SETD7 Controls Intestinal Regeneration and Tumorigenesis by Regulating Wnt/β-Catenin and Hippo/YAP Signaling

Highlights
- SETD7 is required for Wnt-dependent tumorigenesis and regeneration
- SETD7 is a component of the destruction complex
- SETD7-dependent methylation of YAP controls β-catenin function
- Cell density controls subcellular localization of SETD7

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In Brief
The Wnt/β-catenin and Hippo/YAP pathways are intimately linked in structure and function. Oudhoff et al. provide evidence that the lysine methyltransferase SETD7 is a central link between these pathways. SETD7-dependent methylation of YAP is required for optimal β-catenin-dependent gene expression. Accordingly, loss of SETD7 attenuates Wnt-dependent intestinal tumorigenesis and regeneration.
SETD7 Controls Intestinal Regeneration and Tumorigenesis by Regulating Wnt/β-Catenin and Hippo/YAP Signaling

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SUMMARY

Intestinal tumorigenesis is a result of mutations in signaling pathways that control cellular proliferation, differentiation, and survival. Mutations in the Wnt/β-catenin pathway are associated with the majority of intestinal cancers, while dysregulation of the Hippo/Yes-Associated Protein (YAP) pathway is an emerging regulator of intestinal tumorigenesis. In addition, these closely related pathways play a central role during intestinal regeneration. We have previously shown that methylation of the Hippo transducer YAP by the lysine methyltransferase SETD7 controls its subcellular localization and function. We now show that SETD7 is required for Wnt-driven intestinal tumorigenesis and regeneration. Mechanistically, SETD7 is part of a complex containing YAP, AXIN1, and β-catenin, and SETD7-dependent methylation of YAP facilitates Wnt-induced nuclear accumulation of β-catenin. Collectively, these results define a methyltransferase-dependent regulatory mechanism that links the Wnt/β-catenin and Hippo/YAP pathways during intestinal regeneration and tumorigenesis.

INTRODUCTION

Intestinal tumors currently account for approximately 8% of all cancer-related deaths worldwide (Stewart and Wild, 2014). Tumorigenesis in the intestine is a complex process that is thought to initiate in intestinal stem cells (ISCs), and requires multiple subsequent mutations in genes that regulate cell growth, differentiation, and survival (Barker et al., 2009; Vogelstein et al., 2013). The Wnt/β-catenin and Hippo/YAP pathways have emerged as regulators of intestinal tumorigenesis. A large proportion of intestinal cancers carry mutations in the Wnt/β-catenin signaling pathway (Muzny et al., 2012), and deregulation of the Hippo signaling pathway has been associated with a subset of intestinal tumors (Cai et al., 2010; Harvey et al., 2013; Rosenbluh et al., 2012). A better understanding of the cellular and molecular mechanisms regulating these pathways would provide insight into the etiology of intestinal tumorigenesis.

Intestinal homeostasis is regulated by the proliferation and differentiation of a population of “cycling” ISCs that express the surface marker LGR5 (Barker et al., 2007). Subsequent studies have identified several other markers for these cells such as ASCL2 and OLFM4, ultimately leading to a cycling ISC signature, which also includes the Hippo transducer YAP (Barker, 2014; Muñoz et al., 2012; van der Flier et al., 2009). In addition, a second type of ISC, which are termed label-retaining cells (LRCs) or reserve stem cells, co-exists with the cycling ISC population (Buczacki et al., 2013; Montgomery et al., 2011; Powell et al., 2012; Sangiorgi and Cacepchi, 2008; Takeda et al., 2011; Tetteh et al., 2015; Tian et al., 2011; van Es et al., 2012). LRCs do not participate in the day-to-day turnover of the intestinal epithelium, and are normally destined to become a cell of the secretory lineage (Buczacki et al., 2013; van Es et al., 2012). Interestingly, it has recently been found that enterocyte
progenitors can have similar properties as these LRCs (Tetteh et al., 2016). Although the roles of these cell types in homeostasis, regeneration, and tumorogenesis are controversial, it is clear that Hippo/YAP and Wnt/β-catenin signaling are central to the function of intestinal cells in vivo.

Several recent studies have demonstrated that the Hippo/YAP and Wnt/β-catenin pathways are closely related and intimately linked in regulation, structure, and function (Azzolin et al., 2012, 2014; Barry et al., 2013; Byun et al., 2014; Heallen et al., 2011; Imajo et al., 2012, 2014; Rosenbluh et al., 2012; Varelas et al., 2010). However, there have been some conflicting reports concerning the biological and mechanistic function of YAP (or its paralog TAZ) in its role in the Wnt pathway. For example, YAP has been shown to both enhance (Heallen et al., 2011; Rosenbluh et al., 2012) and restrict Wnt signaling (Azzolin et al., 2014; Barry et al., 2013; Imajo et al., 2012; Park et al., 2015; Varelas et al., 2010). Furthermore, knockdown of Yap expression in mouse intestinal cells leads to a dramatic decrease in proliferative cells including Wnt-dependent LGR5hi ISCs (Imajo et al., 2014), which is in contrast with other reports showing that Yap deficiency does not affect intestinal homeostasis but rather is important only during regeneration and tumorogenesis (Azzolin et al., 2014; Barry et al., 2013; Cai et al., 2010, 2015; Gregorieff et al., 2015). These discrepancies can be partially explained by the subcellular localization of these proteins, where cytoplasmic YAP inhibits whereas nuclear YAP promotes Wnt signaling. However, recent reports suggest that nuclear YAP can also inhibit Wnt (Gregorieff et al., 2015; Park et al., 2015). Needless to say, the molecular mechanisms that regulate the interactions between these two pathways have not been fully elucidated.

We have previously reported that SETD7-dependent methylation of YAP mediates its subcellular localization and function in vitro and in vivo (Barsyte-Lovejoy et al., 2014; Oudhoff et al., 2013). SETD7 is a member of the Suppressor of variegation 3-9-Enhancer of zeste-Trithorax (SET) domain-containing family of lysine methyltransferases, and has been shown to methylate and alter the function of a wide variety of proteins in vitro (Herz et al., 2013). The various studies describing interactions between Hippo and Wnt pathways led us to study Wnt-mediated processes in the intestine. We found that SETD7 acts as a facilitator of Wnt-driven tumorogenesis and regeneration. Interestingly, SETD7 is dispensable for normal intestinal homeostasis, but intestinal regeneration is impaired in mice lacking SETD7. Mechanistically, we demonstrate that SETD7 and YAP are associated with the β-catenin “destruction complex” and show that SETD7 activity is required for optimal nuclear accumulation of β-catenin following activation of the Wnt pathway. These results place SETD7 at the intersection of the Wnt and Hippo pathways and identify SETD7 as a potential therapeutic target to inhibit intestinal tumorogenesis.

RESULTS

SETD7 Is Required for Optimal Tumorogenesis in ApcMin/+ Mice

The mouse intestine is a well-established model for studying the Wnt/β-catenin and Hippo/YAP pathways within the biological contexts of homeostasis, regeneration, and tumorogenesis (Ashton et al., 2010; Azzolin et al., 2014; Barker et al., 2007; Barry et al., 2013; Cai et al., 2010, 2015; Camargo et al., 2007; Gregorieff et al., 2015; Imajo et al., 2014; Metcalf et al., 2014; Oudhoff et al., 2013; Sansom et al., 2007; Zhou et al., 2011). We have previously found that mice with an intestinal epithelial cell (IEC)-specific deletion of Setd7 (Setd7IEC mice) had wider and shorter crypts in their large intestines, which was associated with increased IEC turnover, increased nuclear localization of YAP, and heightened Hippo/YAP-dependent gene expression (Oudhoff et al., 2013). As nuclear YAP has been reported to enhance Wnt signaling in the heart and in various cancer cell lines (Heallen et al., 2011; Rosenbluh et al., 2012), we wished to test whether SETD7 is involved in intestinal tumorogenesis in a model that relies on Wnt signaling. We therefore crossed Setd7−/− mice with ApcMin/+ mice that spontaneously develop intestinal adenomas due to increased Wnt/β-catenin activity. We hypothesized that the increased levels of nuclear YAP we observed in Setd7IEC mice would enhance tumorogenesis (Rosenbluh et al., 2012). Surprisingly, ApcMin/+ mice that lack Setd7 (ApcMin/+ Setd7−/− mice) had significantly increased life-spans compared with littermate control ApcMin/+ Setd7+/− mice (Figure 1A), with significantly reduced tumor numbers at endpoint that were of similar size (Figures 1B, 1C, and S1A; and data not shown). Of note, at endpoint most mice were severely anemic, which very likely influenced the lifespan. Treatment of mice with dextran sodium sulfate (DSS) in the drinking water leads to breakdown of the intestinal barrier, inflammatory cell infiltration, and transmural damage to the large intestine (Yan et al., 2009), and promotes rapid tumorogenesis in the large intestine of ApcMin/+ mice (Tanaka et al., 2006). It has been reported that inflammation can induce de-differentiation of non-stem cells to tumor-initiating cells that have stem cell-like properties (Schwittalla et al., 2013), complementing a previous study showing that tumor-initiating cells are LGR5hi cycling ISCs (Barker et al., 2009). Although DSS treatment of ApcMin/+ mice enhanced tumorogenesis in the large intestine, we found that ApcMin/+ Setd7−/− mice developed significantly fewer large intestinal tumors following DSS treatment (Figure 1D). Thus, SETD7 expression is associated with increased susceptibility to tumorogenesis in the context of dysregulated Wnt signaling.

We analyzed gene expression in small intestinal tumor tissue from aged animals and compared it with adjacent normal tissue (Figures 1E and S1B). We found that both Wnt (Axin2, Myc, Lgr5) and Hippo target genes (Ctgf, Cyr61) were upregulated in tumor tissue compared with normal tissue (Figure 1E), supporting recent studies showing that mutation in the Wnt/β-catenin destruction complex components “activates” YAP and/or TAZ (Azzolin et al., 2014; Cai et al., 2015; Gregorieff et al., 2015). However, activation of Wnt/β-catenin but not Hippo/YAP target genes was dependent on Setd7 expression (Figure 1E). Thus, during intestinal tumorogenesis SETD7 regulates Wnt/β-catenin-dependent gene expression.

Next, we examined the levels of β-catenin accumulation and localization in small intestinal tumors (Figures 1F and 1G). Although we found that total amounts of β-catenin in tumor sections from ApcMin/+ Setd7−/− and ApcMin/+ Setd7+/− mice were similar, we observed reduced levels of nuclear β-catenin in tumors from ApcMin/+ Setd7−/− mice (Figure 1G), which is consistent with reduced Wnt/β-catenin-dependent gene expression in these tumors (Figure 1E). We also observed that Wnt-mediated Paneth
SETD7 Is Required for Wnt-Mediated Intestinal Regeneration

Given the key role played by stem cells in both tumorigenesis and the response to damage, we employed DSS-induced inflammation to study regeneration in the large intestine. Following a 5-day treatment with DSS, we did not observe any differences between Setd7fl/fl littermate control mice and Setd7fl/IEC mice in acute damage or inflammation as measured by weight loss, reduced colon length, or intestinal inflammation at day 7/8 (Figures 2A–2C and S2B–S2C). Upon returning to regular water, control Setd7fl/fl mice recovered from injury significantly better than Setd7fl/IEC mice as measured by body weight, colon length, inflammatory cytokine production, histology score, and number of regenerating crypts at day 15 (Figures 2A–2C and S2A–S2C). Gene-expression analysis from IECs isolated from Setd7fl/fl and Setd7fl/IEC mice during regeneration revealed decreased levels of Wnt/β-catenin-dependent genes Lgr5 and Axin2 but no differences in the Notch target gene Hes1 or Hippo/YAP target Ctgf (Figures 2D and S2D). This suggests that SETD7 is required for optimal Wnt/β-catenin-dependent regeneration following DSS-induced inflammation in the large intestine.

We next assessed regeneration in the small intestine following whole-body irradiation, which results in p53-mediated apoptosis of all proliferating IECs, including LGRTgIRG ISCs and, consequently, loss of crypts in the small intestine (Merritt et al., 1994). Following irradiation, the intestine repairs and regenerates itself through a process that requires high levels of Wnt/β-catenin signaling (Ashton et al., 2010; Barry et al., 2013; Metcalfe et al., 2014). Three days after irradiation, we observed similar ablation of crypts in Setd7fl/fl and Setd7fl/IEC mice (Figures 3A and 3C). However, by day 6 post-irradiation, normal crypt regeneration was observed in Setd7fl/fl mice but not Setd7fl/IEC mice (Figures 3B and 3C). Similar to our results during DSS-induced regeneration, we observed significantly reduced levels of Wnt/β-catenin target genes Lgr5 and Axin2 in IECs isolated from Setd7fl/IEC mice compared with Setd7fl/fl mice during regeneration (Figure 3D). These results identify an important role for SETD7 in Wnt-dependent intestinal regeneration.

Generation of intestinal organoids from crypts isolated from the small intestine of SETD7-deficient mice also identified a role for SETD7 in regulation of intestinal regeneration (Figures 3E–3H). We observed that significantly fewer crypts developed from SETD7-deficient crypts compared with heterozygous controls (Figure 3E) despite starting with equivalent numbers of crypt cells (Figure S3A). These cyst-like structures had identical gene-expression patterns including low levels of stem cell markers Lgr5, Axin2, and Olfm4 (Figure 3G). However, the formation of novel crypts that grow from these cysts was impaired in organoids lacking SETD7 (Figures 3F and S3B). Organoids lacking...
SETD7 failed to upregulate the Wnt-dependent genes Lgr5, Axin2, Olfm4, and Myc but displayed increased expression of the Hippo/YAP-dependent genes Ctgf and Areg along with higher expression of the proliferation-associated gene Mki67 (Figures 3G and S3C). Inhibition of the enzymatic activity of SETD7 with the SETD7 inhibitor (R)-PFI-2 during organoid development also resulted in decreased expression of Wnt/β-catenin-dependent ISC-specific genes Lgr5, Axin2, and Ascl2 (Figure 3H). Taken together, these results demonstrate that SETD7 plays a critical role in Wnt/β-catenin-dependent intestinal processes such as tumorigenesis and regeneration.

**SETD7 Is Not Required for Intestinal Homeostasis**

During intestinal homeostasis, LGR5<sup>high</sup> ISCs are constantly replenishing the intestinal epithelium (Barker, 2014; Vermeulen and Snippert, 2014). We found no striking differences in the small intestines of naïve Setd7<sup>f/f</sup> and Setd7<sup>AIEC</sup> mice including nuclear β-catenin in ISCs and Paneth cells at the bottom of crypts (Figures 4A and S4A), or the number of goblet cells per villus (Figure S4B). However, we did observe slightly increased numbers of lysozyme-positive Paneth cells per crypt in the Setd7<sup>AIEC</sup> mice (Figure S4B), which were all normally localized in the bottom of crypts (data not shown). We have previously found differences in crypt width and IEC turnover in the large intestine (Oudhoff et al., 2013). Furthermore, Wnt and Notch target genes (Lgr5 and Hes1) were unaltered whereas Hippo target gene Ctgf was expressed significantly higher in IECs isolated from Setd7<sup>AIEC</sup> mice compared with those from Setd7<sup>f/f</sup> littermates (Figure 4B), consistent with our previous results in the large intestine (Oudhoff et al., 2013). However, gene-expression profiling of all IECs could mask small differences occurring in the stem cell compartment at the bottom of the crypt. We therefore crossed Setd7<sup>−/−</sup> mice with Lgr5<sup>EGFP</sup> (Lgr5-EGFP-IRES-creERT2) mice, isolated crypts, and analyzed the crypt IECs by flow cytometry for LGR5 (GFP) (Figure 4C). Cycling ISCs are LGR5<sup>high</sup>, and Setd7<sup>−/−</sup> mice have equal numbers of LGR5<sup>high</sup> ISCs (Figure 4C). Importantly, these ISCs were equally capable of becoming organoids (Figure 4D), unlike results using whole crypts to generate organoids (Figure 3E). These data show that during homeostasis ISCs do not depend on SETD7 expression; however, SETD7 is critical for intestinal regeneration following chemical- or irradiation-induced damage.

**SETD7 Methyltransferase Activity Regulates Wnt Signaling**

Our in vivo data suggested that SETD7 is important during regeneration and tumorigenesis, processes that rely on “high” levels of Wnt signaling. To determine how SETD7 controls Wnt signaling, we treated confluent HEK293 cells with Wnt3A (Figure 5A) or GSK3 inhibitors (LiCl or CHIR99021) to activate the Wnt/β-catenin pathway. To block SETD7 activity, we treated cells with either the SETD7 inhibitor (R)-PFI-2 or its >500-fold less active enantiomer (S)-PFI-2 (Barsyte-Lovejoy et al., 2014), or siRNA constructs were used to knock down SETD7. Activation of the Wnt pathway leads to cytosolic accumulation of β-catenin, which then translocates to the nucleus to alter gene expression by acting as a transcriptional co-activator (Clevers and Nusse, 2012). We found that accumulation of total β-catenin upon Wnt activation did not depend on SETD7 or its enzymatic activity (Figures 5A, 5C, S5A, S5C, S5E, and S6A). In contrast, Wnt-induced AXIN2 gene expression was abrogated by (R)-PFI-2 treatment (Figures 5B and 5D) or by transient knockdown of AXIN2 (Figure 5E). Furthermore, supplementing (S)-PFI-2 treatment did not promote the exit of β-catenin from the nucleus, but rather blocked further nuclear accumulation (Figure 5J), suggesting that SETD7-dependent methylation is required for optimal nuclear localization—but not nuclear retention—of β-catenin following Wnt activation in vitro.

**SETD7-Mediated Control of Wnt Signaling Requires YAP**

To test whether the effects of SETD7 on Wnt/β-catenin signaling were mediated through YAP (Azzolin et al., 2014; Barsyte-Lovejoy et al., 2014; Oudhoff et al., 2013), we analyzed the effects of SETD7 and YAP knockdown on Wnt/β-catenin pathway activation. Treatment of 293 cells with a pool of two small interfering RNAs (siRNAs) specific for SETD7, YAP, or both, in the absence of Wnt/β-catenin pathway activation had no effect on total or cytosolic β-catenin levels (Figures 6A, S6A, and S6B). However, YAP knockdown itself did result in a slight upregulation of AXIN2 (Figure S6C), consistent with previous reports (Azzolin et al.,...
and S6A). Critically, YAP target gene expression is dependent upon YAP. We further observed that knockdown on LiCl-induced SETD7 affected by knockdown of β-catenin signaling are dependent upon YAP. We further observed knockdown of LiCl- or CHIR-β-catenin-induced CTGF expression (Figures 6A and 6B), demonstrating that the effects of SETD7 on Wnt/β-catenin signaling are dependent upon YAP. We further observed that SETD7 knockdown resulted in increased expression of the YAP target gene CTGF, which, predictably, was also dependent upon Wnt/β-catenin signaling, in agreement with our previous studies (Barsyte-Lovejoy et al., 2014; Oudhoff et al., 2013). These results suggest that SETD7-dependent methylation of YAP is an important control point in Wnt-induced β-catenin function.

**SETD7 Is in a Complex with Components of the Destruction Complex and Mediates YAP/β-Catenin Interactions**

Our results suggest that SETD7 and YAP are critically required for optimal Wnt/β-catenin-mediated gene expression. We next examined whether SETD7 and YAP interacted with components of the destruction complex (Azzolin et al., 2014). We found that SETD7 may be a component of this complex, as immunoprecipitation analyses identified AXIN1 and β-catenin (Figures 6C and 6D) as binding partners of SETD7 in the absence of Wnt activation. Inhibition of the methyltransferase activity of SETD7 with (R)-PFI-2 demonstrated that enzymatic activity is not required for the formation of the SETD7/β-catenin complex in the absence of Wnt3A (Figures 6D and 6E). However, upon Wnt3A stimulation the interaction between SETD7 and β-catenin is lost, and this disassembly is dependent upon SETD7 methyltransferase activity (Figure 6E). Furthermore, we found that the YAP/β-catenin interaction is dependent on SETD7 methyltransferase activity in the presence or absence of active Wnt signaling (Figures 6F and 6G), suggesting that SETD7-dependent methylation of YAP is critical for stabilizing YAP/β-catenin interactions irrespective of Wnt signaling (Azzolin et al., 2014; Imajo et al., 2012). In support, we observed that the YAP/AXIN1 interaction depends on the methyltransferase activity of SETD7 (Figure 6H). Importantly, mutation of the methylation site K494 (but not K497) in YAP also blocks YAP-AXIN1 interactions (Figure 6I). Together, these results identify a role for SETD7 in the cross-regulation of Hippo/YAP and Wnt/β-catenin signaling by mediating interactions within protein complexes. Our results further suggest that methylation of YAP facilitates accumulation of nuclear β-catenin upon Wnt activation or GSK3 inhibition. However, recent studies have shown that YAP-TEAD-mediated gene expression may also directly dampen Wnt-dependent gene expression. For example, a recent study showed that abrogation of YAP-TEAD interactions by verteporfin treatment or knockdown of TEAD induced Wnt target gene expression (Park et al., 2015). As we observed increased YAP-TEAD activity during intestinal homeostasis (Oudhoff et al., 2013) and in organoids (Figures 3 and 4), we tested whether SETD7 mediates Wnt signaling via YAP-TEAD interactions by treating organoids derived from Setd7f/f (black) and Setd7f/f (gray) mice (Figures 6J and 6K). Consistent with results from a different group (Imajo et al., 2014), critically, the changes in gene expression occurred irrespective of the presence or absence of SETD7, thus supporting our hypothesis that SETD7 does not mediate Wnt signaling by altering YAP-TEAD activity.

**SETD7 Localization and Function Are Density Dependent**

As subcellular localization is critical for both Wnt/β-catenin and Hippo/YAP signaling, we next examined SETD7 localization.
We observed that SETD7 localizes to the nucleus in HEK293, Caco-2, and MCF7 cells at low density (sparse), but is found in the cytoplasm and excluded from the nucleus at high cell density (dense) (Figures 7A and 7B). This pattern is strikingly similar to that of YAP (Figures 7A and S7A), further highlighting that YAP and SETD7 are potentially components of a shared complex. Importantly, we did not observe any effects of SETD7 on Wnt-induced AXIN2 expression or nuclear β-catenin accumulation under sparse cell culture conditions in HEK293 and MCF7 cells (Figures 7C and S7B–S7E), suggesting that cytoplasmic and not nuclear SETD7 regulates Wnt/β-catenin signaling. Consistent with this, we found that SETD7 is nuclear in cycling ISCs and Paneth cells that regulate intestinal homeostasis, but is cytoplasmic in the rest of the crypt-villus structure (Figures 7D, S7F, and S7G). Of note, Yap is absent from Paneth cells (Gregorieff et al., 2015; Zhou et al., 2011), whereas SETD7 is nuclear. We did observe more Paneth cells in Setd7AEC mice (Figure S4B), suggesting that perhaps SETD7 plays a distinct role in the absence of YAP. In tumors from ApcMin/+ mice, SETD7 expression was primarily cytoplasmic except for small numbers of cells (Figure 7D), a localization pattern that is similar to LGR5 expression (Barker et al., 2009). Thus, these findings are consistent with a model in which nuclear SETD7 in LGR5high ISCs has no effect on Wnt signaling while cytoplasmic SETD7 plays a critical role in potentiating Wnt/β-catenin signaling through the methylation of YAP, a process required during regeneration and tumorigenesis.

**DISCUSSION**

In this study, we establish a role for SETD7 in the regulation of Wnt-dependent intestinal tumorigenesis and regeneration. We show that in vitro SETD7 is a component of a complex that may contain SETD7, YAP, β-catenin, and AXIN1. Following Wnt stimulation, SETD7-dependent methylation of YAP is required for the dissociation of the complex and nuclear localization of β-catenin. In the absence of SETD7 or its methyltransferase activity, YAP/β-catenin interactions are inhibited, β-catenin fails to translocate to the nucleus, and Wnt/β-catenin-dependent gene expression is abrogated. Thus, our results place SETD7 at the intersection between Hippo/YAP and Wnt/β-catenin signaling and suggest that methylated YAP is important in the nuclear translocation of β-catenin, thereby providing a molecular mechanism linking these two pathways.

Several studies have examined the interplay between the Hippo/YAP and Wnt/β-catenin pathways in intestinal processes, often with contradictory results (Azzolini et al., 2014; Barry et al., 2013; Cai et al., 2010, 2015; Camargo et al., 2007; Gregorieff et al., 2015). For example, two studies show that IEC-intrinsic deletion of Yap abolishes intestinal tumorigenesis in ApcMin/+ mice (Cai et al., 2015; Gregorieff et al., 2015), whereas others indicate that Yap deficiency has negligible effects on tumor formation upon Apc deletion in IECs (Azzolini et al., 2014; Barry et al., 2013). Furthermore, it has been shown that both Yap deletion and activation, by direct overexpression or deletion of Sav, are detrimental for intestinal regeneration (Barry et al., 2013; Cai et al., 2010). We find that in Wnt-driven tumors there is a significant activation of YAP target genes, in accordance with a recent study (Cai et al., 2015). During “active” YAP conditions (tumors in vivo, low cell density in vitro) we do not observe any effects of SETD7 inhibition on YAP function. It has been suggested that YAP may partially drive the development of tumors (Cai et al., 2015; Rosenbluh et al., 2012), explaining why we still observe tumorigenesis in ApcMin/+Setd7−/+ mice. However, Wnt target genes are expressed at significantly lower levels during “high” Wnt conditions in the intestine (regeneration and tumorigenesis) in mice lacking Setd7. Our data are thus in support of a model in which “active” YAP is capable of suppressing Wnt signaling (Gregorieff et al., 2015; Park et al., 2015), although we propose a mechanism that does not depend on YAP-TEAD-dependent transcription.

Mechanistically, the Wnt/β-catenin and Hippo/YAP pathways share many similarities. First, activation of both pathways is controlled by regulating the subcellular localization of the effector proteins of each pathway; Wnt signaling results in the nuclear translocation of β-catenin while activation of the Hippo pathway leads to the cytoplasmic sequestration of YAP. Second, both β-catenin and YAP have been found to be associated with intercellular junctions through associations with α-catenin and E-cadherin (Huber and Weis, 2001; Kim et al., 2011; Pokutta and Weis, 2000; Schlegelmich et al., 2011; Silvis et al., 2011). Furthermore, in addition to sharing general regulatory mechanisms, several studies have identified direct interactions between members of each pathway. For example, YAP has been shown to interact with β-catenin in the cytoplasm (Imajo et al., 2012) as well as in the nucleus, where together they activate tissue-specific gene programs (Heallen et al., 2011; Rosenbluh et al., 2013). For example, two studies show that IEC-intrinsic deletion of Yap abolishes intestinal tumorigenesis in ApcMin/+ mice (Cai et al., 2015; Gregorieff et al., 2015), whereas others indicate that Yap deficiency has negligible effects on tumor formation upon Apc deletion in IECs (Azzolini et al., 2014; Barry et al., 2013). Furthermore, it has been shown that both Yap deletion and activation, by direct overexpression or deletion of Sav, are detrimental for intestinal regeneration (Barry et al., 2013; Cai et al., 2010). We find that in Wnt-driven tumors there is a significant activation of YAP target genes, in accordance with a recent study (Cai et al., 2015). During “active” YAP conditions (tumors in vivo, low cell density in vitro) we do not observe any effects of SETD7 inhibition on YAP function. It has been suggested that YAP may partially drive the development of tumors (Cai et al., 2015; Rosenbluh et al., 2012), explaining why we still observe tumorigenesis in ApcMin/+Setd7−/+ mice. However, Wnt target genes are expressed at significantly lower levels during “high” Wnt conditions in the intestine (regeneration and tumorigenesis) in mice lacking Setd7. Our data are thus in support of a model in which “active” YAP is capable of suppressing Wnt signaling (Gregorieff et al., 2015; Park et al., 2015), although we propose a mechanism that does not depend on YAP-TEAD-dependent transcription.

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et al., 2012). In addition, YAP has also been found to interact with Wnt pathway members Disheveled and AXIN1 (Azzolin et al., 2014; Barry et al., 2013; Varelas et al., 2010). Taken together, these studies suggest that the Wnt/β-catenin and Hippo/YAP pathways may be part of a larger pathway that integrates these signals in tissues. Our results identify SETD7 as a previously undescribed member of this Wnt/Hippo “super-pathway” through methylation of YAP. Azzolin et al. (2014) specifically describe that YAP affects the destruction complex by recruiting β-TrCP to AXIN1, thereby controlling β-catenin protein levels. We fail to observe SETD7 or YAP-mediated control of total β-catenin protein levels with or without Wnt signaling, even though the YAP-AXIN1 interaction is mediated by methylation. Based on our data we would propose a mechanism whereby the continuous cytoplasmic-nuclear shuttling of YAP, and destruction complex members such as APC and AXIN1 (Cong and Varmus, 2004; Dupont et al., 2011; Henderson, 2000; Schmitz et al., 2013) is required for optimal nuclear accumulation of β-catenin.

Furthermore, it has been proposed that YAP may be phosphorylated and retained in the nucleus (Li et al., 2014; Wada et al., 2011), leading us to speculate that methylation and phosphorylation are involved in a complex interplay to establish protein complexes important for the localization and shuttling of β-catenin and YAP. Our studies also highlight the important role of methylation as a post-translational modification (PTM) that can modulate signaling output. Thus, in addition to established PTMs such as phosphorylation and ubiquitination, we propose that methylation of non-histone proteins is an important regulator of signal transduction, which is in line with several recent studies (Fang et al., 2014; Kim et al., 2013; Mazur et al., 2014). Indeed, a recent study identified a role for SETD7 in the direct methylation of β-catenin (Shen et al., 2015). It was proposed that SETD7-dependent methylation of β-catenin was required for optimal degradation in response to oxidative conditions. We fail to find any evidence of direct methylation of β-catenin by SETD7.

Figure 5. SETD7 Is Required for Wnt Signaling In Vitro by Mediating Nuclear Accumulation of β-Catenin
(A) Expression of β-catenin (β-Cat), SETD7, and GAPDH in HEK293 cells following treatment with Wnt3A in the presence of (R)-PFI-2 or its negative enantiomer (S)-PFI-2.
(B) AXIN2 expression analyzed by qPCR after 4 hr of Wnt3A with (S)-PFI-2 (S) or (R)-PFI-2 (R).
(C) Expression of β-catenin (β-Cat), SETD7, and GAPDH in HEK293 cells upon LiCl (20 mM) treatment.
(D) AXIN2 expression analyzed by qPCR following incubation for 4 hr with LiCl (20 mM).
(E) Expression of β-catenin (β-Cat), in cytosolic (cyto.) or nuclear (nucl.) fractions from HEK293 cells.
(F and G) Expression of β-catenin was visualized by confocal microscopy. Magnification, 600.
(H) Nuclear accumulation of β-catenin was visualized by confocal microscopy and quantified 4 hr after Wnt3A treatment.
(J) Nuclear accumulation of β-catenin was visualized by confocal microscopy and quantified after 4 hr of Wnt3A treatment in the presence of (S)-PFI-2 or (R)-PFI-2 for the final 0.5, 1, and 2 hr.

*p < 0.05, **p < 0.01, ***p < 0.001. Data are presented as means ± SEM. See also Figure S5.
following canonical activation of Wnt signaling. Furthermore, we do not observe any changes in the levels of β-catenin in the absence of SETD7, suggesting that degradation is not directly affected by SETD7 deficiency. Thus, although our results do not exclude the possibility that β-catenin is methylated, it is likely that SETD7 is not the relevant methyltransferase. Instead, our results point to an indirect role for SETD7 through its methylation of YAP, a transcription factor that is important for Wnt-induced nuclear stabilization of β-catenin. A genetic deletion of YAP will help to directly define the role of these two pathways. Future studies using mice or organoids with specific genetic deletions of these two pathways will provide additional therapeutic targets to modulate Wnt/Hippo-dependent physiological processes. Indeed, a recent study has identified a demethylase that is important for Wnt-induced nuclear stabilization of β-catenin (Lu et al., 2015). Although this study supports our findings that methylation is a critical component of Wnt signaling, it is likely that distinct mechanisms are in play. For example, Lu et al. (2015) find that degradation of nuclear β-catenin is specifically affected, while we do not detect any differences in β-catenin levels. In addition, these authors show that β-catenin itself is methylated using an antibody specifically recognizing dimethylated lysines. As SETD7 is a monomethylase, our findings do not support that SETD7 is the methyltransferase in this case. Nevertheless, this indicates that methylation is important at several levels in the Wnt signaling pathway.

It is clear that the mechanisms of cross-regulation of the Wnt/β-catenin and Hippo/YAP signaling pathways in vivo are complex and vary between cell types and tissues. Our in vitro results using the inhibitor (R)-PFI2 suggest that SETD7-dependent methylation of YAP is a central regulatory mechanism controlling these two pathways. Future studies using mice or organoids with a genetic deletion of YAP will help to directly define the role of SETD7 and YAP in Wnt-dependent processes in vivo.

In summary, we have shown that SETD7-dependent methylation of YAP is a critical central regulatory mechanism that links the Wnt/β-catenin and Hippo/YAP pathways. The results of these studies have important ramifications for the treatment of
intestinal tumors and provide a pathway to target for the development of novel therapeutics.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

HEK293, MCF7, and Caco-2 cell lines were grown in DMEM supplemented with 10% fetal calf serum and antibiotics. Wnt3A (PeproTech or R&D Systems) was used at 100 ng/ml, LiCl (Sigma) at 20 mM, CHIR-99021 (StemCell Technologies) at 3 μM, and (S)-PFI-2 and (R)-PFI-2 between 1 and 10 μM. Transient knockdown of gene expression was performed using siRNA (Silencer Select [SETD7], S37451/S37452, Positive Control No.1). SETD7 (2D10, Abcam), histone 3 (Abcam), AXIN1 (Cell Signaling), YAP/TAZ (Santa Cruz), SETD7, histone 3 (Abcam), AXIN1 (Cell Signaling), and pYAPS127 (Cell Signaling). HEK293 cells were transfected at 80% confluence with 4% paraformaldehyde, permeabilized with 1% Triton X-100, blocked in 5% BSA, and stained with antibodies against β-catenin, Myc, AXIN1, SETD7, histone 3, AXIN1, and pYAPS127 (Cell Signaling). HEK293 cells were transfected with Lipofectamine RNAiMAX (Life Technologies). Cells were lysed and immunoprecipitations carried out using antibodies against MYC (clone 9E10), AXIN1 (Cell Signaling), YAP (Abcam), SETD7 (Abcam), YAP (Cell Signaling Technology), β-catenin (Santa Cruz Biotechnology), or FLAG (M2; Sigma). Immunoblotting of immunoprecipitates was carried out with antibodies against β-catenin, Setd7, histone 3 (Abcam), AXIN1 (Cell Signaling), and pYAP127 (Cell Signaling). HEK293 cells were transfected at 80% confluency with Lipofectamine 3000 (Life Technologies) using MYC-AXIN1 (Addgene 21287 [Zeng et al., 1997]), YAP, AXIN1, SETD7, and HA-SETD7 as published previously (Ishida et al., 2012; Lehnertz et al., 2011; Oudhoff et al., 2013).

**RNA Extraction and qPCR**

RNA was purified from whole intestine using mechanical disruption followed by TRIzol according to the manufacturer’s instructions, or from IECs or cultured cells using an RNeasy isolation kit (Qiagen). Reverse transcription using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used to generate cDNA, and qPCR was performed using SYBR green with primers from the Primer Bank (http://pga.mgh.harvard.edu/primerbank) using SYBR green chemistry on an ABI 7900 real-time PCR system (Applied Biosystems). Samples were normalized against Actb or Gapdh and are presented as fold over wild-type or relative to housekeeping gene as indicated in the figure legends.

**Cryp Isolation, IEC Preparation, and Organoid Culture**

Cryp isolation, IEC preparation, and organoid culture were performed as described previously (Sato and Clevers, 2013; Sato et al., 2009).

**Statistical Analysis**

Results are presented as mean ± SEM. Statistical significance was determined either using Student’s t test or one-way ANOVA with post hoc tests or, when n < 10, non-parametric testing (Mann-Whitney test). Results with p values of less than 0.05 were considered significant.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2016.03.002.

**AUTHOR CONTRIBUTIONS**

M.J.O., S.A.F., D.W., M.J.S.B., D.G.R., J.W., K.S., M.R.H., M.R.G., K.M.M., I.R., F.M.V.R., T.S., and C.Z. designed and performed the research and/or provided reagents. M.J.O. and C.Z. analyzed the data and wrote the paper. M. Williams (UBC AbLab), T. Murakami (BRC Genotyping), I. Barta (BRC Histology), and all members of BRC mouse facility. This work was supported by the Australian National Health and Medical Research Council (to C.Z.), the Australian National Health and Medical Research Council (to C.Z.), and the Australian and New Zealand Society for Integrative and Developmental Biology. We would like to thank R. Dhesi, L. Rollins (BRC core), A. Johnson (UBCFlow), M. Williams (UBC AbLab), T. Murakami (BRC Genotyping), I. Barta (BRC Histology), and all members of BRC mouse facility. This work was supported by the Australian National Health and Medical Research Council (to C.Z.), and the Australian National Health and Medical Research Council (to C.Z.).
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