The R2TP chaperone cooperates with HSP90 to integrate newly synthesized proteins into multi-subunit complexes, yet its role in tissue homeostasis is unknown. Here, we generated conditional, inducible knock-out mice for \textit{Rpap3} to inactivate this core component of R2TP in the intestinal epithelium. In adult mice, \textit{Rpap3} invalidation caused destruction of the small intestinal epithelium and death within 10 days. Levels of R2TP substrates decreased, with strong effects on mTOR, ATM and ATR. Proliferative stem cells and progenitors deficient for \textit{Rpap3} failed to import RNA polymerase II into the nucleus and they induced p53, cell cycle arrest and apoptosis. Post-mitotic, differentiated cells did not display these alterations, suggesting that R2TP clients are preferentially built in actively proliferating cells. In addition, high RPAP3 levels in colorectal tumors from patients correlate with bad prognosis. Here, we show that, in the intestine, the R2TP chaperone plays essential roles in normal and tumoral proliferation.
The R2TP complex was first discovered in Saccharomyces cerevisiae as an HSP90 co-chaperone. It is a large protein complex composed of six subunits (also called ‘clients’) that act in concert to facilitate the folding of substrates. HSP90 is a highly conserved eukaryotic ATPase that participates in the folding of many proteins, including signaling molecules, cytoskeletal proteins, and transcription factors. HSP90 associates with a heterohexamer of RUVBL1 and RUVBL2, which is a crucial component for the regulation of HSP90 ATPase activity. The chaperone activity of HSP90 is coupled to ATP hydrolysis, and the resulting ATPase cycle is essential for the proper folding of the client proteins.

In mammals, R2TP associates with a heterohexamer of PIH1D1 and RPAP3, which is a crucial component for the regulation of HSP90 ATPase activity. The R2TP complex is composed of a core complex of two subunits, PIH1D1 and RPAP3, and two additional subunits, RUVBL1 and RUVBL2. The R2TP complex is essential for the proper folding of many proteins, including signaling molecules, cytoskeletal proteins, and transcription factors.

RPAP3 has been identified as an HSP90 co-chaperone and is essential for the proper folding of many proteins, including signaling molecules, cytoskeletal proteins, and transcription factors. RPAP3 is a crucial component for the regulation of HSP90 ATPase activity. The R2TP complex is composed of a core complex of two subunits, PIH1D1 and RPAP3, and two additional subunits, RUVBL1 and RUVBL2. The R2TP complex is essential for the proper folding of many proteins, including signaling molecules, cytoskeletal proteins, and transcription factors.

In this work, we generated murine models bearing a conditional KO allele of Rpap3 to study the role of R2TP in intestinal homeostasis. Indeed, Rpap3 is central to R2TP, bridging together HSP90, PIH1D1, and RUVBL1/2 (Fig. 1a). PIH1D1 and RPAP3 are both involved in substrate recognition, while RPAP3 also recruits the chaperones HSP90 and HSP70. RUVBL1 and RUVBL2 are related AAA+ ATPases that also have chaperone activity. Multiple contacts between the PIH1D1:RPAP3 heterodimer and the RUVBL1/2 heterohexamer allow regulation of their ATPase activity. Importantly, the RPAP3:PIH1D1 heterodimer is specific to R2TP whereas RUVBL1/2 are also part of other complexes such as the chromatin remodelers INO80 and SRCAB. In mammals, R2TP also associates with a set of six prefoldins and prefoldin-like proteins, possibly to help protein folding. Altogether, R2TP associated with prefoldins has been termed the PAQosome, for Particle for Arrangement of Quaternary structure.

The first documented R2TP clients were the small nucleolar ribonucleoparticles (snoRNPs), which are required for the maturation of ribosomal RNA. These particles can be grouped into two families, the C/D and H/ACA snoRNPs, each composed of small nucleolar RNAs and proteins, which are required for ribosome biogenesis, DNA repair, and cell growth. The R2TP complex has been shown to be essential for the proper folding of these snoRNPs, which are crucial for ribosome biogenesis, DNA repair, and cell growth.

In the intestine, the most dynamically self-renewing tissue in adult mammals, the R2TP complex has been shown to be crucial for the proper folding of the intestinal crypt cell progenitors. The intestinal crypt cells are divided into two distinct populations, the proliferative progenitors (CBC) and the differentiating enterocytes. The CBC are the only cells that can differentiate into any cell type within the intestinal epithelium. The R2TP complex has been shown to be essential for the proper folding of the intestinal crypt cell progenitors, which are responsible for maintaining the intestinal epithelium.

Among these, the goblet cells are the most represented. These cells produce a lubricant protective mucus layer, and their density increases from the duodenum to the colon. In the small intestine, CBC stem cells also generate long-lived Paneth cells (3–6 weeks), which secrete anti-microbial peptides and maintain crypt niche conditions.

**Results**

**Rpap3 is an essential gene in mice.** To generate Rpap3 murine models, we tested different recombinant murine Embryonic Stem cells (ES cells) available from the KOMP consortium (Supplementary Fig. 1a, b). In the Rpap3<sup>fl/ox</sup> allele, a strong acceptor splice site is introduced before exon 7. The resulting mRNA encodes a Rpap3<sup>fl/ox</sup> protein truncated after the first PPR domain, at amino acid 223, followed by a selection cassette and the bacterial β-galactosidase gene (Supplementary Fig. 1a, b). Site-specific recombination at the Rpap3 locus was confirmed by Southern blot in the H10 clone (Supplementary Fig. 1c). Injection of H10 ES cells into blastocysts generated 3 chimeras with germinal transmission. Intercrossing of Rpap3<sup>fl/ox/+</sup> animals did not yield any homozygous Rpap3<sup>fl/ox/fl/ox</sup> animal, suggesting that this truncated Rpap3 allele is homozygous lethal (0/34 pups; Supplementary Fig. 1a, b).

We used the β-galactosidase cassette to characterize Rpap3 expression. In the small intestine of Rpap3<sup>fl/ox/+</sup> animals, we detected β-galactosidase activity exclusively at the bottom of the crypts, intercalated between lacZ negative cells (Fig. 1b). This could correspond to either CBC stem cells or Paneth cells (see below for a schematic representation of the small intestinal crypts). Pih1d1 interacts with Rpap3, and this interaction is crucial for Pih1d1 stability and R2TP activity. Pih1d1 is detected in CBC stem cells and progenitors but not in Paneth cells nor differentiated cells of the villus (Fig. 1c and below for a schematic representation of the crypts). In the colon, LacZ activity is weak, Pih1d1 is undetectable and both are absent from the stroma (Fig. 1c). Overall, R2TP expression is restricted to proliferative cells.

**Rpap3 is required for small intestine maintenance.** To generate a conditional KO strain, Rpap3<sup>fl/cre/Δ12</sup> mice were crossed with mice carrying a transgene encoding the Flippase Flp33. In the progeny, excision of the Flp-in cassette produced a Rpap3 allele with exon 7 flanked by loxp sites (Supplementary Fig. 1a). Rpap3<sup>fl/lox</sup> mRNA encodes a protein identical to wild-type Rpap3. Indeed, there was no bias in transmission nor any obvious phenotype in Rpap3<sup>fl/lox</sup> mice. Excision of exon 7 by Cre-recombinase produces a Rpap3<sup>Δ7</sup> mRNA with a premature termination codon in exon 8, predicted to be degraded by the nonsense-mediated decay pathway (Supplementary Fig. 1a, b).

To obtain a constitutive deletion of Rpap3 in the intestinal epithelium, Rpap3<sup>fl/lox</sup> mice were crossed with villin-Cre (VilCre) transgenic mice. The VilCre transgene encodes a constitutively active Cre that is specifically expressed in the epithelial cells of the small and large intestine. We were unable to generate homozygous VilCre; Rpap3<sup>fl/lox</sup> animals (0/100...
pups; Supplementary Fig. 1a), showing that the absence of \(Rpap3\) in the intestinal epithelium is lethal. To bypass this problem, we turned to an inducible \(\text{CreERT2}\) under the control of the same promotor (\(\text{VilCreERT2}\)). This Cre-recombinase is also specifically expressed in the intestinal epithelium but needs to be activated by tamoxifen\(^{34}\). \(\text{VilCreERT2}; \ Rpap3^{\text{fl/ox}}\) animals were healthy and did not present any obvious phenotype. We then injected intraperitoneally two doses of tamoxifen separated by an interval of 24 h in 8-week-old mice because the intestine is fully developed at this age. This yielded an efficient recombination, already detectable 1 day after the first injection and only in the small intestine and colon epithelium but not in any of the other organs tested (Supplementary Fig. 1d, e). As a consequence, \(Rpap3\) protein was no longer detected in epithelia from jejunum and...
Fig. 1 Rpap3 deletion compromises the small intestine and mouse survival. a Schematic representation of R2TP with its four subunits (RPAP3, PIH1D1, and the RUVBL1/2 heterohexamer). RPAP3 is the core subunit that contacts directly HSP90, PIH1D1 and RUVBL1/2. b β galactosidase activity in Rpap3fl/fl small intestines (top) and colon (bottom), as compared to negative controls (n = 2). Scale bars = 50 μm are identical for all images. c IHC of Pih1d1 in the small intestine. Counter coloration of DNA with hematoxylin, with magnification (bottom). Top picture: bar represents 50 μm. Inset: arrows point to CBC stem cells, intercalated between Paneth cells with distinctive granules in the cytoplasm; bar is 20 μm. Micrograph is representative of n = 3. d Depletion of Rpap3 after tamoxifen injection. Western blots were revealed with antibodies against the indicated proteins in extracts of the jejunum (left) and the colonic epithelia (Fig. 2b). In contrast, the intestines of the VilCreERT2; Rpap3fl/fl mice (right) lacked any phenotype (Fig. 2b, d). Altogether, these observations confirmed that the loss of Rpap3 induces the rapid disappearance of proliferating stem cells and progenitors, but not of differentiated Paneth cells.

Rpap3 promotes cell proliferation within the epithelium. To understand the basis for this rapid degeneration of the intestinal epithelium, we characterized Ki67 expression. Ki67 marks proliferative cells which are, in the intestine, the CBC stem cells and progenitors from the TA compartment (Fig. 3a, b). Immunostaining revealed a loss of Ki67 in the crypts of VilCreERT2; Rpap3fl/fl mice (Fig. 2b, d). To directly monitor cell cycling, BrdU was injected into the mouse peritoneum 2 h before sacrifice. BrdU is a nucleoside analog incorporated into the DNA during S-phase. BrdU staining was comparable from day 1 to day 6 in the crypts of control and VilCreERT2; Rpap3fl/fl animals. At day 7 however, there were significantly less BrdU+ cells (Fig. 3d, e). This is coherent with the loss of Ki67 staining and confirms cycle arrest in the crypts and the TA compartment of the mutant animals. Overall, such epithelium destruction, with remnants of Ki67+ crypt glands, phenocopies TCF4 loss35. TCF4 is the transcription factor that regulates Wnt signaling to sustain CBC stemness program36. This suggests that R2TP may be required for both CBC and progenitors.

To directly analyze CBC stem cells, we performed immunostaining for Olfm4, a trans-membrane protein commonly used for CBC identification37 (Fig. 4a, b and Supplementary Fig. 4a). Olfm4 staining was similar between control and VilCreERT2; Rpap3fl/fl mice from day 4 to day 6 after tamoxifen injection. However, at days 7 and 8, Olfm4 expression was undetectable in most VilCreERT2; Rpap3fl/fl crypts (Fig. 4a, b and Supplementary Fig. 2a), in agreement with the loss of Ki67+ staining (Figs. 2b, c). In the crypts, CBC stem cells intercalate between larger differentiated cells, called Paneth cells. These cells are Ki67+ and recognizable by the lysozyme marker (Fig. 4c, d) or by their typical supranuclear eosinophilic (cytoplasmatic) granules stained by Hematoxylin/Eosin (HE; Fig. 3c). In marked contrast to CBC stem cells, Paneth cells were detected in a comparable manner in the crypts of control and VilCreERT2; Rpap3fl/fl animals until day 8 (Figs. 3c, 4d). Altogether, these observations confirmed that the loss of Rpap3 induces the rapid disappearance of proliferating stem cells and progenitors, but not of differentiated Paneth cells.

Interestingly, we observed cells with round, dense nuclei, resembling apoptotic cells, in the crypts of small intestines deficient for Rpap3. We verified this by immunostaining for cleaved caspase 3, a well-established marker of apoptosis. In control mice, few cleaved caspase 3-positive cells were visible at the tip of some villi, where they are normally shed. We detected significantly more cleaved caspase 3+ cells in the crypts of tamoxifen-injected VilCreERT2; Rpap3fl/fl mice (Fig. 4e). These apoptotic cells were observed in the crypts and TA compartment, but not at the villi tips, as observed in normal epithelia (Fig. 4f). Thus, Rpap3 deletion eventually induces apoptosis of CBC stem cells and progenitors.

Rtap3 stabilizes clients of diverse families. A prominent R2TP client is Rpb1, the largest subunit of RNA polymerase II (RNA PolII)19,20. R2TP incorporates neo-translated Rpb1 within RNA PolII in the cytoplasm, after which it is imported into the nucleus19. As expected, immunohistochemistry (IHC) for Rpb1 displayed a nuclear staining in control intestines (Fig. 5a). In VilCreERT2; Rpap3fl/fl animals, 6 days after the first tamoxifen injection, when the epithelial architecture was still preserved, Rpb1 accumulated in the cytoplasm of CBCs and TA progenitors, but remained nuclear in differentiated epithelial cells, including Paneth cells (Fig. 5a). This suggested that Rpap3 is necessary to assemble RNA PolII in the proliferative compartment (Fig. 5b).

Misfolded HSP90 clients are usually degraded38, so we determined the expression levels of R2TP clients in VilCreERT2; Rpap3fl/fl animals at day 6. R2TP was initially characterized as an assembly factor for the box C/D snoRNPs, and in particular it chaperones NOP58, one of the four core proteins in these particles15,39. Western blot analysis of crypt cell lysates showed a near twofold reduction of NOP58 levels in extracts from VilCreERT2; Rpap3fl/fl animals, as compared to controls (Fig. 5c). R2TP stabilizes other non-coding RNPs in HeLa cells,
including the U5 snRNP, a splicing ribonucleoparticle that includes the proteins PRPF8 and EFTUD217,18. Expression of these proteins was reduced by nearly twofold in KO animals, showing their dependency on R2TP in the small intestine (Fig. 5c). HSP90 and R2TP are thought to stabilize clients only before their assembly, and the extreme stability of snoRNPs and snRNPs may explain this moderate but consistent decrease observed here for their components40.

In mammals, PIKKs consist of six structurally related proteins (ATR, ATM, DNA-PK, mTOR, TRRAP, and SMG1). PIKKs are stabilized by the trimeric co-chaperone TTT32,41–43 and in murine fibroblasts, TTT recruits R2TP to assemble PIKKs with their partners22. By Western blot, we observed again a strong diminution of ATR and ATM, the primary sensors of DNA damage, as well as of mTOR, which activates translation and cell proliferation in response to nutrient availability (Fig. 5d). These results show that R2TP participates in the stabilization of ATR, ATM, and mTOR. Yet, staining for γH2AX, a marker of DNA damage, was similar in control and KO tissues (Supplementary Fig. 3a). In contrast to the other PIKKs, TRRAP level did not vary, either because R2TP does not chaperone TRRAP or because it is a very stable protein.

Interestingly, in control tissues, these R2TP substrates are concentrated in the crypts rather than in the villi. This was observed for the U3 box C/D snoRNP (Supplementary Fig. 3b) and components of the U5 snRNP, as well as ATR.

**Fig. 2 Rtap3 is necessary to small intestine integrity.** a Schematic representation of the experimental setting: 8-week-old mice of the indicated genotype received two sequential injections of tamoxifen 24 h apart, and were analyzed 6 to 8 days after the first injection. b Pictures of jejunum tissue sections stained with HE (left) or by IHC with anti-Ki67 antibodies (right panels—Ki67 signal is brown) at different days after the first tamoxifen injection. Black arrowheads indicate remnants of crypt glands with Ki67+ cells observed at day 8. Scale bar is identical for all panels, and is 50 μm as shown in control HE panel. Each panel is representative of 8 to 12 animals from three independent experiments.
Altogether, these data suggest that R2TP assembles cellular machineries mainly in the CBCs and progenitors. R2TP KO triggers p53-dependent and independent apoptosis. Cell stress, including DNA damage and ribosome biogenesis defects, stabilize p53, which in turn arrests cell cycle and induces apoptosis. We observed a strong increase of p53 levels at day 6 in the crypts and TA compartment of KO mice (Fig. 6a, Supplementary Fig. 4a). Mutually exclusive staining for p53 or lysozyme confirmed that the induction of p53 occurs in CBCs and progenitors but not in Paneth cells (Fig. 6b and Supplementary Fig. 4b). To address the role of p53 in epithelium degeneration following the loss of R2TP activity, we generated double KO VilCreERT2; Rpap3fllox/fllox; Trp53fllox/fllox mice. We verified that adding floxed alleles did not alter the kinetics of Rpap3 recombination, which was already detected 16 h after tamoxifen injection (Supplementary Fig. 4c). Accordingly, the phenotype of Rpap3 deletion was unaffected by hemizygous deletion of Trp53 as VilCreERT2; Rpap3fllox/fllox; Trp53fllox/+ mice phenocopied VilCreERT2; Rpap3fllox/fllox; Trp53+/+ mice (hereafter referred to as “Rpap3 KO”). As reported before, invalidation of Trp53 alone did
Fig. 3 Rpa3 is required for proliferation in the small intestine. a Schematic representation of the experimental setting: 8-week-old mice of the indicated genotype received two sequential injections of tamoxifen 24 h apart, and were analyzed 5 to 8 days after the first injection. 2 h before each sacrifice, BrdU was injected intraperitoneally to detect cells in S-phase (thin arrows). b Schematic representation of a crypt from the small intestine, with CBC stem cells (in green) sandwiched between Paneth cells (in brown) and progenitors forming the TA on top (purple). c Representative pictures of jejunum tissue sections stained with HE (top) or by IHC with anti-Ki67 antibody (bottom, Ki67 signal is brown) at indicated days after the first tamoxifen injection. Pink arrows point towards Paneth cells and black arrows towards CBC stem cells. Scale bar is shown in control HE panel. Each panel is representative of 8 to 12 animals analyzed in three independent experiments. Scale bar is identical for all panels and is 15 μm. d Representative pictures of jejunum taken from control (boxed in blue) and VilCreER<sup>T2</sup>; Rpa3<sup>flox/lox</sup> animals (boxed in red), stained by IHC with anti-BrdU antibodies (brown arrows). n = 3–6 animals/tissue point from two independent experiments. Scale bar is shown in control panel and is 50 μm. e Graph shows mean number of BrdU<sup>+</sup> cells/crypt and cell proliferation in controls and VilCreER<sup>T2</sup>; Rpa3<sup>fllox/lox</sup> animals (p = 0.0073; n = 3). Source data are provided as a “Source Data file”.

not cause any noticeable phenotype<sup>14,45</sup> (Fig. 6c and Supplementary Fig. 6d). However, double KO mice showed a transient rescue. At day 6, the intestinal epithelium from VilCreER<sup>T2</sup>; Rpa3<sup>flox/lox</sup> mice appeared normal, with regular Ki67<sup>+</sup> crypt staining, unlike that of Rpa3 single KO mice (Fig. 6c). At day 7, the epithelium from double KO mice started shrinking, with partial loss of Ki67<sup>+</sup> staining, while that of single Rpa3 KO mice were strongly disorganized (Fig. 6c). Finally, at day 8, the epithelium from both single Rpa3 and double KO showed similar villus blunting (Fig. 6c). Staining for p53 and Rpb1 confirmed the near total penetrance of Trp53 and Rpa3 deletion (Supplementary Fig. 4d and Fig. 6d). We detected cleaved caspase 3 and typical round apoptotic cells in both the double Trp53; Rpa3<sup>ko</sup> and the single Rpa3<sup>ko</sup> crypts, but not in control nor p53<sup>ko</sup> crypts (Fig. 6e). Altogether, these results show that inactivation of Rpa3<sup>ko</sup> induces p53 expression, cell cycle arrest and apoptosis. Still, sustained defective R2TP activity results in epithelium degeneration even in absence of p53.

Rpa3 functions in the colon as in the small intestine. Depletion of the Rpa3 protein was effective in both the small intestine and the colon (Fig. 1d). Yet, colonos were shorter not in Rpa3<sup>ko</sup> KO mice (Fig. 1f), nor was their architecture modified until day 8, as observed by staining with Periodic Acid Schiff (PAS), Ki67, BrdU, and CD44<sup>v6</sup> labeling of colon stem cells<sup>46</sup> (Fig. 7a and Supplementary Fig. 5a, b). We analyzed R2TP chaperone activity in the colon by performing Western blots. This revealed a small diminution of R2TP substrates in the colon, mostly visible for mTOR and ATM. This diminution was less pronounced than that in the small intestine (Fig. 7b and Supplementary Fig. 5c). It was not accompanied by any detectable accumulation of Rpb1 in the cytoplasm (Supplementary Fig. 5d), nonetheless p53 was induced at the bottom of the crypt, as well as apoptosis (Fig. 7a, c and Supplementary Fig. 5e). This suggests that the small defects occurring in the colon are sufficient to trigger p53 activation, but not to induce a visible phenotype at the tissue level.

To analyze the role of R2TP in the differentiation of the small intestine and the colonic crypts, we simultaneously generated organoids from both tissues, using mice treated with tamoxifen to delete Rpa3<sup>ko</sup>, as previously described<sup>47</sup>. Crypts prepared from control small intestines grew to form organoids that budded after 72 h in culture. In contrast, organoids prepared from KO mice degenerated from 48 h on, with no observable budding, confirming the essential role of Rpa3<sup>ko</sup> for the survival and differentiation of the small intestinal crypts (Fig. 7d). In parallel, control colonic organoids started differentiating at 72 h and budded after 96 h in culture. Remarkably, colonic organoids from Rpa3<sup>ko</sup> KO mice also died without reaching the budding stage, at 72 h (Fig. 7d). Thus, R2TP is necessary for the survival and differentiation of both, small intestinal and colonic organoids.

Rpa3 phenotypes mirrors cellular turnover. To reassess the role of Rpa3<sup>ko</sup> in the colon, we generated Lgr5<sup>flox/lox</sup>-IRES-CreER<sup>T2</sup>; Rpa3<sup>flox/lox</sup> animals (Supplementary Fig. 6a). In these mice, GFP and the tamoxifen-inducible Cre are both under the control of the CBC-specific Lgr5 promoter. This construct has a mosaic expression restricted to a few GFP<sup>+</sup> crypts<sup>48</sup>, allowing the animals to survive and the comparison of Rpa3-deficient- and -expressing crypts in the same intestines. Indeed, tamoxifen treated Lgr5<sup>flox/lox</sup>-IRES-CreER<sup>T2</sup>; Rpa3<sup>flox/lox</sup> mice did not show any visible physiological alteration and could survive for 3 weeks at least. Co-staining for GFP and Rpb1 showed that, at day 7, in the small intestine, Rpb1 accumulated in the cytoplasm of CBC stem cells and progenitors from GFP<sup>+</sup> crypts, but not in GFP<sup>−</sup> crypts, nor in non-recombinant GFP<sup>+</sup> crypts from Lgr5<sup>flox/lox</sup>-IRES-CreER<sup>T2</sup>; Rpa3<sup>flox/lox</sup> control mice (100% of GFP<sup>+</sup> crypts had cytoplasmatic Rpb1; Fig. 8b and Supplementary Fig. 6a). At day 10, GFP<sup>+</sup> crypts had been eliminated from the small intestine of Lgr5<sup>flox/lox</sup>-IRES-CreER<sup>T2</sup>; Rpa3<sup>flox/lox</sup> mice, as expected from ex vivo organoid experiments (Fig. 7d). In the colon, Rpb1 was nuclear in GFP<sup>−</sup> and GFP<sup>+</sup> colonic crypts from Lgr5<sup>flox/lox</sup>-IRES-CreER<sup>T2</sup>; Rpa3<sup>flox/lox</sup> mice at day 7 (Supplementary Fig. 6a, b). At day 10–12, however, Rpb1 accumulated in the cytoplasm of CBC stem cells and progenitors of GFP<sup>+</sup> crypts, but not in GFP<sup>−</sup> and GFP<sup>+</sup> crypts from control animals (Fig. 8b and Supplementary Fig. 6b). Thus, Rpa3<sup>ko</sup> is necessary for Rpb1 biogenesis in both the small intestine and the colon, yet the effect on Rpb1 appears later in the colon than in the small intestine.

To understand the reason for this delay, we compared the cellular turnover between these tissues. For this, we injected the nucleoside analog BrdU as a cell tracer into wild-type mice and sacrificed them subsequently at different time points (Fig. 8c). Two hours after injection, BrdU was incorporated exclusively by CBC stem cells and progenitors in both the small intestine and the colon (Fig. 8c). After one day, BrdU<sup>+</sup> cells were detectable above the TA compartment in the small intestine, while some remained at the bottom of the crypt. BrdU<sup>+</sup> cells then migrated to the tip of the villus at day 3, and were eliminated at day 4, in accordance with the previously described kinetics<sup>49</sup>. In contrast, in the colon, migrating BrdU<sup>+</sup> cells did not reach the crypt tip before day 4 and were still detectable at day 5 (Fig. 8c). This illustrates a slower cellular turnover in the colon than in the small intestine, which correlates with a slower accumulation of Rpb1 in the cytoplasm of colonic cells, smaller effects on client levels and milder phenotypes at the tissue level (Fig. 7 and Supplementary Fig. 5).

RPA3 expression correlates with colorectal cancer prognosis. Our results so far suggest an intimate link between R2TP activity and cell proliferation in the intestine. To test if R2TP is also involved in pathogenic proliferation, we took advantage of the CODEREAD dataset (available at https://xenabrowser.net/).
Transcriptomic analyses showed a significant enrichment of mRNAs encoding RUVBL1, RUVBL2, and RPAP3 in human primary colorectal tumors (n = 380) as compared to normal tissue (n = 51), in agreement with a previous report on a smaller cohort. This observation prompted us to test the expression of RPAP3 in CRC patient samples, using immunohistochemistry on Tissue Microarrays (TMAs) sections from CRC patients and anti-RPAP3 antibodies, which only detects human RPAP3 but not its murine homolog. We analyzed TMAs containing core tissues...
Fig. 4 Rpap3 invalidation induces CBC stem cells loss and apoptosis in the proliferative compartment of the small intestine. a, b Staining for Olfm4 in the jejunum from control (top panel) and VilCreERT2; Rpap3lox/lox animals 7 days (a) or 6 to 8 days after the first tamoxifen injection. Panels are representative for 2 to 4 animals/time point from at least two independent experiments. Scale bar is 20 μm in (a) and 50 μm in (b). c Schematic representation of a crypt from the small intestine, with CBC stem cells (in green) sandwiched between Paneth cells (in brown) and progenitors forming the TA on top (purple). d Representative micrographs of tissue sections immuno-stained for lysozyme, a specific marker of Paneth cells, in the jejunum of control (top) and VilCreERT2; Rpap3lox/lox mice from day 6 to day 8. Panels are representative for 2 to 3 animals/time point from two independent experiments. Scale bar, identical in all pictures, is 50 μm. e Total number of apoptotic cells identified by cleaved caspase 3 (cleaved cas3+) per surface (mm²) of jejunum for each mouse analyzed, at day 6. Mean values with S.E.M are indicated for each experimental group. Unpaired two-tailed Welch’s correction indicates significant difference between controls and VilCreERT2; Rpap3lox/lox animals (p = 0.0384; t = 3.538, df = 3, n = 4). f Micrographs are tissue sections stained for cleaved caspase 3 in the jejunum. In control animals, cleaved caspase 3+ cells (brown arrows) are mainly detected at the tip of the villi, as a result of epithelial turnover (top panel). In the jejunum from VilCreERT2; Rpap3lox/lox animals, at day 6 and 7, cleaved caspase 3+ cells were detected within the crypts (brown arrows). Panels are representative from 2 to 4 animals/time point from two independent experiments. Scale bar, identical in all pictures, is 50 μm. Source data are provided as a “Source Data file”.

from patients diagnosed with CRC without pathological evidence of nodal involvement and distant metastasis. 157 out of 177 (88.7%) cases expressed RPAP3 in the tumor cell cytoplasm. The proportion of RPAP3-positive cases was in the range of 4–100%, with a mean ± S.E. of 62.6% ± 2.6. To dichotomize the RPAP3 expression level in RPAP3high and RPAP3low, an optimal cut-off value of 26% of positive tumor cells was chosen based on the Receiver Operating Characteristic (ROC) analysis (AUC = 0.593; Fig. 9b). The relationships between RPAP3 expression and clinicopathological parameters were investigated by Pearson’s χ² test: RPAP3 expression negatively correlated with the tumor stage of CRC (p = 0.049; Supplementary Tables 1, 2). Compared with stage I tumors, the expression of RPAP3 was decreased in stage II tumors (p = 0.049). Furthermore, compared with mucinous carcinoma, the expression of RPAP3 was increased in CRC of the adenocarcinoma type (p = 0.025). In addition, high RPAP3 expression was positively correlated with the occurrence of tumor relapse (p = 0.003) and patients’ mortality (p = 0.036). Since mTOR can influence CRC outcome52, we addressed its contribution by IHC in tumors from the same patient cohort. RPAP3 contributed only to 22% of mTOR expression level (r̂ho = 0.226, p = 0.007), which was not correlated with DFS (Supplementary Fig. 7).

39 out of 123 (31.7%) patients with RPAP3high tumors and 6 out of 54 (11.1%) patients with RPAP3low tumors had disease relapse. Analysis of Kaplan–Meier curves showed that patients with RPAP3high tumors had a lower DFS rate than patients with RPAP3low tumors (p = 0.037; Fig. 9b). Multivariate analyses of DFS adjusted for other prognostic factors confirmed that RPAP3 expression was a significant prognostic parameter influencing disease relapse (HR = 2.7: 95% CI, 1.2–6.5; p = 0.023; Supplementary Table 3), but not the overall survival (OS) of patients. These results provide evidence that high RPAP3-expression levels in CRC tissues are associated with poor patient prognosis.

Discussion

Since its discovery in 2005, biochemical and structural studies highlighted a chaperoning role for R2TP.13 RMAP3 is a central subunit within R2TP: it recruits the chaperones HSP70/90 via its TPR domains, PIH1D1 through a short peptide domain and the PIKs22. Overall, R2TP appears as a central hub for assembling multi-subunit complexes by coordinating the activity of HSP70/90 with other existing machineries. Indeed, in Hela cells, many R2TP clients Rpb1 accumulate in the cytoplasm prior to its nuclear translocation19. Remarkably, invalidation of Rpap3 triggers p53 stabilization, cell cycle arrest, apoptosis and destruction of the intestinal epithelium. Transcription inhibition, DNA damage and defects in ribosome biogenesis all activate p5354–57, yet p53 induction was observed before any detectable alteration in R2TP client activity, such as DNA damage (as monitored by yH2AX; Supplementary Fig. 3a). This was even more striking in the colon, where p53 was activated while R2TP clients were only mildly affected (Fig. 7). Indeed, at day 6 and 7, p53 removal rescued the alterations in small intestine, further showing that, at this point, loss of R2TP client per se was not deleterious. To explain this result, we hypothesize that unassembled R2TP clients trigger p53 activation. For instance, exposed protein surfaces that are normally buried into complexes could sequester the p53-E3 ligase MDM2, as observed for unassembled ribosomal proteins58. And indeed, a subset of ribosomal proteins was recently shown to bind Rpap359. These ribosomal proteins could trigger p53 activation following Rpap3 deficiency. In any case, the rescue of the Rpap3 KO phenotype by Trp53 removal was only transient. Defects in ATR, RNA PolII, snoRNPs, and ribosome biogenesis might induce p53-independent apoptosis and lead to small intestine degradation, as described for ATR60 and proteins involved in housekeeping cellular machineries such as transcription and translation51.

Ex vivo experiments showed that crypts from both small intestine and colon depended on Rpap3 to form organoids, confirming the essential role of Rpap3 in proliferating cells. Interestingly, we found that differentiated cells in the small intestine are barely affected by Rpap3 removal, in contrast to the rapidly dividing CBC stem cells and TA progenitors. Indeed, Rpb1 accumulates in the cytoplasm of proliferating cells, while it remains fully nuclear in the differentiated cells of the epithelium. We propose that R2TP preferentially assembles the basic cellular machineries in the proliferative compartment: CBC stem cells and progenitors (Fig. 9c). Indeed, the high division rate of TA and CBC stem cells (–10–12 h) likely requires high rates of protein synthesis, folding and assembly, and accordingly, high R2TP activity62. In contrast, non-dividing differentiated cells may rely on the pre-existing machineries. Indeed, in Hela cells, many R2TP clients (NOP58, PRPF8, EFTUD2, ATR, and mTOR) have an half-life that is too stable to be measured (–24 h)63. Additional data support this model of a preferential role of R2TP in proliferating cells. First, R2TP is concentrated in CBC stem cells and progenitors of the small intestine. Second, several R2TP clients are more abundant in
Fig. 5 Rpap3 deletion decreases expression of R2TP clients and leads to cytoplasmic accumulation of RNA polymerase II in intestinal crypts and TA compartment. **a** Images are tissue sections of small intestines stained by immunohistochemistry (IHC) for Rpb1, the catalytic subunit of RNA polymerase II, from control Rpap3^{fl^{ox}/fl^{ox}} mice (blue frame, left panel), or VilCreER^T2; Rpap3^{fl^{ox}/fl^{ox}} animals at day 6 (red frame, right panel), with magnifications of crypts (scale bars used for the two magnification insets are identical between blue and red frames). Note that the staining in stromal cells is nuclear in both wild-type and VilCreER^T2; Rpap3^{fl^{ox}/fl^{ox}} animals (stromal cells do not express the Cre) and control epithelium, while it becomes cytoplasmic in the mutant epithelium. Panels are representative for n = 6 animals from three independent experiments. Scale bar is 50 and 10 μm for insets, as shown in control panels. **b** Schematical interpretation of the micrographs in (a). In control epithelial cells (blue), R2TP incorporates Rpb1 into RNA PolII, which is then imported into the nucleus. In the absence of Rpap3 (red), neo-synthesized Rpb1 accumulates in the cytoplasm. **c** Western blot analysis of preparations enriched for epithelial crypt cells from the jejunum of animals, sacrificed 6 days after the first tamoxifen injection. NOP58, EFTUD2, and PRPF8 (c), mTOR, ATM, ATR, and TRRAP (d) were detected with specific antibodies. Tubulin and GAPDH were used as loading controls. Quantification of the signal ratios are indicated on top of each lane (average for the control ratios was arbitrarily set to 100). Each lane was loaded with the lysate obtained from one animal of the indicated genotype (n = 3 per genotype). Similar results were obtained with animals from at least two independent experiments. Apparent molecular weights are indicated on the right. Source data are provided as a “Source Data file.”
the intestinal proliferative compartment, including ATR, U3 snoRNP and U5 snRNPs (Supplementary Fig. 3b, c). Third, markers of RNA PolI activity, a potential R2TP client, accumulate in the crypts and TA compartment with decreasing levels along the crypt-villus axis\(^5\). Fourth, a recent transcriptomic analysis along the crypt-villus axis detected mRNAs encoding ribosomal, splicing, and transcription components at the very bottom of the villus, but not along the villus or at the tip\(^6\).

A correlation between proliferation and sensitivity to \(Rpap3\) removal further extends to the colon. Indeed, R2TP substrates showed milder destabilization in the colon than in the small intestine, while \(Rpb1\) accumulation in the cytoplasm of
Fig. 6 R2TP invalidation triggers p53-dependent and independent apoptosis. a Micrographs are tissue sections stained for p53 by immunofluorescence in controls (top) and VilCreERT2; Rpap3fl/fl (bottom) at day 6, representative of n = 7 animals from three independent experiments. Nuclei were stained with DAPI. Scale bar is 50 μm and is identical for all pictures. b Micrographs are tissue sections stained by immunofluorescence for p53 (Cy5, red) and lysozyme (Alexa 488, green), a marker of Paneth cells, in VilCreERT2; Rpap3fl/fl mice at day 6 (n = 4). Nuclei were stained with DAPI. Scale bar, 50 μm, is identical for all pictures. c Pictures of jejunal sections stained with HE (top) or by IHC with anti-Ki67 antibodies (bottom) in PS3 KO (VilCreERT2; Rpap3fl/fl/+; Trp53fl/fl/+), Rpap3 KO (VilCreERT2; Rpap3fl/fl/+; Trp53fl/fl/+), and double Rpap3 KO; P53 KO (VilCreERT2; Rpap3fl/fl/+; Trp53fl/fl/+; P53 KO) at day 6 to 8 after the first tamoxifen injection. Pictures are representative for each single KO (n = 3) and double KO (n = 5–7) mice, from three independent experiments. Scale bar, 50 μm, is identical for all pictures. d Pictures of jejunal sections stained by IHC with anti-Rpb1 antibody in control, PS3 KO (VilCreERT2; Rpap3fl/fl/+; Trp53fl/fl/+), Rpap3 KO (VilCreERT2; Rpap3fl/fl/+; Trp53fl/fl/+; P53 KO) and double Rpap3 KO; P53 KO (VilCreERT2; Rpap3fl/fl/+; Trp53fl/fl/+; P53 KO) at day 6 following tamoxifen injection. Pictures are representative of n = 3 for each single KO, n = 7 for double KO, from two different experiments. Mean values with S.E.M are indicated for experimental groups with n > 3. One-way ANOVA analysis (p = 0.0006) with Bonferroni’s multiple comparison post-test (*p < 0.001). Source data are provided as a “Source Data file”.

recombinant colonic crypts was detected several days later than in the small intestine. This correlates with the rate of epithelial turnover, which is slower in the colon than in the small intestine (Fig. 8c). In Drosophila, the RPAP3 ortholog, Spag, is required for essential for spermatogenesis28 diminishes lung metastasis in a murine model69. Yet, in clinical trials, HSP90 inhibitors have – inhibition65 another case where RPAP3/Spag affects dividing stem cells, and dependency on RPAP3 in the proliferative compartment, and (iv) supported by several lines of evidences: (i) a higher expression of therapeutic target, as a role for R2TP in intestinal carcinogenesis is – and PIH1D1 were both identiﬁed during the experiment in an SPF animal facility. Naive mice were minimum 6- weeks old and euthanized by CO2 and isoflurane. To activate the CreERT2, controls and animals of interest received two intra-peritoneal (IP) injection of 2 mg tamoxifen each. For BrdU incorporation assays, mice were intraperitoneally injected with 100 μg Bromodeoxyuridine (BrdU) per gram of body weight. The entire small intestine (cut in three parts) and colon were flushed with PBS, then with neutral buffered formalin 10% and ﬁxed in it for 24 h, dehydrated, and embedded in paraffin.

Mice generation and treatments. Mouse experiments were performed in strict accordance with the guidelines of the European Community (86/609/EEC) and the French National Committee (87/848) for care and use of laboratory animals, and comply the ARRIVE guidelines and were approved by the French Ministry of Higher Education, Research and Innovation (reference APAFIS#18665) to be performed in the institute animal facility (agreement # F3417216). Mice were housed in temperature-controlled ventilated cages (20 °C – 22 °C) with a 12 h light-dark cycle, with percentage of humidity between 45 and 55%, and maintained in pathogen-free conditions in the institute animal facility. Rpap3tm1a(KOMP)Wtsi mice were generated from ES cells (JMBAL-N3. C57BL/6 N genetic background) generated by the trans-NIH KnockOut Mouse Project (KOMP) and obtained from the KOMP Repository (www.komp.org). Genotyping was performed by PCR ampliﬁcation using primers F5, R5 and R6, on mouse tail genomic DNA (gDNA—Supplementary Fig. 1a). All mice strains Rpap3tm1a(KOMP)Wtsi; Trp53tm1a(CAG-flpo)1Aft (TG-CAG-flpo)1Aft, Tg(Vil1-cre)-205yr, Tg(Vil1-cre;ERERT2)235yr, Lgr5tm1a(CAG-flpo)1Aft, Tg(CAG-flpo)1Aft, Tg(Vil1-creERT2), Lgr5-GFP-IREs-CreERT2, Trp53fl/fl/+; LRPAP3fl/fl/+ and compounds lines were maintained on C57BL/6 background and bred in an SOPF animal facility, and during the experiment in an SPF animal facility. naive mice were minimum 6-week-old and euthanized by CO2 and isoflurane. To activate the CreERT2, controls and animals of interest received two intra-peritoneal (IP) injection of 2 mg tamoxifen each. For BrdU incorporation assays, mice were intraperitoneally injected with 100 μg Bromodeoxyuridine (BrdU) per gram of body weight. The entire small intestine (cut in three parts) and colon were flushed with PBS, then with neutral buffered formalin 10% and ﬁxed in it for 24 h, dehydrated, and embedded in paraffin.

Histology and immunostainings. TMA tissue sections were stained with a mouse monoclonal antibody raised against RPAP3 (proprietary 19B11 antibody at 1:10 dilution), an antibody against human mTOR (Clone 7C10, EMD Millipore, overnight incubation; cat. number #2983; Cell Signaling). Antigen retrieval was performed by microwave treatment at 750 W (10 min) in 10 mMl/l sodium citrate buffer (pH 6.0). The polymer kit (EnVision kit, K4003, Agilent) was used for signal amplification. DAB (3,3-Diaminobenzidine) was used as chromogen. Anti-human RPAP3 has been validated and previously published145. See Supplementary Table 4.

For histological analysis on murine tissues, tissue sections (4 μm thick) were deparaffinized and rehydrated. They were stained with hematoxylin and eosin (H&E) or Periodic Acid Schiff staining (PAS) for preliminary analysis. For Ki67, Cleaved-caspase 3 and yH2-A.X immunostainings, tissue sections were deparaffinized, rehydrated, and subsequently subjected to heat induced antigen retrieval in 10 mM sodium citrate buffer (pH 6) or 10 mM Tris HCl-1 mM EDTA buffer (pH 9), depending on the antibody. Immunohistochemistry was performed using a Dako autostainer (Dako, Glostrup, Denmark) with the RHEM primary antibodies overnight at 4°C. For immunohistochemistry (Rpb1, Lysozyme, Ofm4, Pih1d1), tissue slides were incubated in 10 mM sodium citrate pH 6 (T0050, DiaPATH) or 10 mM Tris HCl 1 mM EDTA buffer pH 9 for 20 min at 100 °C for antigen retrieval, depending on the antibody. Endogenous peroxidase activity was inactivated with PBS–0.3% hydrogen #H10099, Sigma). After blocking in 2.5% blocking serum–5% BSA–5% nonfat milk for 30 min at room temperature, tissue slides were incubated with primary antibodies overnight at 4 °C. Then corresponding secondary antibody

Methods

Human colorectal sample collection and analysis. A total of 177 CRCs were collected from patients surgically treated at the University “G. D’Annunzio”, Chieti, Italy, between 1996 and 2010. Only the CRC patients who did not receive adjuvant systemic therapy were included in the study. The median follow-up was 53 months (range 3–238 months). During the follow-up, 25.4% of CRC patients (45 out of 177) had a disease relapse, while deaths were observed in 18.6% of CRC patients (33 out of 177). Tumor stage was determined according to the American Joint Classification of tumors of the digestive system (4th edition). Patients and tumor characteristics are summarized in Supplementary Tables 1, 2. The study was reviewed and approved by the Institutional Research Ethics Committee Comitato Etico delle Province di Chieti e Pescara and dell’Università degli studi “G. D’Annunzio” di Chieti e Pescara and written informed consent was obtained from all patients. The study complied with all relevant regulations regarding the use of human study participants, including the criteria set by the Declaration of Helsinki.

TMAs were constructed by extracting 2-mm diameter cores of histologically confirmed neoplastic areas from the 177 CRC cases using a manual Tissue Arrayer (MTA, Beecher Instruments, WI), as previously detailed1. It was conducted following REMARK guidelines, as detailed in the REMARK checklist (see Supplementary Note).

For immunohistochemistry (Rpb1, Lysozyme, Ofm4, Pih1d1), tissue slides were incubated in 10 mM sodium citrate pH 6 (T0050, DiaPATH) or 10 mM Tris HCl 1 mM EDTA buffer pH 9, depending on the antibody. Immunohistochemistry was performed using a Dako autostainer (Dako, Glostrup, Denmark) with the RHEM primary antibodies overnight at 4°C. For immunohistochemistry (Rpb1, Lysozyme, Ofm4, Pih1d1), tissue slides were incubated in 10 mM sodium citrate pH 6 (T0050, DiaPATH) or 10 mM Tris HCl 1 mM EDTA buffer pH 9 for 20 min at 100 °C for antigen retrieval, depending on the antibody. Endogenous peroxidase activity was inactivated with PBS–0.3% hydrogen #H10099, Sigma). After blocking in 2.5% blocking serum–5% BSA–5% nonfat milk for 30 min at room temperature, tissue slides were incubated with primary antibodies overnight at 4 °C. Then corresponding secondary antibody

in the intestinal epithelial homeostasis by promoting proliferation of stem cells and progenitors (Fig. 9c).

In conclusion, we show that the R2TP chaperone plays a crucial role in the intestinal epithelial homeostasis by promoting proliferation of stem cells and progenitors (Fig. 9c).
Reagents (ImmPRESSTM kit, Vector Laboratories), directed against mouse, goat or rabbit were used for detection. Incorporation of BrdU in proliferating intestinal epithelial cells was detected using an anti-BrdU antibody (Biolegend, 1:100) after deparaffinization of the tissues, antigen retrieval in 10 mM citrate buffer pH 6, as described above, and DNA denatured using 2N HCl for 1H at 37 °C followed by an incubation in 0.1M borax buffer pH 9. Revelation was performed using the Avidin/Biotin Vectastain System kit (Vectorlab, USA) according to the manufacturer protocol. After neutralization of the endogenous peroxidase activity, the sections were incubated with the primary antibodies. Antibody were visualized using the Envision® system (Dako). 3,3’-Diaminobenzidine (Dako) was used as the chromogen and the sections were lightly counterstained with hematoxylin. Histological slides were scanned using the Nanozoomer 2.0 HT scanner with a ×40 objective, and visualized with the NDP. view2 software (both from Hamamatsu).

For p53 immunofluorescence, paraffin-embedded tissues were cut into 3-µm-thick sections, mounted on slides, then dried at 37 °C overnight. Staining was performed on the Discovery Ultra Automated IHC staining system from Roche Ventana. Following deparaffinization with the Discovery EZ Prep solution at 75 °C for 24 min, antigen retrieval was performed at 95 °C for 16 min using the Discovery CC1 buffer. After blocking in TBS, 10% goat serum, 5% BSA, 5% milk, 0.3% triton X100, the slides were incubated with a rabbit anti-p53 antibody 37 °C for 60 min (Leica, P53-CM5P-L, 1:250). Signal enhancement was performed using the OmniMap anti-Rabbit HRP kit (Roche, 760-4457) then with the Cy5 Kit (Roche, 760-238). For double immune-stainings, slides were stripped by heating before incubation with anti-lysozyme antibody.
For Rbp1/GFP immunofluorescence, antigen retrieval with 1 mM EDTA was performed for 30 min at 99°C. Slides were blocked with 5% goat serum-PBS—0.3% Triton X-100 for 1 h at RT then incubated with primary antibodies overnight at 4°C. Samples were incubated in a DAPI solution combined with the secondary antibodies for 1 h at RT. Slides were mounted with cover slip in ProLong Gold Antifade Mounting medium or Vectashield. Fluorescent images were acquired by the Axioscan 40x camera and analyzed with ZEN (both from Zeiss), brightfield upright Zeiss Axioimager Z1 using the Metamorph software, or on the inverted Confoal SP5 (Leica) using the Leica LAS AF software.

All antibodies used for immunostainings are described in Supplementary Table 4 and have been validated by the manufacturers, independent groups (see https://www.citeab.com/), the RHEM platform, our previous work (Rbp1) or this study.

For β-galactosidase activity, small intestines and colons cryo-preserved were cut to slices of 10 μm thickness. Samples were treated with 0.5% glacial acetic acid for 10 min at room temperature, followed by 24 h of incubation at 37°C in a staining solution containing 1 mg/ml X-gal, 5 mM potassium ferrocyanide (K3Fe(CN)6), 5 mM potassium ferrocyanide (K4Fe(CN)6) and 2 mM MgCl2, 0.1% Triton X100 in PBS. Samples were washed in PBS for 5 min, counterstained with Nuclear Fast Red and briefly rinsed with D2O before mounting using an aqueous mounting solution (Aquaphotex, 108562; Merck).

U3 snoRNA FISH. Small intestines and colons cryo-preserved were cut to slices of 10 μm thickness. The frozen sections were fixed in PBS/paraformaldehyde 4% at room temperature for 30 min, followed by permeabilization with ethanol 70%, overnight at 4°C. In situ hybridization was performed overnight with Cy3 labeled oligonucleotides against U3 (AT*AGACGATTTCAACCATCAACCGCGGGG*GCACCTTGGCTTCTC*A).

Microscopy and imaging. Histological slides were scanned using the Nanozoomer 2.0 HT scanner with a x40 objective, and visualized with the NDP.view2 software (Hamamatsu). Fluorescent images were acquired with Axioscan using ZEN software (Zeiss), brightfield Axioimager Z1 (Zeiss) using the Metamorph software or on the inverted Confoal SP5 (Leica) using the Leica LAS AF software. Images were processed with Adobe Photoshop CS6.

Isolation of epithelial cells from the intestine. Small intestines and colons were isolated and flushed with cold PBS. Colon and the three intestine fragments were cut open length-wise, then kept in 10 ml cold wash buffer (PBS, 2% FBS and antibiotics). Tubes were shaken several times to wash the fragments and put into 15 ml Falcon tube containing 10 ml CE buffer (PBS, 1% BSA, 1 mM DTT, 1 mM EDTA, 5.6 mM glucose). Tubes were placed on a vertical shaker at 37°C for 30 min. After removing tissue, cells were collected by centrifugation at 1410 rpm for 7 min. They were washed in 10 ml wash buffer. Cells collected are split in different tubes and stored at −80°C.

To separate villi and crypt cells, after PBS wash, villi were gently scraped off using a glass cover slip. Free the villi fractions were collected by pipetting in 1 ml of cold PBS. The rest of the tissue (the crypts were still attached) was incubated with 10 ml CE lysis buffer to dissociate and collect crypt cells.

Organoids. Colons and small intestines were dissected, inverted on a stick, cut longitudinally then washed extensively in cold PBS-supplemented with penicillin/streptomycin. After cutting them in 0.3 cm long pieces, they were incubated in 10 ml cold PBS. Pieces were washed 5 to 10 times in cold PBS with antibiotics and then treated for 30 min at 4°C with PBS-25mM EDTA for the colon and PBS-2mM EDTA for the small intestine. After sedimentation, the pieces were washed four times in 10 ml of cold PBS. For each wash, corresponding to Fraction 1 to 4, the supernatant was strained in a 70 μm mesh filter. Each fraction was then centrifuged at 390 g for 7 min and checked for the presence of well-formed crypts under the microscope. Usually fractions 3 and 4 were used for the organoid culture. 200 crypts were suspended in 50 μl of Matrigel in WENR (DMEM-F12 media supplemented by WNs3a, EGF, Noggin, R-spondin-1) media for the colon (DMEM-F12 media supplemented by EGF, Noggin, R-spondin-1) media for the small intestine and microscopically inspected once a day, as described15.

Western blots. Protein extracts were prepared from epithelial cell preparations by lysis in cold RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP40, 0.5% deoxycholate) supplemented with inhibitors. Protein lysates were separated on a gradient 4–15% SDS-PAGE (BioRad) and transferred to a nitro-cellulose membrane (Amersham Protran 0.2 μm NC). Membranes were blocked with TBS—2.5% fat milk (w/v)−0.05% Tween and incubated with the appropriate primary antibodies, followed by an incubation with secondary antibodies conjugated to the horseradish-peroxidase or a fluorophore. ECL was revealed with Amersham ECL 600 camera and fluorescence with the Amersham Typhoon scanner.

Primary antibodies from Cell Signaling: AT3 clone D2E8 (CST 2873S) rabbit at 1/1000, ATR clone E1S3S (CST 13934S) rabbit at 1/1000, MTOR (CST 2972S) rabbit at 1/1000, p53 clone IC12 (CST2524S) mouse 1/1000, PRP8 (sc-30207, Santa Cruz) rabbit 1/200, from Abcam: EFTUD2 (ab72456) rabbit 1/2000, GAPDH (ab8245 6CS) mouse 1/10000; from Sigma: NOPS8 (HPA018472) rabbit 1/100, RPAP3 (SAB141438) rabbit 1/1000, α-Tubulin (IZG10) mouse 1/500; from Proteintech PHH1D1 (14251-2) rabbit 1/1000; from Birtarr TRRAP (GI0103 murine clone 2DS) mouse 1/1000 (a generous gift from L. Tora). We validated anti-PRRP8 in17 and anti-TRRAP using a proprietary cell line with degradable TRRAP. All other antibodies have been validated by the manufacturers, as well as by independent groups which reported their use in several publications, notably in the CiteAb platform (https://www.citeab.com/) and are described in Supplementary Table 4.

DNA extraction. Samples were incubated in 100 μl of alkaline lysis buffer (NaOH 25 mM, EDTA 0.2 mM) at 92°C for 20 min, kept on ice for 10 min. Then 100 μl of neutralizing reagent (40 mM Tris HCl pH 5) was added to the samples. 2 μl were used for each PCR.

Southern blot. Briefly, ES cells were incubated overnight at 65°C in 100 mM Tris HCl pH 8.5–5 mM EDTA—0.2% SDS—200 mM NaCl with 0.1 ng/ml proteinase K in a humid chamber. On the following morning, lysates were extracted twice with phenol pH 7.0–8.0 and once with chloroform then precipitated in NaCl/ETOH. Following recovery, 10–15 μg of genomic DNA were digested for 24 to 48 h with appropriate highly concentrated restriction enzymes (40–20 U/l/10 μl/10 μg of DNA). The analysis was performed by Southern blot as described in74.

PCR for genotyping. Initial denaturation: 95°C (2 min), Denaturation 95°C (30 s), Annealing 55°C (45 s), Extension 72°C (45 s), and Final extension: 72°C (2 min) for 35 cycles. Primers: RPAP3-Fs (GTTGCGCAGCATGTTGAG), R5 (TGGC TCGTACTACATCAG, R6 (ACCGTGTCGCTAGTACGTC), CREERT2-R (TATGCGAGGCGCCACTAGT), CREERT2-F (TCTTACCCGCTGCAAGTC), CREERT2-R (AAGGGCCCTGCGTGTCCTTCT), P3-F (TGC TAG ATG CTT AGG GCT GC), P3-R (GAC TGC CCC TCT TGG TGT CT), Primers are reported in the Supplementary Table 5.

Statistical analysis. Transcriptomic analysis of R2TP subunits in human samples were extracted from the CODEREAD cohort available at UCSC Xena platform for
**Fig. 8 Rpap3 activity in intestinal crypts correlates with tissue turnover.**

**a** Schematic representation of the experimental setting. Eight-week-old Lgr5-GFP-ires-CreERT2; Rpap3flox/flox mice received five sequential intra-peritoneal injections of tamoxifen and were analyzed 7 or 10 days after the first injection. In this genetic model, the Cre is expressed in the Lgr5+ CBC stem cells labeled by GFP (see scheme on the right).

**b** Representative images of tissue sections labeled by immunofluorescence with antibodies against GFP (green) and Rpb1 (red), with DAPI counter-staining of nuclei (blue). Please note the mosaic expression of GFP. Panels are representative for 5 animals/time point from two independent experiments. White arrow: GFP+ crypts. Asterisks: GFP− crypts. Scale bars (40 or 20 μm) are identical for matching panels.

**c** Images are intestine tissue sections of wild-type animals stained for BrdU. Animals received one BrdU injection and were sacrificed at the indicated time point. The experiment was repeated twice (2 to 4 animals/time point from two different experiments). Scale bars (50 μm) are identical for all pictures.
One-way ANOVA test was performed using GraphPad Prism 5.0.

For the outcome endpoints of human data, the DFS was defined as the measure of time after treatment during which one of the following events occurred: relapse at local or distant sites, or intercurrent death without recurrence. OS was defined as the time between surgery and death from any cause. Survival curves were analyzed by the Kaplan–Meier method and compared using the log-rank test. Cox’s proportional hazards model, adjusted for other prognostic factors (i.e., gender, tumor location, tumor grade, tumor stage, and RPAP3 status), was used to evaluate the association of RPAP3 expression with outcome. Spearman correlation was used to analyze the...
Fig. 9 R2TP expression correlates with pathological cell proliferation. a The graph depicts transcript levels of R2TP components in human primary colorectal tumor samples \((n = 380)\), as compared to normal solid tissues \((n = 51)\) from COADREAD cohort. y-axis: Log2 normalized counts for the indicated transcript. Distributions are presented as box-and-whisker plots (center line: median; box limits: first and third quartiles; whiskers: 10th and 90th percentiles). Statistical significance was determined by one-way ANOVA \((p < 0.001)\). b Kaplan–Meier analysis of disease-free survival among 177 CRC patients according to the proportion of RPAP3-expressing cells in tumor tissues. Solid green line and dashed blue line indicate high and low proportion of RPAP3-expressing tumoral cells, respectively. Statistical significance was determined by log-rank test \((p = 0.037)\). Right panels show examples of CRC tissues with low (top) or high (bottom) RPAP3 expression, with scale bar. c Proposed model for R2TP activity in the small and large intestine. R2TP assembles cellular machineries such as RNA polymerases, snRNPAs, snRNPs, and PIKKs-complexes in CBCs and progenitors in the proliferative compartment (blue cells). Differentiated cells (including Paneth cells from the small intestine crypts, in pink) mostly rely on the complexes assembled during the proliferative phase. A defect in R2TP activity induces client dysfunction, cell cycle arrest and apoptosis via p53, and eventually, epithelium degradation.

correlation between the expression of RPAP3 and mTOR. Statistical analysis were performed with the SPSS 15.0 (SPSS Inc., Chicago, IL).

All other statistical analysis were performed employing GraphPad Prism 5.0.

Sample availability

Mouse sperm for the different strains are available upon request, as well as proprietary antibodies.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The datasets analyzed during the current study for the transcriptomic analysis of R2TP subunits in human samples (Fig. 9a) used the UCSC Xena platform for public and private cancer genomics data visualization and interpretation available at https://xenabrowser.net\(^\text{\textregistered}\). The datasets concerning the patient biopsies (Fig.9b, Supplementary Fig. 7) are subject to restrictions due to legal and privacy reasons. It must be authorized by the subject’s legally authorized representative, and a specific request must be issued to Direzione Medica/Presidio Ospedaliero “SS. Annunziata”, ASL02 di L’Aquila-Vasto-Chieti/Via Dei Vestini/66100 Chieti (Italy). Timeframe for response is usually 1 month. The remaining datasets that support the findings of this study are available in the Article, Supplementary Information, or, together with details about the experimental procedures, are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Received: 10 January 2020; Accepted: 5 July 2021; Published online: 10 August 2021

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Acknowledgements
We thank Solange Morera and EMBl facility for help with the anti-RPAP3 antibody. Laxtila Tora for the gift of anti-TTRAP. The ES cells were generated by the trans-NIH KnockOut Mouse Project (KOMP) and obtained from the KOMP Repository (www.komp.org). NIH grants to VelociCigen at Regenomer Inc (U10HG004085) and the CSD Consortium (U10HG004086) funded the generation of gene-targeted ES cells for 8500 genes in the KOMP Program and archived and distributed by the KOMP Repository at UC Davis and CHORI (U44RR024424). We thank the CIGM team in Institut Pasteur for microinjection experiments and animal husbandry and Laurent Le Cam for help with Trp5flox model. We thank the RAM, PCEA, and ZEFI animal facilities, the MRI imaging facility, and the RHEM histology facility. MIR is member of the national infrastructure France-BioImaging supported by the French National Research Agency (ANR-10-INBS-04, ‘Investments for the future’). RHEM is supported by SIRIC Montpellier Cancer and funded by grant INCa_Inserm_JDG08_12553, the European Regional Development Fund, and the Occitanie region. The work was supported by La Ligue Nationale Contre le Cancer (equipes labellisées à E.B., P.J.) and the INCa grants PLBIO 2016-161 to D.H., E.B., M.H., P.B.B., and PLBIO 2018-158 to P.J. C.M. and C.A. had fellowships from the Ligue Nationale Contre le Cancer.

Author contributions
C.M., C.A., B.L., M.G., C.L., V.P., M.F., C.P., J.B., F.G., C.V., and B.P.B. performed research. All authors approved the content of the paper. C.M., C.A., B.L., M.G., C.L., V.P., M.F., C.P., J.B., F.G., C.V., and B.P.B. designed the experiments, analysed the data, and wrote the paper. B.P.B. supervised the research. All authors approved the content of the paper.

Competing interests
The authors declare no competing interest.

Additional information
Supplementary information
The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-24792-4.

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Peer review information
Nature Communications thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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