Redeployment of Myc and E2f1–3 drives Rb-deficient cell cycles

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Robust mechanisms to control cell proliferation have evolved to maintain the integrity of organ architecture. Here, we investigated how two critical proliferative pathways, Myc and E2f, are integrated to control cell cycles in normal and Rb-deficient cells using a murine intestinal model. We show that Myc and E2f1–3 have little impact on normal G1–S transitions. Instead, they synergistically control an S–G2 transcriptional program required for normal cell divisions and maintaining crypt–villus integrity. Surprisingly, Rb deficiency results in the Myc-dependent accumulation of E2f3 protein and chromatin repositioning of both Myc and E2f3, leading to the ‘super activation’ of a G1–S transcriptional program, ectopic S phase entry and rampant cell proliferation. These findings reveal that Rb-deficient cells hijack and redeploy Myc and E2f3 from an S–G2 program essential for normal cell cycles to a G1–S program that re-engages ectopic cell cycles, exposing an unanticipated addiction of Rb-null cells on Myc.

Spatial and temporal control of cell proliferation is vital for organogenesis and maintenance of tissue integrity. Normal proliferative stimuli impinge on cell surface receptors and engage multiple intracellular signalling cascades that converge on cell cycle control. Growth factor receptor activation culminates in the accumulation of Myc and E2f transcription factors, two central components believed to link external proliferative signals to early phases of the cell cycle1,2.

In response to mitogenic signals such as Wnt, Notch, TGFβ and activation of receptor tyrosine kinases3–7, Myc transcriptionally orchestrates a broad range of biological processes, including macromolecule biosynthesis, energy production, induction of cyclin-dependent kinases8–17 (Cdks), and physically interacts with the DNA pre-replication complex18 to collectively prepare cells for S phase entry. Several studies have also linked Myc to the regulation of E2f expression and activity9,19,20. The E2f family consists of eight related members that have transcription activation and repression functions21. Phosphorylation of Rb by Cdks leads to the accumulation of E2f1–3 late in G1 and activation of a G1–S transcriptional program that licenses the entry into S phase and commitment through cell division22. E2f6–8-mediated transcriptional repression late in S phase contributes to the oscillatory expression of target genes as cells march through the cell cycle23. The fact that MYC and E2F1–3, or components that regulate them such as RB, Cdks and p16INK4a, are invariably disrupted in human cancer highlights their central role in the control of cellular proliferation23,24.

Surprisingly, knockout mouse models of Myc and E2f’s reveal a paucity of major cell cycle defects25–29. This has been attributed to redundancy within these two families of transcription factors30,31. Recent work showed that mouse retinal precursors can proliferate in the absence of N-Myc or E2f1–3, but not in the absence of both sets of factors27, suggesting further redundancy among the two transcription factor families. Whether Myc and E2fs collaborate in other cell types and tissues, and how they might do so, remains to be determined.

Here we evaluated the roles of Myc and E2f1–3 in the control of cell proliferation in the small intestine of mice. The functional unit of the small intestine consists of pouch-like invaginations called crypts and finger-like projections called villi. Pluripotent stem cells at the base of crypts continuously divide to generate transit-amplifying progenitor cells, which undergo several rounds of proliferation before they exit
the cell cycle, differentiate and repopulate villi. Here we show that the combined loss of Myc and E2f1–3 has little impact on G1–S transitions in the small intestine of mice. Rather, Myc and E2f1–3 engage an S–G2 transcriptional program required for the completion of S phase and progression through mitosis. When Rb is inactivated, however, we show that Myc and E2fs are redeployed and engage a distinct G1–S program that promotes ectopic cell cycles. These findings distinguish how Myc and E2f control the proliferation of normal versus Rb-deficient cells, and expose a molecular mechanism for the unexpected dependency of Rb-deficient cells on Myc.

RESULTS
Combined ablation of Myc and E2f1–3 results in disruption of crypt–villus integrity
To explore whether Myc and E2f activities collaborate in the control of normal cell cycles in vivo, we examined the small intestine of mice containing an inducible intestinal-specific Cre transgene (Ah-cre) and conditional alleles of Myc and/or E2f3 (ref. 32). Owing to the potential functional redundancy between E2f3 and the other two E2f activators, we also introduced null alleles of E2f1 and E2f2 into experimental animals. Ah-cre expression in crypts was induced by intraperitoneal administration of β-naphthoflavone (β-NF) and tissue histopathology was examined 7 days later by haematoxylin-and-eosin staining. Ablation of either E2f1–3 (Ah-cre; E2f1−/−; E2f2−/−; E2f3loxP/loxP, referred to as E2f TKO) or Myc (Ah-cre; MycloxP/loxP, referred to as Myc KO) had little effect on intestinal architecture (Fig. 1a), consistent with recent studies showing active crypt cell proliferation in the absence of either E2f1–3 or Myc (refs 25,28,33). The simultaneous deletion of E2f1–3 and Myc (Ah-cre;E2f1−/−;E2f2−/−;E2f3loxP/loxP;MycloxP/loxP, referred to as E2f/Myc QKO), however, resulted in the complete collapse of crypt–villus structure, at each indicated time point. (d) Quantification of average number of crypt cells. Data are presented as mean ± s.d., n = 3 mice per genetic group at each indicated time point. (e) E2f/Myc QKO tissue sections stained by H&E and IHC of Myc to show the regeneration of intestinal epithelium by Myc-positive cells at 7 and 14 days after induction of Ah-cre expression (arrows). Data are representative images from n = 3 mice at each time point. Scale bars represent 100 µm (a,e), 50 µm (b) and 25 µm (c).
preceded the changes observed in associated villi (Fig. 1b). By two days post β-NF injection, E2f/Myc QKO crypt cells had enlarged nuclei with reduced basophilic staining, and seemed overall larger than controls (Fig. 1b,c). By four days post β-NF injection, the number of cells in E2f/Myc QKO crypts decreased to less than 50% of control animals, leading to marked crypt atrophy and deterioration of villus...
Figure 3  Synergistic regulation of an S–G₂ transcriptional program by Myc and E2f1–3. (a) Heatmap representation for clustering of differentially expressed genes between mutant genetic groups compared with control samples. Crypts were collected 2 days after induction of Ah-cre expression. n = 4 for control and Myc KO mice, n = 5 for E2f TKO and E2f/Myc QKO mice. P < 0.05, Student’s t-test. (b) RT–qPCR analysis for a subset of Group III (S–G₂-related) genes. Normal expression levels are illustrated as grey dotted lines and dysregulated expression levels in E2f/Myc QKO crypts are illustrated as red dotted lines. (c) RT–qPCR analysis for a subset of G₁–S-related genes. Normal expression levels are illustrated as grey dotted lines. In b and c, expression levels from individual mice are plotted (4 per genetic group) and error bars represent mean ± s.d. from n = 3 technical replicates. (d) Immunofluorescence staining of Pcna, Mcm3, Ccna2 and Cdc2. Note that degenerating E2f/Myc QKO crypts with less dense cells have comparable protein levels of Pcna and Mcm3, yet significantly less Ccna2 and Cdc2, compared with other genetic groups. Data are representative images from n = 3 mice per genetic group. Scale bars in d represent 50 µm.

Although mice became moribund within 1–2 weeks of β-NF treatment, they subsequently recovered, groomed and seemed healthy. Inspection of their small intestines showed that residual crypts escaping Cre-mediated deletion had repopulated the intestinal epithelium (Fig. 1e), as similarly observed in other studies using this system.33,34
Figure 4 Chromatin binding of E2f3 and Myc in wild-type tissues. (a) Heatmap of tag intensity for all E2f3- and Myc-binding locations in wild-type intestines. Data collected from pooled crypts (n=32 mice) or villi (n=7 mice). (b) E2f3 ChIP-PCR validation: control (Rb KO, n=3 villi) and E2f1-3 deficient (Rb/E2f QKO, n=3 villi); Myc ChIP-PCR validation: control (wild type, n=4 crypts) and Myc deficient (Myc KO, n=3 crypts). Data are presented as mean ± s.d. For detailed peak location and primer sequence information see Supplementary Table 11. (c) Genomic spatial distribution of all E2f3 and Myc peak summits in wild-type intestines. The number of summits in each tissue compartment is shown in parentheses. 5′ distal: 5′ proximal: 5′ region within −50 kb to −5 kb of TSSs. Promoter: −5 kb to +2 kb of TSSs. Gene body: from +2 kb of TSSs to the end of transcripts. 3′ region: 3′ region starting from end of transcripts. (d) Density plots of all E2f3 and Myc peak summits across genomic regions in wild-type intestines. Gene bodies for individual genes were divided into 100 bins and summit locations were accordingly assigned a genomic position. Data in c and d were collected from pooled crypts (n=32 mice) or villi (n=7 mice). (e) IHC staining of E2f3a and Myc in wild-type intestines. Data are representative images from n=3 mice. Scale bar, 50 µm. (f) Heatmap of differential expression of cell-cycle-related genes, as annotated in the Cyclebase database, in wild-type crypts (n=5 mice) and villi (n=5 mice). P < 0.01, empirical Bayes method. (g) Genomic spatial distribution of E2f3 and Myc peak summits associated with differentially expressed cell-cycle-related genes in wild-type intestines. The data represent a subset of c and classification of genomic regions is the same as in c. (h) IHC-exo-seq track examples showing E2f3 and Myc binding to selected G1–S-related genes (h) and S–G2-related genes (i) in wild-type crypts. E2f3 and Myc peaks are highlighted in blue and red, respectively. Examples are derived from pooled crypts (n=32 mice). Scale bars in h and i represent 1 kb.
**Figure 5** Rb-deficient cells require Myc to drive ectopic cell cycles. (a) H&E-stained tissue sections from control, Rb KO, Rb/E2f QKO and Rb/Myc DKO intestines. Note the hyperplastic feature of Rb KO villi. (b) Immunofluorescence staining of BrdU and P-H3. Note the nonspecific staining of blood cells in the lumen of villi. Data in a and b are representative images from n = 3 mice (BrdU) or n = 4 mice (H&E, P-H3) per genetic group. (c) Quantification of BrdU and P-H3 staining. Data are presented as mean ± s.d., BrdU (n = 3 mice), P-H3 (n = 4 mice). (d) RT–qPCR analysis for indicated cyclins in control, Rb KO, Rb/E2f QKO and Rb/Myc DKO villi. Expression levels from individual animals are plotted (4 per genetic group) and error bars represent mean ± s.d. from n = 3 technical replicates. (e) IHC staining of Ccna2. Data are representative images from n = 3 mice per genetic group. Scale bars in a, b and e represent 50 µm.

**Combined Myc and E2f1–3 deficiency leads to S–G2 cell cycle arrest**

We reasoned that the acute degeneration of E2f/Myc QKO crypts could be due to decreased cell proliferation. Surprisingly, DNA synthesis was unaffected in progenitor cells at a time when Myc and E2f1–3 proteins were clearly depleted (Fig. 2a,b and Supplementary Fig. 1a–c). Expression of geminin, a protein involved in blocking the re-replication of the genome late in S phase and
G2 (ref. 35), was also normal in E2f/Myc QKO cells (Fig. 2a,b and Supplementary Fig. 1a,b). However, progression through cell division was severely impaired in E2f/Myc QKO cells as indicated by the absence of mitotic figures and Ser-10-phosphorylated histone 3 (P-H3) staining (Fig. 2a,b). Fluorescence-activated cell sorting analysis showed an accumulation of E2f/Myc QKO crypt cells in S phase and a reduction in G1–M compared with control littermates (Fig. 2c). Despite the late cell cycle arrest in E2f/Myc QKO samples, cell type-specific marker analysis revealed an appropriate number of paneth and goblet cells along the crypt–villus unit (Supplementary Fig. 1d), probably reflecting pre-existing non-deleted cells that persist beyond the experimental time frame analysed here (that is, paneth cells live for several weeks)36. Together, these findings suggest that E2f/Myc QKO progenitor cells were able to enter S phase but failed to fully progress through S–G2.

DNA integrity was compromised in E2f/Myc QKO progenitor cells as indicated by increased phosphorylated H2AX (P-H2AX) staining (Fig. 2d,e). This increase in DNA damage was a consequence of the specific ablation of E2fS because E2f TKO, but not Myc KO intestines, exhibited higher levels of P-H2AX. To determine whether cell death, possibly due to incurred DNA damage, contributed to E2f/Myc QKO crypt degeneration, tissue sections were processed for immunohistochemistry (IHC) using cleaved caspase-3-specific antibodies. This analysis showed that E2f TKO crypts, but not Myc KO or E2f/Myc QKO crypts, contained apoptotic cells (Fig. 2d,e).

We considered the possibility that loss of Myc might accelerate the elimination of E2f1–3-deficient apoptotic cells in E2f/Myc QKO crypts; however, this seems unlikely because a similar analysis at one and two days following β-NF injection also failed to detect apoptotic cells in these samples. Thus, the execution of programmed cell death in E2f1–3-deficient crypts is dependent on Myc. Interestingly, Myc was recently shown to be required for DNA-damage-induced apoptosis of crypt cells37. From these findings, we conclude that cell cycle arrest in S–G2 underlies crypt atrophy caused by combined E2f–Myc deficiency.

Synergistic regulation of an S–G2 transcriptional program by Myc and E2f1–3

Given the established roles of Myc and E2f as transcription factors, we reasoned that changes in gene expression might underlie the observed S–G2 block in E2f/Myc QKO cells. We thus examined the transcriptome from β-NF-treated control, E2f TKO, Myc KO and E2f/Myc QKO crypts using an Affymetrix platform. This comparison revealed three main categories of differentially expressed genes (Fig. 3a and Supplementary Table 1). One category (group I) includes genes dysregulated in all three mutant genetic groups, indicating a requirement for E2f1–3 and Myc in regulating the expression of these genes. A second category (group II) includes genes dysregulated in E2f/Myc QKO samples and either E2f TKO or Myc KO samples, suggesting that these target genes are uniquely regulated by E2f1–3 or Myc. The third and perhaps most interesting category (group III) includes genes that are unaffected or only marginally dysregulated in E2f TKO and Myc KO cells, but are profoundly dysregulated in E2f/Myc QKO crypts, suggesting that this group of genes are synergistically regulated, albeit not necessarily directly, by E2f1–3 and Myc. Gene ontology (GO) analysis of each group failed to detect any significant enrichment for classic G1–S-regulated messenger RNAs. However, group III genes were markedly enriched for mitotic-related functions (Supplementary Table 2). Quantitative gene expression and immunofluorescence assays confirmed the acute and specific downregulation of S–G2-related genes in E2f/Myc QKO crypts (Fig. 3b–d). We conclude that Myc and E2f synergistically regulate an S–G2 expression program required for normal cell cycle progression in vivo.

Myc and E2f3 bind to both G1–S and S–G2 target genes in crypts

We then queried the genome-wide occupancy of E2f3 and Myc on chromatin of intestinal cells using chromatin immunoprecipitation (ChIP) combined with exonuclease digestion (exo), followed by next-generation sequencing (seq)38,39. The ChIP-exo-seq approach provides enhanced sensitivity and specificity over traditional ChIP-seq methods, improving the detection of chromatin occupancy by non-abundant transcription factors, such as E2fs and Myc. E2f3 and Myc DNA-binding peak summits were identified using two algorithms (MACS2 and GEM) with a false discovery rate of less than 1%. A summary of the ChIP-exo-seq data is presented in Fig. 4a and Supplementary Table 3. Twenty randomly selected E2f3- and twenty Myc-specific peak summits were validated by ChIP-PCR assays using control, E2f3-deficient and Myc-deficient tissues (Fig. 4b). Peak summits were then mapped to genes on the basis of the minimal distance between summits and proximal transcription start sites (TSSs; Fig. 4c). The patterns of E2f3 and Myc chromatin binding in crypts were distinct, with E2f3 occupancy biased towards promoter regions and Myc occupancy distributed more broadly across genes (Fig. 4d). E2f3 and Myc binding was reduced in villi, consistent with lower levels of these two proteins in this compartment (Fig. 4e and Supplementary Fig. 2a).

We then profiled and compared gene expression in wild-type crypts and villi. As expected, cell-cycle-related genes were preferentially expressed in crypts and differentiation-related genes were preferentially expressed in villi (Fig. 4f and Supplementary Fig. 2b and Supplementary Tables 4 and 5). Integration of ChIP-exo-seq and gene expression data sets revealed a marked enrichment of E2f3 binding to promoter regions of differentially expressed cell-cycle-related genes (compare Fig. 4c and 4g)40. In crypts, E2f3 and Myc bound to both G1–S- and S–G2-related genes (Fig. 4h,i and Supplementary Fig. 2c and Supplementary Table 5). As the expression of only S–G2-related genes was disrupted in E2f/Myc QKO crypts, we suggest that the regulation of S–G2 targets requires direct binding by E2f or Myc, whereas the regulation of G1–S targets most likely involves additional factors beyond E2f and Myc.

Myc drives ectopic proliferation of Rb-deficient intestinal cells

Previous work suggested that distinct mechanisms regulate the proliferation of normal and cancer cells41–43. Rb loss results in excessive proliferation of transit-amplifying progenitor cells, even after they migrate, differentiate and begin to populate the length of the villus44. To explore the roles of E2f and Myc in an abnormal hyper-proliferative context, we initially queried expression profiles derived from β-NF-treated Rbflp/flp (control) and Ah-cre;Rbflp/flp (Rb KO) villus-enriched fractions. In addition to the expected
upregulation of E2f target genes in Rb-deficient villi, there was a striking increase in the expression of many known Myc target genes, as defined by previous gene expression, reporter and ChIP assays (Supplementary Table 6). We thus tested whether Myc may be playing a role in driving the ectopic proliferation caused by Rb deficiency. Remarkably, ablation of Myc suppressed the ectopic proliferation of Rb-deficient villus cells to a similar extent as loss of E2f3-deficient villi cells to a similar extent as loss of E2f1–3 (390 from a total 701 genes; Fig. 6c,d and Supplementary Table 3. In crypts, the number of E2f3 peak

Individual genes (701) dysregulated in Rb KO villi

(701 genes) is ameliorated in Rb/E2f QKO and Rb/Myc DKO villi. (c) Venn diagram showing the overlap between genes with expression levels rescued by loss of E2f1–3 or Myc (complete and partial rescue). Data in a–c were collected from n = 3 mice per genetic group for E2f-rescue experiments and n = 5 mice per genetic group for Myc-rescue experiments. (d) RT–qPCR analysis for a subset of G1–S-related genes. Expression levels for individual mice are plotted (2 or 3 per genetic group as indicated) and error bars represent mean ± s.d. from n = 3 technical replicates.

Redeployment of Myc and E2f3 in Rb-deficient cells

We then profiled Myc and E2f3 chromatin occupancy in Rb-deficient crypts/villi. A summary of the ChIP-exo-seq data is presented in Fig. 7a and Supplementary Table 3. In crypts, the number of E2f3 peak
Figure 7 Rb loss redefines the chromatin-binding landscape of E2f3 and Myc. (a) Heatmap representation of tag intensity for all E2f3- and Myc-binding locations in Rb KO intestines. Data were collected from pooled crypts (n=27 mice) or villi (n=7 mice). (b) Genomic spatial distribution of all E2f3 and Myc peak summits in control and Rb KO tissues (data for control tissues from Fig. 4c are included here for comparison). The number of summits in each tissue compartment is shown in parentheses. Data were collected from pooled crypts (n=32 mice for control, n=27 mice for Rb KO) or villi (pooled from n=7 mice). Gene bodies for individual genes were divided into 100 bins and summit locations were accordingly assigned a genomic position. (c) Genomic spatial distribution of E2f3 and Myc peak summits associated with dysregulated genes in Rb KO villi. The number of summits in each compartment is shown in parentheses. Data represent a subset of b and classification of genomic regions is the same as in b. (e,f) Peak summit-distance plots for E2f3 summits (e) and Myc summits (f) in control crypts and Rb KO villi that are associated with the 701 dysregulated genes in Rb KO villi. (g) Heatmap representation of tag intensity for all E2f3- and Myc-binding locations in control crypts and Rb KO villi. (h) Tag intensity plots (tags per base pair per peak per 100 M reads) around the peak summits associated with genes dysregulated in Rb KO villi. The canonical DNA-binding motifs for E2f3 (TTCGCCGC) and Myc (CACGTG) are highlighted in blue and red rectangles, respectively. Data in d–h were collected from pooled crypts (n=32 mice for control, n=27 mice for Rb KO) or villi (n=7 mice for control, n=7 mice for Rb KO).
summits was unaffected by loss of Rb, whereas the number of Myc summits was significantly decreased (Fig. 7b, left panel). In villi, the number of E2f3 peak summits was increased by loss of Rb but the number of Myc summits was decreased (Fig. 7b, left panel). The spatial distribution of peak summits across gene regions is illustrated in Fig. 7c.

**Figure 8** Myc regulates E2f3a expression in Rb-deficient villi. (a) IHC staining of β-catenin. (b) IHC staining of Myc. Data in a and b are representative images from n=3 mice for each genetic group. (c) RT-qPCR analysis for E2f3a and E2f3b in control, Rb KO and Rb/Myc DKO villi. Expression levels for individual mice are plotted (4 per genetic group) and error bars represent mean ± s.d. from n=3 technical replicates. (d) IHC staining of E2f3a in control, Rb KO, Rb/Myc DKO and Rb/E2f QKO samples. Note the nonspecific staining of blood cells in the lumen of the villi. Data are representative images from n=3 mice per genetic group. (e) ChIP-PCR analysis showing E2f3 loading to target genes (Pbk and Rrm1) in control, Rb KO and Rb/Myc DKO villi. n=4 mice per genetic group. The 5’ region ∼1 kb away from the TSS of Gapdh was used as the negative control. (f) ChIP-exo-seq tracks showing E2f3 and Myc occupancy on the E2f3 locus. Distinct promoter regions for E2f3a and E2f3b are shaded in red and yellow, respectively. Data were collected from pooled crypts (n=32 mice for control, n=27 mice for Rb KO) or villi (n=7 mice for control, n=7 mice for Rb KO). (g) RT-qPCR analysis for E2f1 and E2f2 in control and Rb KO villi. Expression levels for individual mice are plotted (4 for each genetic group) and error bars represent mean ± s.d. from n=3 technical replicates. (h) Diagrams summarizing the regulation of cell cycles by Myc and E2f1–3 in wild-type and Rb-deficient cells. Scale bars represent 25 μm (a) and 50 μm (b–d). Scale bars in f represent 1 kb.

*P < 0.05, one-tailed Student’s t-test. Errors bars represent mean ± s.d. from n=3 mice for each genetic group. (f) ChIP-exo-seq tracks showing E2f3 and Myc occupancy on the E2f3 locus. Distinct promoter regions for E2f3a and E2f3b are shaded in red and yellow, respectively. Data were collected from pooled crypts (n=32 mice for control, n=27 mice for Rb KO) or villi (n=7 mice for control, n=7 mice for Rb KO). (g) RT-qPCR analysis for E2f1 and E2f2 in control and Rb KO villi. Expression levels for individual mice are plotted (4 for each genetic group) and error bars represent mean ± s.d. from n=3 technical replicates. (h) Diagrams summarizing the regulation of cell cycles by Myc and E2f1–3 in wild-type and Rb-deficient cells. Scale bars represent 25 μm (a) and 50 μm (b–d). Scale bars in f represent 1 kb.
Three major parameters were analysed and compared between wild-type and \(Rb\)-deficient crypts and villi: DNA-binding location, DNA-binding strength and DNA-binding sequence motifs.

We first focused on the DNA-binding location of E2f3 and Myc to target genes dysregulated by \(Rb\) deficiency (Supplementary Table 9). E2f3 binding, but not Myc binding, was highly enriched in promoter regions (Fig. 7d). We then compared E2f3 binding in the two proliferative compartments, wild-type crypts and \(Rb\)-deficient villi, by measuring the distance between a given summit in wild-type crypts (as a reference; Fig. 7e, light blue line) and its corresponding summit in \(Rb\ KO\) villi; the same comparison was performed with the \(Rb\ KO\) villi as the reference compartment (Fig. 7e, dark blue line). A distance of 0–100 base pairs (bp) between corresponding summits in crypts and villi indicates overlapping peaks and suggests that binding of E2f3 was not altered between compartments; a distance greater than 100 bp between corresponding summits suggests a movement or disappearance of E2f3 occupancy from its original position. The ‘peak summit-distance plots’ shown in Fig. 7e revealed that approximately half of the E2f3 summits in control crypts had corresponding summits in \(Rb\)-deficient villi at distal locations greater than 100 bp (light blue line). Consistent with this analysis, heatmaps of sequence tags showed that a portion of E2f3 summits in control crypts and \(Rb\)-deficient villi were identical, whereas a large portion of summits present in control crypts were reduced/absent in the corresponding positions in \(Rb\)-deficient villi (Fig. 7g). Moreover, a significant number of E2f3 summits in \(Rb\)-deficient villi were reduced/absent in control crypts. Summit-distance plots and heatmaps comparing E2f3 peak summits across other possible compartment and genotype combinations are shown in Supplementary Figs. 4a–d. Examples of E2f3 binding to specific genes are shown in Supplementary Fig. 5a–c. Together, these findings show that \(Rb\) deficiency in villi results in the recruitment of E2f3 to some of the same chromatin positions it normally occupies in control crypts, but also to new chromatin positions. A similar analysis failed to show a redistribution of Myc binding from wild-type crypts to \(Rb\)-deficient villi (Fig. 7f,g). However, comparison of control and \(Rb\)-deficient crypts revealed the appearance of ‘new’ Myc-specific summits in \(Rb\)-deficient crypts (Supplementary Fig. 4e–h; also see below). Thus, E2f3 is redistributed in \(Rb\)-deficient villi and Myc is redistributed in \(Rb\)-deficient crypts.

We then focused on the DNA-binding strength of E2f3 and Myc to target genes dysregulated by \(Rb\) deficiency. The intensity plots shown in Fig. 7h and Supplementary Fig. 6a illustrate an increase in the E2f3- and Myc-sequence tag intensity in \(Rb\ KO\) versus control tissues, particularly in villi, indicating that E2f3 and Myc binding strength is increased on \(Rb\) loss.

Finally, E2f3 and Myc DNA-binding sequence motifs on target genes were analysed by de novo motif algorithms. This analysis shows that E2f3 predominantly utilizes E2f canonical DNA-binding elements irrespective of \(Rb\) deletion status or tissue compartment being analysed (Fig. 7h top panels and Supplementary Figs 6 and 7). In contrast, Myc utilizes a variety of non-canonical motifs in villi and control crypts but utilizes canonical E-box elements in \(Rb\ KO\) crypts (Fig. 7h bottom panels and Supplementary Figs 6 and 7).

From the combined analysis described above, which includes changes in the number of binding events as well as changes in DNA-binding location/distribution, DNA-binding strength, and DNA-binding sequence motifs, we conclude that loss of \(Rb\) results in a major spatial redeployment of E2f3 binding in villi and a refocusing of Myc binding in crypts.

**Myc is required for the accumulation of E2f3 in \(Rb\)-deficient villi**

We then explored the mechanistic relationship between Myc and E2fs in regulating gene expression. Previous studies established Myc as a critical downstream effector of Apc/\(\beta\)-catenin signalling. Several nodes of crosstalk between canonical Apc/\(\beta\)-catenin/Myc and Cdk/Rb/E2f pathways have also been described. Thus, we investigated the possibility that Apc/\(\beta\)-catenin/Myc signalling might be increased in \(Rb\)-deficient intestinal cells and contribute to their ectopic proliferation. Immunostaining of intestinal sections showed identical levels and localization of \(\beta\)-catenin protein in control and \(Rb\ KO\) small intestines (Fig. 8a). Moreover, comparison of global mRNA profiles from control and \(Rb\ KO\) villi (Fig. 6a) showed no change in the expression of a large cadre of known \(\beta\)-catenin target genes, including Myc itself (Supplementary Fig. 8a and Supplementary Table 10). Indeed, IHC of tissue sections showed that Myc protein levels were unchanged by loss of \(Rb\) (Fig. 8b). These results suggest that loss of \(Rb\) does not cause a general increase in Apc/\(\beta\)-catenin/Myc signalling in villi.

We then considered the possibility that Myc may regulate E2f expression as previous cell culture studies have shown. The E2f3 locus encodes two isoforms, E2f3a and E2f3b, driven by distinct promoters. Quantitative polymerase chain reaction with reverse transcription (RT-qPCR) and IHC assays showed that E2f3a (but not E2f3b) expression was markedly increased in \(Rb\ KO\) villi compared with wild-type controls, and that this increase was suppressed by loss of Myc (Fig. 8c,d). Furthermore, E2f3 ChIP assays showed increased recruitment of E2f3 to target promoters in \(Rb\ KO\) villi compared with wild-type controls, and that this increase was attenuated by loss of Myc (Fig. 8e).

We then evaluated Myc binding to the E2f3 locus in both crypts and villi. ChIP-exo seq data from both control and \(Rb\)-deficient crypts revealed an abundance of Myc protein residing close to the TSS of the E2f3a gene (Fig. 8f). Myc binding to the E2f3 locus was absent in villi. Binding of E2f3 to a region just upstream of the E2f3a TSS (Fig. 8f), which contains E2f-binding elements that positively regulate its expression, was increased in \(Rb\ KO\) villi compared with controls. Furthermore, the expression of E2f1 and E2f2, whose promoters are occupied by E2f3 and are also E2f auto-regulated, was increased in \(Rb\)-deficient villi (Fig. 8g and Supplementary Fig. 8b). Together, these findings suggest that Myc binding to the E2f3a promoter in \(Rb\)-deficient crypts contributes to the ectopic accumulation of ‘free’ E2f3a protein in villi. We propose that Myc-mediated E2f3a accumulation engages a positive feedback loop culminating in the execution of an E2f gene expression program that includes G1–S genes and drives unregulated cell proliferation.

**DISCUSSION**

Cell proliferation is orchestrated by developmental and environmental mitogenic cues that are transmitted through signalling pathways controlling key events in the cell cycle. The convergence of multiple pathways provides cells with the necessary robustness (redundancy).
to withstand perturbations in any single pathway that might otherwise compromise cell cycle progression. We show here that Myc and E2f converge on the control of S–G1 in normal proliferating cells of the small intestine, but switch to the control of G1–S when Rb is inactivated (Fig. 8h). These experiments uncover a molecular mechanism to explain how normal Myc and E2f pathways may be exploited and redirected by inactivation of the Rb tumour suppressor to foster unregulated cell proliferation, and highlight the unprecedented addiction of these Rb-deficient cells on Myc.

We show an unexpected collaboration between Myc and E2f in the synergistic regulation of an S–G2 transcriptional program required for the cell cycle progression of progenitor cells. The immediate consequence of ablating Myc and E2f1–3 from progenitor cells is an arrest in S and/or G2. Whether intestinal stem cells have a similar requirement for Myc and E2f1–3 requires further rigorous evaluation. The exact mechanisms that underlie the cooperative roles of Myc and E2f in regulating S–G2 transcription remain to be determined but most likely involve the co-recruitment of Myc and E2f1 to target promoters and interactions with chromatin remodelling factors as shown previously in vitro.31,32

The observation that G1–S programs are refractory to the combined loss of Myc and E2f1–3 is surprising. Recently, the combined ablation of N-Myc with E2f1–3 in mouse embryos was shown to impede DNA replication in retinal progenitor cells.37 The retina is a non-regenerating tissue where cell proliferation is restricted to a narrow developmental window late in embryogenesis. The caveat in studying non-regenerating tissues is that in the absence of a continuous replenishing pool of progenitor cells, an arrest at any cell cycle stage would exhibit reduced expression of G1–S target genes and score negative by assays that measure DNA replication (that is, BrdU), precluding an understanding of the underlying nature of the arrest. Thus, it remains possible that Myc and E2f may also converge to regulate S–G2 in the mouse retina as we show here in the small intestine. Although G1–S transcription is insensitive to the combined loss of Myc and E2f1–3, we show that Myc and E2f do indeed bind to G1–S-related targets. Thus, it is possible and perhaps likely that Myc and E2f may contribute to G1–S transcriptional control, but only in collaboration with additional transcriptional inputs yet to be identified.33 Despite extensive functional redundancy, the Myc and E2f pathways also seem to have unique roles. For example, we show here that E2f1–3 helps maintain genomic integrity, whereas Myc supports the necessary biosynthetic capacity to fuel robust cell proliferation.33

In contrast to normal cells, ectopic cell cycle progression of Rb-deficient intestinal cells is acutely dependent on Myc and E2f1–3. The analysis described here provides molecular insights into how loss of Rb subverts the existing Myc transcription machinery to support unregulated proliferation. While physiological levels of Myc in normal crypts utilize a variety of non-canonical DNA binding sequences,34 the loss of Rb in crypts leads to the recruitment of Myc to target genes through the classical E-box sequence motif, as recently described for overexpressed or amplified Myc (refs 55–58). The mechanisms for how this shift to the use of E-box DNA-binding elements in crypts is established and how this might impact transcription in villi remain to be determined. It is possible that loss of Rb results in a global reconfiguration of chromatin59–62, which exposes cryptic DNA elements that enhance Myc binding and activity. Myc may contribute to the transcriptional output in Rb-deficient villi in two ways that are not mutually exclusive. On one hand, Myc binding to regulatory sequences of E2f3a in crypts may facilitate its expression, which in the absence of Rb-mediated repression would lead to the accumulation of ‘free’ E2f3a protein. This event would then engage a positive feed-forward loop to activate expression of all three E2f activators and increase total E2f transcriptional activity. In this view, Myc in crypts indirectly participates to amplify the output of the E2f program. On the other hand, the redirecting of Myc binding to E-box elements in Rb-deficient crypts may impose lasting chromatin structural changes that facilitate increased binding of E2f and possibly other transcription factors to their target sites, even in subsequent cell cycles. This may provide an additional molecular mechanism for how the function of Rb in crypts is essential to suppress ectopic proliferation in villi.

Loss of Rb also subverts the existing E2f transcription machinery to support unregulated proliferation. ChIP-exo-seq analysis shows that Rb deficiency results in the accumulation and recruitment of E2f3 in villi to some of the same chromatin positions it normally occupies in control crypts, but also to new chromatin locations not normally used in proliferating crypts. Binding of E2f3 to chromatin, like that of other transcription factors, is a dynamic process that is continuously in flux owing to on–off binding kinetics. In addition, E2f3 protein acutely decreases in late S–G2 and must be remade and reloaded on chromatin at the next G1–S. The broad ectopic redeployment of E2f3 to ‘old’ and ‘new’ chromatin locations in Rb-deficient villi culminates in the precocious expression of a transcriptional program that drives unregulated proliferation. In summary, we show that Myc conspires with inactivation of Rb to regulate and redeploy E2f3 across the entire genome, providing a molecular mechanism that explains how Rb-deficient cells rely on or become addicted to Myc. □

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H.L. and G.L. designed the experiments. H.L., P.D., B.H., S.R., N.F., A.B., R.K., C.K. and M.T.S.-R. performed the experiments, collected and analysed data. X.T., A.S., T.P., K.H., R.M. and P.C. performed bioinformatic and statistical analysis for this manuscript with inputs from all authors.

COMPETING FINANCIAL INTERESTS

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1. Method: Mice. Mouse usage has been approved by the Institutional Animal Care and Use Committee at the Ohio State University. Mouse strains used in this study (Ah-cre, Rb<sup>1641/1641</sup>, E2f3<sup>−/−</sup>, E2f3<sup>−/−</sup> Ah<sup>Cre</sup> and Myc<sup>521/521</sup>) have been described before and were maintained in a mixed background (C57BL/6 x 129 x FVB/N). Primers were used for genotyping the mice are listed in Supplementary Table 11. Both male and female mice were used in the studies. Within each mutant genetic group, the animals were randomly used. Lartermates with appropriate genotypes were used as the control group. The number of mice used for analysis is described in the legends and no statistical method was used to determine sample size. The investigators were blinded to group allocation during experiments and outcome assessment. No criteria were used to exclude samples or animals from the analysis.

2. β-naphthoflavone, BrdU and EDU injection. To induce Ah-cre expression, 80 mg kg<sup>−1</sup> body weight of β-naphthoflavone (Sigma-Aldrich; N3633) dissolved in corn oil (Sigma-Aldrich; C8267) was administrated into 2-month-old mice with 5 injections within 30h. For DNA synthesis detection, 100 mg kg<sup>−1</sup> body weight of BrdU (Sigma-Aldrich; B5002) or 5 mg kg<sup>−1</sup> body weight of EDU (Life Technologies; C10337) dissolved in sterile phosphate-buffered saline (PBS) was intraperitoneally injected 2h or 1h before the mice were euthanized, respectively. EDU staining was performed following the manufacturer’s protocol (Life Technologies; C10337).

3. Tissue preparation and histology. The tissue used for RNA and histology was collected from the stomach-proximal 10-cm region of the intestine for chromatin immunoprecipitation (ChIP), the intestine was divided into three parts of equal length and the first third proximal to the stomach was used. Intestinal tissues were divided into ~1 cm pieces and fixed with 10% pH-buffered formalin (Fisher Scientific; 23-245-685) for 48 h at room temperature, embedded in paraffin and cut into 4 μm sections for histological staining. H&E images were representative of n = 3 or 4 animals per genetic group with minimal 3 fields per animal.

4. Infection Diseases (NIADC). NIH. The degree of rescue (D) for mRNA level of a given gene in the waterfall plot in Fig. 6b is defined as:

\[
D = \frac{A - B}{A} \times 100
\]

A: mRNA level fold change between Rb KO and control; B: mRNA level fold change between Rb/E2f QKO (or Rb/Myc DKO) and control.

5. Immunostaining. After deparaffinization, antigen retrieval was performed with boiling citrate buffer (Dako; S619984) for 30 min except that proteinase K was used for BrdU and lysozyme. Specifically, 2 N HCl was further applied to tissue sections for exposing and detecting BrdU and then neutralized with 0.1 M sodium tetraborate (pH 8.0). For immunohistochemistry staining, the endogenous peroxidase was inactivated by incubation with 3% H<sub>2</sub>O<sub>2</sub> for 15 min at room temperature. Normal horse serum was applied as the blocking reagent except that Mouse on Mouse (M.O.M.) Blocking Reagent (Vector Labs; MKB-2213) was applied for primary antibodies raised in mice. Antibodies and dilutions used in this study were as follows: BrdU (Dako; M0744, 1:50), enterin (Santa Cruz; sc-13015, 1:100), Ser-10 phosphorylated histone 3 (Millipore; 06-750, 1:250), cleaved caspase-3 (Cell Signaling; 9661, 1:100), phosphorylated H2AX (Cell Signaling; 9718, 1:100), Ccn2 (Santa Cruz; sc-596, 1:250), Cdc2 (Santa Cruz; sc-54, 1:100), Mcm3 (Santa Cruz; sc-9850, 1:500), Pena (Santa Cruz; sc-56, 1:500), lysozyme (Dako; A0099, 1:100), Myc (Santa Cruz; sc-764, 1:500), E2f 3 (Millipore; 05-551, 1:100) and β-catenin (AbD Serotec; OBT1683, 1:250). Proteins were detected with biotinylated goat secondary anti-rabbit IgG antibody (Vector Labs; BA-1000, 1:250) and DAB substrate kit (Vector Labs; SK-4100). donkey anti-mouse Alexa Fluor 488 or Alexa Fluor 594, donkey anti-goat Alexa Fluor 594, donkey anti-rabbit Alexa Fluor 594 (Invitrogen, 1:250). TSA Plus Fluorescin kit (Perkin Elmer; NEL741001KT) was used for visualizing geminin signals. The nuclei were counterstained with haematoxylin or 4',6-diamidino-2-phenylindole (DAPI). The images were collected using Nikon Eclipse 50i (immunohistochemistry) or Zeiss Axioskop 40 (immunofluorescence) microscopes with a Zeiss AxioCam HRC camera and AxioVision software. IHC and immunofluorescence images were representative of n = 3 or 4 animals per genetic group with minimal 3 fields per animal, except n = 2 animals per genetic group were used for Supplementary Fig. 2a.

6. ChIP-PCR. Freshly isolated crypts/villi were incubated in PBS with 1% formaldehyde at 37 °C for 15 min. The DNA-protein crosslink reaction was terminated by incubation with 0.125 M glycine for 5 min. The sample was washed in ice-cold PBS, resuspended in cytosolic lysis buffer (5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% NP-40 and proteinase inhibitors) and nuclear lysis buffer (50 mM Tris (pH 8.0), 10 mM EDTA, 1% SDS and proteinase inhibitors). The chromatin was sonicated to obtain DNA fragments between 100–300 bp. The fragmented chromatin was diluted 1:10 with IP dilution buffer (16.7 mM Tris (pH 8.0), 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100 and protease inhibitors) and pre-cleared by incubating with Protein G Plus/Protein A beads (Calbiochem; IP05) at 4 °C for 1 h on a rotator before adding antibodies. Antibodies used were E2f 3 (Santa Cruz; sc-878 X), Myc (Santa Cruz; sc-764 X) and normal rabbit IgG (Santa Cruz; sc-2027). The DNA–protein–antibody–bead complex was washed twice with each following buffer in the presence of proteinase inhibitors: low-salt buffer (20 mM Tris (pH 8.0), 2 mM EDTA, 1% Triton X-100, 0.1% SDS and 150 mM NaCl), high-salt buffer (20 mM Tris (pH 8.0), 2 mM EDTA, 1% Triton X-100, 0.1% SDS and 500 mM NaCl), LiCl buffer (10 mM Tris (pH 8.0), 1 mM EDTA, 250 mM LiCl, 1% NP-40 and 1% deoxycholic acid) and Tris–EDTA buffer (pH 8.0). Immunoprecipitated chromatin was retrieved from Protein G Plus/Protein A beads in 1% SDS plus 0.1 M NaHCO<sub>3</sub> and reverse-crosslinked with 0.5 M NaCl and 0.1 mg ml<sup>−1</sup> Proteinase K and the DNA was purified with Qiagen kits (Qiaprep Purification Kit (Qiagen; 28104)) and DNA detected by quantitative PCR. Primers used for ChIP quantitative PCR are listed in Supplementary Table 11. One-tailed Student’s t-test was performed to determine whether E2f 3 binding to target genes was decreased in Rb/Myc DKO villi compared with Rb KO villi in Fig. 8e.

7. ChIP-exo-seq library construction and Illumina sequencing. Cysts from 32 control mice and 7 Rb KO mice and villi from 7 control mouse and 7 Rb KO mice were used for ChIP-exo-seq. Cysts collected from every 2–5 mice of the same genotype were combined and processed as one sample. The crosslink, cytosolic lysin, nuclear lysin, sonication, dilution with IP dilution buffer, pre-clean and immunoprecipitation steps were performed as described above except Pierce Protein A/G Magnetic Beads (Thermo Scientific; 88002) were used. Before immunoprecipitation, the pooled chromatin derived from each genetic/tissue compartment was divided into two halves for E2f 3- and Myc-ChIP, respectively. The library construction steps including all on-bead enzymatic reactions (end polishing, P7 exo-adapter ligation, nick
repair, λ-exonuclease digestion, RecJ, exonuclease digestion), elution and reverse crosslinking, primer extension and P5 exo-adaptor ligation were performed as described previously\(^3\). The resulting DNA was enriched by 13 cycles of PCR using NEBNext High-Fidelity PCR Master Mix (NEB; M0541S) before measuring the library concentration and DNA size distribution. Cluster generation on Illumina cBot and single-end high-throughput sequencing on Illumina HiSeq 2500 platform were performed at the Ohio State University Shared Resources.

ChIP-exo-seq data analysis. The 50 bp sequencing reads were de-multiplexed on the basis of the barcodes in index adaptors used in the library construction, followed by mapping the reads to a reference mouse genome (GRCm37/mm9) with the Bowtie2 aligner (version 2.2.1; ref. 65). Only the reads uniquely mapped to the genome were used for further analysis. E2f3 and Myc DNA-binding peaks were identified using the Genome wide Event finding and Motif discovery algorithm GEM (version 2.4.1; ref. 66) with a false discovery rate (FDR) less than 1% and the Model-based Analysis of ChIP-Seq algorithm MACS2 (version 2.0.10; ref. 67) with a FDR less than 5%. The intersection of GEM and MACS2 results (when the distance between corresponding summit coordinates predicted by these two algorithms was less than 1 kb) was used for all downstream analysis. The DNA-binding peaks were associated to genes on the basis of the minimal distance between peak summits and proximal transcription start sites. Motif discovery and matching de novo to known motifs were performed with HOMER algorithm (version 4.6; ref. 68) using DNA sequences around peak summits (±50 bp). Integrative Genomics Viewer algorithm IGV (ref. 69) was used to visualize the binding events and reads coverage. The BED files with detailed information for identified peaks have been deposited in the GEO database with accession number GSE56009. The computer code for ChIP-exo-seq analysis can be accessed on request.

Flow cytometry. Isolated crypts were homogenized using a Dounce homogenizer in ice-cold suspension buffer (25 mM Tris (pH 7.3), 50 mM KC1, 2 mM MgCl\(_2\), 1 mM EDTA and 1 mM phenylmethylsulphonyl fluoride). After brief centrifugation, the pellet was washed twice with the suspension buffer and once with PBS. A single-cell suspension was then prepared in PBS containing 0.1% Triton X-100, 25 μg ml\(^{-1}\) propidium iodide (Roche; 11348639001) and 20 μg ml\(^{-1}\) RNase A (Invitrogen; 12091-021) and incubated 1 h at room temperature in the dark. The suspension was then filtered through 35 μm cell strainer caps (BD Falcon; 352235). The DNA content measured by the fluorescence intensity of propidium iodide was analysed using a BD LSR II Flow Cytometer at the Ohio State University Shared Resources. The data were analysed with FlowJo cytometric analytical software.

Statistics. Student's \(t\)-test and empirical Bayes method were performed. The data have normal distributions that meet the assumptions of the tests. Whether the variance is similar between the groups that are being statistically compared was not estimated. All data are presented as mean ± s.d.

Data deposition. Affymetrix gene expression and ChIP-exo-seq data have been deposited in the GEO database with accession number GSE56009.

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Supplementary Figure 1 Loss of E2f1-3 and/or Myc in crypts. (a) Dual staining of EdU (red) and geminin (green, included here for the composition of merged images) in crypts from intestines harvested 2 days after induction of Ah-cre expression. Nuclei were stained with DAPI (blue). Data are representative images from n=3 mice per genetic group. (b) Quantification of EdU and geminin staining. Data presented as mean ± s.d., n=3 mice per genetic group. (c) IHC staining of Myc in intestines harvested 2 days after induction of Ah-cre expression. Data are representative images from n=3 mice for each genetic group. (d) Alcian Blue staining for goblet cells (blue) and IF staining of lysozyme for paneth cells (red) in intestines harvested 2 days after induction of Ah-cre expression. Nuclei were counterstained with Nuclear Fast Red or DAPI. Data are representative images from n=3 mice per genetic group. Scale bars in a, c and d represent 50 μm.
Supplementary Figure 2 DNA binding of E2f3 and Myc in wild type crypts. (a) IHC staining of E2f3 using the antibody (Millipore; 05-551) in control, E2f3a−/−, E2f3b−/− and Ah-cre;E2f1−/−;E2f2−/−;E2f3loxP/loxP intestines. Data are representative images from n=2 mice per genetic group. E2f3a−/− but not E2f3a−/− intestines show positive-stained cells, indicating the specific recognition of E2f3a isoform by this antibody. Note the non-specific staining of blood cells. (b) Heatmap representation of differentially expressed genes in wild type crypts (n=5) versus wild type villi (n=5), P<0.01, empirical Bayes method. (c) ChIP-exo-seq track examples showing E2f3 and Myc binding to selected G1-S and S-G2 related genes in wild type crypts. Examples are derived from pooled crypts (n=32 mice). Scale bars in a represent 25 µm. Scale bars in c represent 1kb.
Supplementary Figure 3 Loss of either E2f1-3 or Myc corrects aberrant transcription in Rb KO villi. RT-qPCR analysis of mRNA levels for a subset of genes in control, Rb KO, Rb/E2f QKO and Rb/Myc DKO villi. Expression levels from individual mice are plotted (2 or 3 per genetic group as indicated) and error bars represent mean ± s.d. from n=3 technical replicates. The aberrant expression of these genes in Rb KO villi were normalized by loss of either E2f1-3 or Myc. The average expression level of control samples was set as 1.
Supplementary Figure 4  Peak summit-distance plots and tag intensity heatmaps across tissue compartments and genetic groups. (a) Peak summit-distance plots for E2f3 summits that are associated with the 701 dysregulated genes in Rb KO villi. E2f3 binding is compared between crypts and villi of the same genetic group. (b) Heatmap representation of tag intensity for all E2f3 binding locations. E2f3 binding is compared between crypts and villi of the same genetic group. (c) Peak summit-distance plots for E2f3 summits that are associated with the 701 dysregulated genes in Rb KO villi. E2f3 binding is compared between control and Rb KO intestines. (d) Heatmap representation of tag intensity for all E2f3 binding locations. E2f3 binding is compared between control and Rb KO intestines. (e) Peak summit-distance plots for Myc summits that are associated with the 701 dysregulated genes in Rb KO villi. Myc binding is compared between crypts and villi of the same genetic group. (f) Heatmap representation of tag intensity for all Myc binding locations. Myc binding is compared between crypts and villi of the same genetic group. (g) Peak summit-distance plots for Myc summits that are associated with the 701 dysregulated genes in Rb KO villi. Myc binding is compared between control and Rb KO intestines. (h) Heatmap representation of tag intensity for all Myc binding locations. Myc binding is compared between control and Rb KO intestines. Data in this figure (a-h) are derived from pooled crypts (n=32 mice for control, n=27 mice for Rb KO) or villi (n=7 mice for control, n=7 mice for Rb KO).
**Supplementary Figure 5** E2f3 DNA binding in wild type and Rb KO villi. 

(a-c) Three main categories of target genes having different patterns of E2f3 DNA binding in wild type and Rb KO villi are illustrated: Group 1 includes target genes with E2f3 peaks present in Rb KO villi but absent in control villi; Group 2 includes target genes with distinct E2f3 peaks in control and Rb KO villi (either due to additional peak summits or peak position changes); Group 3 includes target genes with E2f3 peaks similarly positioned in control and Rb KO villi (but possibly with different magnitude of binding strengths). ChIP-exo-seq track examples are shown for group 1 (a), group 2 (b) and group 3 (c). Data in this figure are derived from pooled villi (n=7 mice for control, n=7 mice for Rb KO). Scale bars in a-c represent 1kb.
Supplementary Figure 6 DNA binding strength and DNA binding motifs of all Myc and E2f3 peaks. (a) Tag intensity plots (tags per bp per peak per 100M reads) around all peak summits identified in crypts and villi, as indicated. (b) Canonical DNA binding motifs (TTCCGCC motif for E2f3, underlined with blue lines; CACGTG motif for Myc, underlined with red lines) or strongest non-canonical motifs identified from all peak sequences in indicated genomic regions. Data in this figure are derived from pooled crypts (n=32 mice for control, n=27 mice for Rb KO) or villi (n=7 mice for control, n=7 mice for Rb KO).
### Crypts (peaks associated with dysregulated genes in Rb KO villi)

| Region                  | Motif     | % of peaks | p-value |
|-------------------------|-----------|------------|---------|
| **Control crypts E2f3 peaks** |           |            |         |
| 5' proximal             | TTCCCCGC  | 12.42%     | 1e-14   |
|                         | CCA       |            |         |
| Promoter                | TTCCCCGC  | 64.20%     | 1e-188  |
| Gene body               | TTCCCCGC  | 3.72%      | 1e-9    |
| 5' distal and 3' regions| TTCCCCGC  | 3.58%      | 1e-9    |
| **Rb KO crypts E2f3 peaks** |           |            |         |
| 5' proximal             | TTCCCCGC  | 21.28%     | 1e-16   |
|                         | CCA       |            |         |
| Promoter                | TTCCCCGC  | 49.83%     | 1e-210  |
| Gene body               | TTCCCCGC  | 6.53%      | 1e-9    |
| 5' distal and 3' regions| TTCCCCGC  | 4.90%      | 1e-9    |
| **Control crypts Myc peaks** |           |            |         |
| 5' proximal             | ACCGACCT  | 14.37%     | 1e-9    |
|                         | GAT       |            |         |
| Promoter                | TTCCCCGC  | 10.87%     | 1e-17   |
| Gene body               | TTCCCCGC  | 4.30%      | 1e-23   |
| 5' distal and 3' regions| TTCCCCGC  | 2.50%      | 1e-18   |
| **Rb KO crypts Myc peaks** |           |            |         |
| 5' proximal             | ACCGACCT  | 7.14%      | 1e-10   |
|                         | GAT       |            |         |
| Promoter                | TTCCCCGC  | 34.42%     | 1e-39   |
| Gene body               | TTCCCCGC  | 14.54%     | 1e-10   |
| 5' distal and 3' regions| TTCCCCGC  | 4.95%      | 1e-12   |

### Villi (peaks associated with dysregulated genes in Rb KO villi)

| Region                  | Motif     | % of peaks | p-value |
|-------------------------|-----------|------------|---------|
| **Control villi E2f3 peaks** |           |            |         |
| 5' proximal             | TACCCGCA  | 19.35%     | 1e-7    |
|                         | A          |            |         |
| Promoter                | TTCCCCGC  | 64.20%     | 1e-132  |
| Gene body               | TTCCCCGC  | 10.00%     | 1e-6    |
| 5' distal and 3' regions| AGTGGACAC  | 6.93%      | 1e-10   |
| **Rb KO villi E2f3 peaks** |           |            |         |
| 5' proximal             | TTCCCCGC  | 10.61%     | 1e-13   |
|                         | A          |            |         |
| Promoter                | TTCCCCGC  | 50.44%     | 1e-170  |
| Gene body               | TTCCCCGC  | 3.85%      | 1e-12   |
| 5' distal and 3' regions| TTCCCCGC  | 8.08%      | 1e-6    |
| **Control villi Myc peaks** |           |            |         |
| 5' proximal             | TGCTAGAGT  | 15.15%     | 1e-7    |
|                         | G          |            |         |
| Promoter                | TTCCCCGC  | 41.67%     | 1e-8    |
| Gene body               | TTCCCCGC  | 14.81%     | 1e-12   |
| 5' distal and 3' regions| TTCCCCGC  | 3.57%      | 1e-12   |
| **Rb KO villi Myc peaks** |           |            |         |
| 5' proximal             | ACCGACCT  | 29.41%     | 1e-8    |
|                         | GAT       |            |         |
| Promoter                | TTCCCCGC  | 57.14%     | 1e-8    |
| Gene body               | TTCCCCGC  | 14.29%     | 1e-8    |
| 5' distal and 3' regions| TTCCCCGC  | 25.0%      | 1e-8    |

**Supplementary Figure 7** DNA binding motifs of Myc and E2f3 peaks associated with the 701 dysregulated genes in Rb KO villi. Canonical DNA binding motifs (TTCCGC motif for E2f3, underlined with blue lines; CACGTG motif for Myc, underlined with reds lines) or strongest non-canonical motifs identified from peak sequences in indicated genomic regions of the 701 dysregulated gene in Rb KO villi. Data in this figure derived from pooled crypts (n=32 mice for control, n=27 mice for Rb KO) or villi (n=7 mice for control, n=7 mice for Rb KO).
Supplementary Figure 8 Expression of Wnt/β-catenin targets in Rb KO villi and auto-regulation of E2fs. (a) Venn diagram showing the overlap between the 701 genes dysregulated in Rb KO villi and the 111 Wnt/β-catenin target genes. (b) ChIP-exo-seq tracks showing E2f3 binding to the E2f1 and E2f2 loci. The differential binding between control and Rb KO villi is highlighted in blue. Examples are derived from pooled villi (n=7 mice for control, n=7 mice for Rb KO). Scale bars in b represent 1kb.
**Supplementary Table Legends**

**Supplementary Table 1** | Affymetrix gene expression profiles in control, *E2f TKO, Myc KO, E2f/Myc QKO* crypts

**Supplementary Table 2** | Gene ontology (GO) analysis for genes regulated by E2f1-3 and Myc in crypts

**Supplementary Table 3** | Summary of ChIP-exo-seq statistics

**Supplementary Table 4** | Differentially expressed genes between wild type crypts and villi and associated peaks

**Supplementary Table 5** | Differentially expressed cell cycle related genes between wild type crypts and villi and associated peaks

**Supplementary Table 6** | Myc target genes and their expression levels in control and *Rb KO* villi

**Supplementary Table 7** | Affymetrix gene expression profiles in control, *Rb KO, Rb/E2f QKO* and *Rb/Myc DKO* villi

**Supplementary Table 8** | Gene ontology for 390 dysregulated genes in *Rb KO* villi that were commonly rescued by loss of either *Myc* or *E2f1-3*

**Supplementary Table 9** | Dysregulated genes in *Rb KO* villi that were bound by E2f3 and Myc in different compartments/genetic groups and GO analysis of genes bound by E2f3

**Supplementary Table 10** | List of Wnt target genes

The original list of target genes defined by previous studies is from: http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes (Roel Nusse lab website). For the target genes identified in other species, corresponding mouse orthologs (one-to-one relationship based on Ensembl database) were identified and utilized to compare with Affymetrix gene expression profiles derived from *Rb KO* villi.

**Supplementary Table 11** | List of primers used in this study for genotyping mice, RT-qPCR analysis of mRNA levels and ChIP-PCR