag, pcDNA-HA or pcDNA-MYC vectors. Various mutants were generated by PCR into pcDNA-MYC or pcDNA-HA. Mutants include NEDD4-CS (C854S) and NEDD4L-CA (C962A). pRK5-HA-ubiquitin-ated by PCR into pcDNA-MYC or pcDNA-HA. Mutants include LGR4 and LGR5 were cloned into XhoI site of pcDNA-Flag, pcDNA-HA or pcDNA-MYC vectors. Various mutants were generated by PCR into pcDNA-MYC or pcDNA-HA. Mutants include NEDD4-CS (C854S) and NEDD4L-CA (C962A). pRK5-HA-ubiquitin-ated by PCR into pcDNA-MYC or pcDNA-HA. Mutants include LGR4 and LGR5 were cloned into XhoI site of pcDNA-Flag, pcDNA-HA or pcDNA-MYC vectors. Various mutants were generated by PCR into pcDNA-MYC or pcDNA-HA. Mutants include

Gene editing with CRISPR/Cas9 system
To generate NEDD4 and NEDD4L mutants, HEK293T cells were transfected with plasmids encoding Cas9 (41815, Addgene) modified to add puromycin resistance and guideRNAs (gRNAs) (gRNA-GFT-T1 was, #41819, Addgene) as previously described (Novellasademunt et al, 2017). For NEDD4 targeting, gRNA1, 5′-AAGTCCCGCAGTCACCAAT-3′ or gRNA2, 5′-GACTCTTACCGGAGAATTAT-3′ was used. For NEDD4L targeting, gRNA1, 5′-TATGGACATTTCGAAAGACGA-3′ or gRNA2, 5′-AAGAGATTTCAACCCTAC-3′ was used.

To generate DVL2 CRISPR mutants, HEK293T cells were transfected with plasmids encoding Cas9 (pSpCas9(BB)-2A-Puro (PX459) V2.0) in which we introduced our guideRNA (gRNA). This plasmid was a gift from Feng Zhang. For DVL2, gRNA1, 5′-GACCAAGGT-GATTTACCACC-3′ was used. The gRNAs were targeting specific genomic loci. We screened the potential targeted cells by immunoblotting and then confirmed by genomic DNA sequencing.

SNAP-surface immunofluorescence
HEK293T cells were grown on laminin-coated glass coverslips. Cells were co-transfected with 100 ng SNAP-LGR5 or SNAP-FZD5 and 150 ng myc-NEDD4, HA-NEDD4-CS, myc-NEDD4L, HA-NEDD4L-CA or pcDNA4 as a control with Fugene according to the manufacturer’s instructions. After 24 h of transfection, cells were labelled with 1 μM SNAP-Surface Alexa 488 (NEB) for 15 min at 4°C to block endocytosis. Cells were then immediately washed with fresh RPMI media and fixed in 4% paraformaldehyde. Cells were incubated with primary antibodies NEDD4 (Santa Cruz) or NEDD4L (Cell Signaling) for 1 h at RT followed by a secondary antibody conjugated to Alexa-568 (Invitrogen) and DAPI (Sigma) in 2% BSA-PBS (Roche). Cells were mounted in Prolong Diamond (Life technologies) and imaged using a DeltaVision Core system.

Real-time quantitative RT–PCR
RNA was extracted according to the manufacturer’s instructions (Qiagen RNAeasy). cDNA was prepared using Maxima first strand cDNA synthesis (#1672, Thermo Scientific). Quantitative PCR detection was performed using iQ SYBR Green Supermix (#172-5121, Bio Rad) using specific primers to: mAscl2: F: 5′-GGAAGCCCAAGTTTACCAGC-3′; mLgr5: F: 5′-GGCTCATCATCTTGGCATCT-3′; mCD44: F: 5′-GGCTCATCATCTTGGCATCT-3′; mAxin2: F: 5′-GATTTACCACC-3′. The gRNAs were targeting specific genomic loci. We screened the potential targeted cells by immunoblotting and then confirmed by genomic DNA sequencing.
When indicated, cells were pre-treated with 10 nM Bafilomycin A1 overnight or 10 μM MG132 proteasome inhibitor for 4 h prior to lysis collection. Cells were washed and collected with cold PBS and lysed in cold lysis buffer containing 150 mM NaCl, 30 mM Tris (pH 7.5), 1 mM EDTA, 1% Triton X-100, 10% glycerol, 0.5 mM DTT, protease and phosphatase inhibitor cocktail (Thermo Scientific, 78446). After clarification by centrifugation (18,800 g for 30 min at 4°C), the cellular lysates were pre-cleared with IgG-agarose beads (Millipore, 16-266) for 1 h at 4°C and the supernatants were immunoprecipitated with the indicated antibodies or anti-Flag-M2 affinity beads (A220, Sigma) at 4°C overnight. Immunocomplexes were washed with cold lysis buffer six times, resuspended in SDS sample buffer, and subjected to SDS-PAGE and Western blot analysis.

IP under denaturing conditions

When indicated, indicated, cells were treated with 10 μM MG132 proteasome inhibitor for 4 h prior to lysis collection. Cells were collected in denaturing lysis buffer (1% SDS, 5 mM EDTA, 10 mM DTT, 5 mM NEM, protease and phosphatase inhibitor cocktail (Thermo Scientific, 78446)). Then, cells were mixed by vortexing for 2–3 s at maximum speed and the samples were boiled at 95°C for 5 min to denature. The cells suspension was then diluted 1/10 in the IP lysis buffer, and the lysate was passed through a needle several times to fragment the DNA followed by immunoprecipitation protocol as described before.

Immunohistochemistry and Edu staining

For analysis of small intestine and colon by immunohistochemistry and Edu staining, tissues were fixed in 10% formalin and embedded in paraffin. For small intestinal tissues, same proximal part of the small intestine from all genotypes was used throughout the study. Immunohistochemistry was performed as described (Novellasdemunt et al., 2017). The buffers used for antigen retrieval were citrate (Srex and Caspase-3) or Tris-EDTA (β-catenin and lysozyme). Edu staining was performed following manufacturer’s instructions (C10338, Invitrogen).

RNAscope in situ hybridisation

In situ hybridisation (ISH) for Lgr5, Olfm4, Nedd4 and Nedd4l was performed using the RNAscope FFPE assay kit (Advanced Cell Diagnostics) according to the manufacturer’s instructions. Briefly, 4 μm formalin-fixed, paraffin-embedded tissue sections were pre-treated with heat and protease digestion before hybridisation with the target probe. Then, an HRP-based signal amplification system was hybridised to the target probes (Lgr5, 312171; Olfm4, 3111831) before colour development with 3,30-diaminobenzidine tetrahydrochloride (DAB). 20 crypts from 3 mice per group were used to quantitate the number of Olfm4+ cells.

Intestinal organoid culture

Organoids were established from freshly isolated wild-type, Nedd4 cKO, Nedd4l cKO and DKO small intestine. Tissues were incubated in cold PBS containing 2 mM EDTA for isolating epithelial crypts and culture as previously describe (Sato et al., 2009) except that Matrigel was replaced with Cultrex® BME, Type 2 RGF PathClear (Amsbio 3533-010-02). In brief, the organoid basal media contain EGF (Invitrogen PMG8043), Noggin and R-spondin (ENR) (5%). For the R-spondin withdrawal experiment, when indicated, R-spondin was used at 1%. Noggin and R-spondin-conditioned media (CM) were generated from HEK293T cells. For all the other organoid experiments, Intesticult™ Organoid Growth media (06005, Stem Cell Technologies) was used. The Rho kinase inhibitor Y-27632 (Sigma) was added to the culture when trypsinised.

Edu staining in organoids

Organoids were grown in 15 μl of RGF BME into an 8-well chamber side (Lab-Tek II, 154534). When indicated, 10 μM Edu was added to the growth media for 2 h before fixing. Edu staining was performed as described (Novellasdemunt et al., 2017).

Organoid colony formation assay

Organoids were trypsinised and counted. 2,000 single cells were seeded in BME per 48 wells and placed in a 37°C incubator to polymerise for 20 min. 250 μl of IntestiCult™ Organoid Growth media plus Y-27632 was then added and cultured for 5 days. Number of spheres formed in each well was counted as plating efficiency. Experiments were performed in triplicate.

Generation of Lgr5-HA knock-in mouse intestinal organoids

The knock-in of Lgr5-HA tag was performed by electroporation using an oligo donor (TTACCCCATGACTGAAGAACGTGTCATCTCCTCTTCAGTTGCAATTGCTCCATGTCTCTACCCATAGTGTTCAGATTACGCGCAGCCCATAGTACATAGGAGAGGACGTGGTTTTTAAAGCATTGAAAACCTGAAAAGTGATTTCTATCAGAGCAGTAGCTAAGAAACGCTGAGCTGAG 3’. After cDNA amplification (40 cycles), samples were normalised to Hprt1 and data were expressed as mean ± SD.

Animal procedures

All animal regulated procedures were carried out according to Project License constraints (PEF3478B3 and 70/8560) and Home Office guidelines and regulations. In accordance with the 3Rs, the smallest sample size was chosen that could show a significant difference. Nedd4fl/fl and Nedd4lfl/fl mice were obtained from Max-Planck-Institute of Experimental Medicine, Goettingen, Germany, Department of Molecular Neurobiology. Nedd4fl/fl mouse was
generated as detailed (Kawabe et al., 2010). Nedd4lR/fl mouse was generated as explained (Shi et al., 2008). Animals of both sexes at age 6–7 weeks on C57/BL6J background were used for the different experimental conditions and harvested as indicated.

Tamoxifen was injected intraperitoneally for 3 consecutive days (1.5 mg/10 g of mouse weight) from a 20 mg/ml stock solution. 5-ethyl-20-deoxouridine (EdU) (Life Technologies) was injected intraperitoneally (0.3 mg/10 g of mouse weight) from a 10 mg/ml stock solution.

For the Rspo3 experiments, mice were injected with tamoxifen as detailed above, and 7 days later, Rspo3 (10 mg/kg in PBS) was injected intraperitoneally for three consecutive days. Mice were culled 24 h later, and the tissue was collected to be analysed.

Quantification and statistical analysis

Statistical analyses were performed using GraphPad Prism 8 software. Statistical details and sample numbers are specified in the figure legends. For parametric data, statistical significance was determined using student’s unpaired, two-tailed t-test. For survival experiments, Log-rank (Mantel–Cox) test was used. P values are represented as \*P < 0.05; **P < 0.01; ***P < 0.001.

Conflict of interest

The authors declare that they have no conflict of interest.

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