Elp3 drives Wnt-dependent tumor initiation and regeneration in the intestine

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Tumor initiation in the intestine can rapidly occur from Lgr5+ crypt columnar stem cells. Dclk1 is a marker of differentiated Tuft cells and, when coexpressed with Lgr5, also marks intestinal cancer stem cells. Here, we show that Elp3, the catalytic subunit of the Elongator complex, is required for Wnt-driven intestinal tumor initiation and radiation-induced regeneration by maintaining a subpool of Lgr5+/Dclk1+/Sox9+ cells. Elp3 deficiency dramatically delayed tumor appearance in Apc-mutated intestinal epithelia and greatly prolonged mice survival without affecting the normal epithelium. Specific ablation of Elp3 in Lgr5+ cells resulted in marked reduction of polyp formation upon Apc inactivation, in part due to a decreased number of Lgr5+/Dclk1+ cancer stem cells. Consequently, Elp3 or Sox9 depletion led to similar defects in Dclk1+ cancer stem cells in ex vivo organoids. Finally, Elp3 deficiency strongly impaired radiation-induced intestinal regeneration, in part because of decreased Sox9 protein levels. Together, our data demonstrate the crucial role of Elp3 in maintaining a subpopulation of Lgr5-derived and Sox9-expressing cells needed to trigger Wnt-driven tumor initiation in the intestine.

The intestinal epithelium is characterized by a repetitive architecture made of crypt–villus units and is seen as a powerful experimental model to study adult stem cells in health and diseases (Clevers, 2013; Barker, 2014). Each villus is covered by a single layer of postmitotic cells and is surrounded at its base by multiple epithelial invaginations, referred to as crypts of Lieberkühn. Each crypt–villus unit is composed of six differentiated epithelial cell types. These are lysozyme and defensin-secreting Paneth cells located at the bottom of the crypts, absorptive enterocytes, Goblet and enteroendocrine cells that secrete mucus or hormones, microfold (M) cells that act as a specific tuft cell marker because of its strong expression in Paneth cells (Bastide et al., 2007; Mori-Akiyama et al., 2007). Tuft cells specifically express Doublecortin-like kinase 1 (Dclk1; also referred to as Dcamkl-1) as well as the transcription factor Gfi1B (Bjerknes et al., 2012; Gerbe et al., 2012). Sox9 is also expressed in Tuft cells, but is not seen as a specific tuft cell marker because of its strong expression in Paneth cells (Bastide et al., 2007; Mori-Akiyama et al., 2007). Identifying molecular determinants for the specification and differentiation of Tuft cells is therefore critical to shed more light on their poorly understood biological functions.

The intense self-renewal kinetics of the intestinal epithelium relies on crypt base columnar (CBC) stem cells located at the bottom of intestinal crypts together with Paneth cells. Cycling CBC cells express the Wnt target gene Leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5; Barker et al., 2007). A second pool of quiescent stem cells has also been described in the intestine. Indeed, DNA label–retaining cells (LRCs), also referred to as +4 stem cells, are located at the cell apex exposed to the luminal environment (Gerbe et al., 2011, 2012). They are distinct from intestinal secretory cells, as transcription factors such as Neurog3, Sox9, and Spdef are dispensable for their generation (Gerbe et al., 2011; Bjerknes et al., 2012). Tuft cells specifically express Doublecortin-like kinase 1 (Dclk1; also referred to as Dcamkl-1) as well as the transcription factor Gfi1B (Bjerknes et al., 2012; Gerbe et al., 2012). Sox9 is also expressed in Tuft cells, but is not seen as a specific tuft cell marker because of its strong expression in Paneth cells (Bastide et al., 2007; Mori-Akiyama et al., 2007). Identifying molecular determinants for the specification and differentiation of Tuft cells is therefore critical to shed more light on their poorly understood biological functions.

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Abbreviations used: CBC, Crypt base columnar; Dclk1, Doublecortin-like kinase 1; Hes1, Hairy/enhancer of split 1; LRCs, label-retaining cells; Mcm5, 5-methoxycarbonyl-methyl; SAM, S-adenosylmethionine.
above Paneth cells and express various markers such as Bmi1, Hopx, mTERT, and Lrig1 (Potten et al., 1978; Sangiorgi and Capecchi, 2008; Montgomery et al., 2011; Takeda et al., 2011; Powell et al., 2012). Importantly, a high level of plasticity occurs between Lgr5+ and LRC stem cells as Hopx-expressing +4 cells can generate Lgr5+ cells, whereas the latter can also express +4 stem cell markers (Takeda et al., 2011; Muñoz et al., 2012). Similarly, Lgr5+ cells mark a heterogeneous population as Lgr5high stem cells are mitotically active, whereas other Lgr5low progenitors exit the cell cycle (Basak et al., 2014).

Wnt signaling fuels the stem cell compartment and also critically controls tumor development in the intestine by promoting crypt proliferation through transcriptional activation of Tcf target genes (Clevers and Nusse, 2012). Although it is not totally clear how cancer stem cells are controlled by Wnt signaling, it has nevertheless been demonstrated that Lgr5+ stem cells critically drive tumor initiation (van de Wetering et al., 2002; Barker et al., 2009; Schepers et al., 2012; Clevers, 2013). The tuft cell marker Dclk1 also appears to distinguish cancer versus normal stem cells (Nakanishi et al., 2013). Dclk1+ cells have been demonstrated to sustain tumor growth in the intestine, but it is currently unclear how Dclk1 expression is regulated (Westphalen et al., 2014). A better characterization of proteins acting downstream of the oncogenic Wnt signaling pathway is required for the maintenance of intestinal cancer stem cells is therefore required.

The intestine, as a proliferative and self-renewing organ, is highly sensitive to DNA-damaging agents, but regenerates when subjected to high doses of radiation. Indeed, intestinal stem cells that escaped from massive p53-mediated apoptosis undergo cell proliferation and repopulate the intestine within a few days, a process that critically relies on Wnt signaling (Withers and Elkind, 1970; Merritt et al., 1994; Ashton et al., 2010; Cordero and Sansom, 2012). The mitotically active Lgr5+ cells, although dispensable for intestinal homeostasis, are critical for intestinal regeneration upon damage. Notably, upon caloric restriction or expression of the Wnt agonist R-spondin1, the increased number of Lgr5+ cells promotes protection against intestinal damage (Bhana et al., 2009; Tian et al., 2011; Yilmaz et al., 2012; Metcalfe et al., 2013; Zhou et al., 2013). Similarly, a population of quiescent LRCs, defined as Paneth cell precursors that express Paneth and +4 markers, can revert back into proliferating Lgr5+ cells upon radiation (Buczacki et al., 2013). Another study also defined an intestinal Dclk1+ quiescent cell population required for regeneration as the genetic ablation of Dclk1 led to defects in recovery after intestinal injury (Westphalen et al., 2014). Moreover, the transcription factor Sox9 is also critical for epithelial regeneration after high-dose irradiation highlighted (Roche et al., 2015). Still, the molecular mechanism governing stem cell plasticity during radiation-induced intestinal regeneration remains poorly characterized.

Elp3 is the catalytic subunit of Elongator (Elp1–Elp6), a protein complex initially identified as a component of a hyperphosphorylated RNA polymerase II holoenzyme isolated from budding yeast chromatin. Elp3 sequence harbors motifs found in the GNAT family of histone acetyltransferases (HATs; Otero et al., 1999; Wittschier et al., 1999; Kim et al., 2002) and a radical S-adenosylmethionine (SAM) domain at the N-terminal region that includes a FeS cluster critical for Elongator integrity (Greenwood et al., 2009). Elongator promotes transcriptional elongation in the nucleus through histone H3 acetylation but also, as a cytoplasmic complex, in translational efficiency by adding 5′-methoxy carbonylmethyl (mcm5) and 5′-carbamoylmethyl (mcm5) groups on uridines at the wobble position of some tRNAs (Kristjúhan et al., 2002; Winkler et al., 2002; Huang et al., 2005; Close et al., 2006; Esberg et al., 2006; Bauer et al., 2012; Lin et al., 2013). Elongator promotes cell migration in a variety of primary and transformed cells (Close et al., 2006, 2012; Creppe et al., 2009; Lee et al., 2009). However, it is unclear whether Elongator is involved in tumor development in vivo.

We show here that Elp3 expression is induced by Wnt signaling, promotes Tuft cell differentiation, and is essential for Wnt-driven tumor development in the intestine, as well as for radiation-induced intestinal regeneration. Mechanistically, our data support a model in which Lgr5+ cells rely on Elp3 to initiate tumor development upon constitutive Wnt signaling at least through the maintenance of a pool of Lgr5+/Dclk1+/Sox9+ cells. We show that Elp3 regulates Sox9 protein levels through tRNA modification. Therefore, we define Elp3 as a downstream effector of Wnt signaling that controls Sox9 translation and maintains a pool of Lgr5+/Dclk1+/Sox9+ cells to drive tumor initiation in the intestine.

RESULTS

ELP1 and ELP3 are Wnt target genes
Elongator-deficient melanoma cells fail to form colonies in soft agar (Close et al., 2012). The anchorage-independent growth of two colon cancer–derived cell lines, HCT116 and HT29, is also strongly impaired after Elp3 depletion (unpublished data). Both HCT116 and HT29 cells harbor enhanced Wnt signaling as a result of β-catenin or APC mutations, respectively (Morin et al., 1997; Wang et al., 2003), suggesting a crucial role of Elongator in Wnt-driven tumorigenesis. As β-catenin critically drives colony formation in soft agar (Verma et al., 2003), we next investigated whether Elongator is functionally connected to Wnt- and β-catenin–dependent signaling pathways. β-catenin nuclear levels, as well as expression of described Wnt target genes, remained unchanged upon ELP3 deficiency in HT29 cells, indicating that Elongator is dispensable for Wnt signaling activation (unpublished data). We then impaired the Wnt pathway by generating β-catenin–depleted HT29 cells and noticed that ELP1 and ELP3 mRNA levels decreased upon β-catenin deficiency, similarly to other Wnt target genes, such as Lgr5, Axin 2, Cyclin D1, and c-Myc (Fig. 1 A). Conversely, Wnt activation in RKO cells, which have low intrinsic Wnt signaling, increased ELP3 protein levels (Fig. 1 B), suggesting that ELP3 expression is β-catenin–dependent in colon cancer–derived cells. The
Elp3 promoter harbors multiple TCF4-binding sites, and we detected a specific recruitment of TCF4 on two sites located 3,400 and 3,200 bp upstream from the transcription start site by ChIP assays (Fig. 1 C). To investigate whether this finding is also relevant in vivo, we assessed Elp3 mRNA levels in control versus Apc+/min mice, which spontaneously develop adenocarcinomas as a result of constitutive Wnt signaling (Su et al., 1992). Similarly to c-Myc, Elp3 mRNA dramatically increased over time in intestinal epithelial cells (IECs) from Apc+/min but not from control mice (Fig. 1 D). Elp3 expression was also higher in human intestinal carcinomas than in normal adjacent tissues, similar to c-MYC (Fig. 1 E). Consistently, in human samples of colon cancer, Elp3 protein expression was enriched in parts enclosing adenomas and carcinomas compared with normal intestinal epithelium, as shown by anti-ELP3 immunohistochemistry analyses (Fig. 1 F). Collectively, our data indicate that Elp3 expression is Wnt and β-catenin dependent in the intestine and is enhanced upon constitutive Wnt signaling.

**Elp3 promotes Tuft cell differentiation in the intestine**

Having defined Elp3 as a Wnt target, we next assessed its role in intestinal homeostasis. We first generated a genetically engineered mouse model in which the exon 2 of the Elp3 gene was flanked with LoxP sites to create the conditional knockout (KO) allele (referred hereafter to as Elp3Control). Deletion of exon 2 upon Cre expression results in Elp3 loss of function by generating a frameshift to all downstream exons (Fig. 2 A). The constitutive knockout alleles generated by crossing our Elp3Control strain with the Villin-Cre mouse (referred hereafter to as Elp3ΔIEC) was indeed identified in IECs through PCR analysis (Fig. 2 B). As a result, Elp3 expression was severely decreased in IECs of Elp3ΔIEC mice (Fig. 2 C and D). The absence of Elp3 in IEC did not impact on the overall gain of weight of mice over lifetime (Fig. 2 E). Moreover, decreased hematocrit levels, another feature of Apc+/min mice, was not observed upon Elp3 deficiency, as spleen weight of Apc+/min Elp3ΔIEC mice were almost identical to those seen in Elp3Control or in Elp3ΔIEC mice (Fig. 3, F and G). Moreover, decreased hematocrit levels, another feature of Apc+/min mice, was not observed upon Elp3 deficiency (Fig. 3 H). Additionally, Elp3 deletion strongly impaired spheroid structure formation from ex vivo culture of Apc–mutated intestinal crypts (Fig. 3 I). Together, these data demonstrate that Elp3 promotes Wnt-driven tumor initiation in the intestine.

**Wnt-driven tumor initiation requires Elp3**

Because Elp3 is required for colony formation in colon cancer cell lines, we next evaluated the putative role of Elp3 in Wnt-driven intestinal tumor development by crossing Elp3Control or Elp3ΔIEC mice with the Apc+/min strain. As expected, Elp3 expression was severely impaired in intestinal crypts of the resulting mouse model (Apc+/min Elp3ΔIEC), as confirmed by real-time PCR analysis (Fig. 3 A). Strikingly, Elp3 deficiency in Apc+/min mice dramatically extended their life span due to a severe decrease in tumor number in proximal, middle, and distal intestines (Fig. 3, B–E). Splenomegaly, a typical feature of Apc+/min mice (Lane et al., 2010), did not occur upon Elp3 deficiency, as spleen weight of Apc+/min Elp3ΔIEC mice were almost identical to those seen in Elp3Control or in Elp3ΔIEC mice (Fig. 3, F and G). Moreover, decreased hematocrit levels, another feature of Apc+/min mice, was not observed upon Elp3 deficiency (Fig. 3 H). Additionally, Elp3 deletion strongly impaired spheroid structure formation from ex vivo culture of Apc–mutated intestinal crypts (Fig. 3 I). Together, these data demonstrate that Elp3 promotes Wnt-driven tumor initiation in the intestine.

**Lgr5+ cells require Elp3 to promote Wnt-dependent tumor initiation in the intestine**

Given the critical role of Lgr5+ cells in tumor initiation (Barker et al., 2009; Schepers et al., 2012), we next wondered whether the genetic inactivation of Elp3 in Lgr5+ cells would also impact on Wnt-driven tumor initiation. To address this issue, we first crossed the stem cell–specific Lgr5-EGFP-IRES-creERT2 knock-in mouse with our Elp3lox/lox strain and subsequently deleted Elp3 in the resulting mice through tamoxifen administration (referred to as Lgr5-EGFP-Elp3lox/lox). Tamoxifen was also administered in the Lgr5-EGFP-IRES-creERT2 strain as control (referred to as Lgr5-EGFP-Elp3lox/lox). Notably, Elp3 expression was enriched in cycling Lgr5+ (EGFP+) cells, which provides an additional demonstration for Wnt signaling acting as a driver of Elp3 expression (unpublished data). Impaired Elp3 expression was specifically observed in sorted EGFP+ cells upon tamoxifen injection (unpublished data). Importantly, Elp3 deficiency did not impact on the number of Lgr5+ cells (unpublished data). Therefore, Elp3 is dispensable for the maintenance of Lgr5+ stem cells in healthy crypts.
We next crossed the Apc<sup>lox/lox</sup> strain with the Lgr5-EGFP-IRES-creER<sup>T2</sup> knock-in mouse and with our Elp3<sup>lox/lox</sup> strain. Both Apc and Elp3 were simultaneously inactivated in Lgr5<sup>+</sup> cells of the resulting mouse through tamoxifen administration (referred to as Apc<sup>ΔCBC</sup> Elp3<sup>ΔCBC</sup>). Tamoxifen was also administered in mice resulting from the breeding of Apc<sup>lox/lox</sup> mice with the Lgr5-EGFP-IRES-creER<sup>T2</sup> strain (referred to as Apc<sup>ΔBC</sup>). As expected, numerous hyperplasias that resulted from constitutive Wnt signaling in Lgr5<sup>+</sup> cells were observed 15 d after tamoxifen administration in Figure 1.
Figure 2. *Elp3* promotes Tuft cell differentiation. (A) LoxP sites flanking exon 2 of the *Elp3* gene were inserted. Deletion of exon 2 upon Cre expression results in loss of function of *Elp3* by generating a frameshift to all downstream exons (red boxes). Primers used for detection of the wild-type or mutated alleles are depicted by arrows. (B) Genomic DNA was extracted from IECs of *Elp3*<sup>ΔIEC</sup> (<em>Elp3<sup>ΔIEC</sup></em>) or *Elp3*<sup>ΔIEC</sup> Villin<sup>Cre+</sup> (<em>Elp3<sup>ΔIEC</sup></em>) mice and subjected to PCR analysis using primers 1 and 2 to amplify the wild-type or recombinant allele (1,341- or 172-bp fragments, respectively). (C) mRNAs from *Elp3* Control and *Elp3<sup>ΔIEC</sup>* mice were subjected to quantitative real-time PCR analysis to assess *Elp3* mRNA levels (mean values ± SD; Student's t test; ***, P < 0.001). (D) Anti-Elp3 IHC analyses were performed with intestinal crypts from 120-d-old *Elp3* Control and *Elp3<sup>ΔIEC</sup>* mice. (E) The weight of five male *Elp3* Control and *Elp3<sup>ΔIEC</sup>* mice (8–55 wk old) was quantified and plotted. Weights were normalized at 8 wk of age for each genotype. (F) Intestinal epithelial cells (IECs) were
ApCΔIEC mice, as demonstrated by H&E and Ki67 IHC analyses (Fig. 4, A and B). Those areas of hyperplasia also showed elevated numbers of Dclk1+ cells (Fig. 4 C). Elp3 deficiency in Lgr5+ and Apc-deleted cells did not impact the total number of Lgr5+ cells, but severely impaired tumor initiation, as judged by decreased numbers of hyperplastic foci (not depicted and Fig. 4, A and B, respectively). The number of Dclk1+ cells was also decreased in the rare Elp3-deficient hyperplastic foci (Fig. 4 C). Importantly, Dclk1+ cells found in these hyperplastic foci were largely negative for acetylated-α-tubulin staining, as compared with adjacent crypts, which supports the notion that they are not Tuft cells (unpublished data). Therefore, Elp3 expression in Lgr5+ cells is required for Wnt-dependent intestinal tumor initiation. Intestinal crypts extracted from ApCΔIEC Elp3ΔIEC mice also failed to efficiently generate spheroid structures ex vivo (Fig. 4 D). To further demonstrate that Elp3 expression is required for the self-renewal capacity of Lgr5+ cancer stem cells, FACS-sorted Lgr5+ cells from both ApCΔIEC and ApCΔIEC Elp3ΔIEC mice were subjected to an in vitro limiting dilution assay. Elp3 ablation strongly affected the Lgr5+ cells cancer stem cell potential (Fig. 4 E). Therefore, the cancer stem cell potential of Lgr5+ cells relies on Elp3 in the intestine.

**Elp3 maintains the pool of Dclk1+ cells in the intestine**

We next explored the mechanisms underlying the role of Elp3 in Wnt-driven intestinal tumor initiation. Nuclear β-catenin protein levels, as well as mRNA levels of Wnt target genes, such as c-Myc, EphB2, and Tcf-1, were unchanged in 4-month-old ApCΔIEC Elp3ΔIEC mice (unpublished data) as compared with ApCΔIEC Elp3Control mice. Moreover, crypt cell proliferation and cell apoptosis at the top of the villi remained unchanged upon Elp3 deficiency in ApCΔIEC mice (unpublished data), as were the number of goblet cells, apical enterocytes, Paneth, and enteroendocrine cells (unpublished data). Of note, Atoh1 and Hes1 mRNA levels were also unchanged in Elp3-deficient ApCΔIEC mice, as well as several markers of cell differentiation, namely Krt20, Anpep, and Prss7 (unpublished data). In agreement with a dispensable role of Elp3 in the maintenance of Lgr5+ cells in ApCΔIEC mice, Lgr5, but also Ascl2, mRNA levels did not change upon Elp3 deficiency in intestinal tumors of ApCΔIEC mice (Fig. 5 A). Interestingly, in line with the fact that Dclk1+ cells in tumors are distinct from tuft cells, both Dclk1 and Gf11B, but not COX1, were decreased at the mRNA level upon Elp3 deficiency in the ApCΔIEC tumors (Fig. 5 B). Dclk1, whose protein levels were higher in cystic lesions than in adenocarcinomas (Fig. 5 C), was also less expressed in IECs extracted from ApCΔIEC Elp3ΔIEC mice, as shown by IHC and WB analyses (Fig. 5, D and E). Consistently, the number of Dclk1+ cells in both tumors and adjacent regions significantly decreased in the intestine of ApCΔIEC Elp3ΔIEC mice (Fig. 5 F). Importantly, although the number of Tuft cells (Dclk1+ and acetylated α-tubulin+ cells) slightly decreased, the number of Dclk1+/acetylated α-tubulin+ cells dramatically decreased in ApCΔIEC Elp3ΔIEC mice (Fig. 5 G). Importantly, the depletion of Dclk1 in ex vivo organoid cultures with intestinal crypts extracted from ApCΔIEC mice did not impact on Elp3 expression, but interfered with the generation of spheroid structures, similar to Elp3 deficiency (Fig. 5 H). Therefore, Elp3 promotes Wnt-driven tumor initiation in the intestine, at least by maintaining the pool of Dclk1+ cells.

**Elp3 promotes Sox9 expression through tRNA modifications**

Constitutive Wnt signaling induces significant changes in gene and protein expression to promote tumor development (Sansom et al., 2007). Sox9 is required downstream of Wnt/β-catenin signaling for the long-term self-renewal of oncogene-expressing cells in a genetic mouse model of basal cell carcinoma (Larsimont et al., 2015). Moreover, Sox9 is expressed in intestinal crypts as a Wnt target gene and is consequently overexpressed upon constitutive Wnt signaling (Blache et al., 2004). Therefore, we hypothesized that the blockage of Wnt-driven tumor development upon Elp3 deficiency may be due, at least in part, to a defective Sox9 expression. As expected, Sox9 levels were robustly increased in ApCΔIEC intestinal crypts as a result of constitutive Wnt signaling (Fig. 6 A). Importantly, its expression was severely decreased in ApCΔIEC Elp3ΔIEC mice to reach similar levels as in normal intestinal crypts (Fig. 6 A). Consistently, Sox9 protein levels dramatically decreased in ApCΔIEC Elp3ΔIEC versus ApCΔIEC Elp3Control mice (Fig. 6 B). Igfbp4, a stem cell marker whose expression is Sox9-dependent in the intestine (Merlos-Suárez et al., 2011; Shi et al., 2013), was consequently less expressed upon Elp3 deficiency in intestinal crypts from ApCΔIEC mice (Fig. 6 C). Thus, Elp3 is critical for the expression of Sox9 seen upon ApC loss. This conclusion was further supported by the fact that although Wnt 3a increased ELPS expression in intestines (Fig. 6 C), the inhibition of Wnt 3a increased ELPS expression in the intestine (Fig. 6 D and not depicted). Importantly, the number of ex
Figure 3. Elp3 is required for tumor development in a mouse model of colon cancer. (A) IECs were isolated from Apc<sup>min</sup> Elp3<sup>Control</sup> and Apc<sup>min</sup> Elp3<sup>ΔIEC</sup> 120-d-old mice and the resulting total mRNAs were subjected to quantitative real-time PCR analysis to assess Elp3 mRNA levels (mean values ± SD; Student’s t test; ***, P < 0.001; n = 4). (B) A Kaplan–Meyer curve (P < 0.001; n ≥ 16) was established with the indicated genotypes. (C and D) The histograms show the total number of adenomas in the entire intestine (C) or in the indicated areas (D) of 120-d-old control [Apc<sup>min</sup> Elp3<sup>Control</sup>] and Elp3-deficient mice [Apc<sup>min</sup> Elp3<sup>ΔIEC</sup>; mean values ± SD; Student’s t test; ***, P < 0.001; n ≥ 11]. (E) Representative pictures of jejunal section of 120-d-old Apc<sup>min</sup> Elp3<sup>Control</sup> and Apc<sup>min</sup> Elp3<sup>ΔIEC</sup> mice. H&E and K67 staining are also illustrated. (F) Representative pictures of the spleen from the indicated genotypes. (G) Quantification of the spleen weight of 120-d-old mice from the indicated genotypes (mean values ± SD; Student’s t test; ***, P < 0.001; n ≥ 7). (H) Hematocrit levels in the indicated genotypes of 3-mo-old mice (Elp3<sup>Control</sup>, n = 4; Apc<sup>min</sup> Elp3<sup>Control</sup>, n = 9; Elp3<sup>ΔIEC</sup>, n = 3) and Apc<sup>min</sup> Elp3<sup>ΔIEC</sup> (n = 9) was assessed and plotted (mean...
vivo spheroid structures generated from ApcΔCRC mice similarly decreased upon Sox9 or Elp3 depletion, suggesting that Elp3 promotes intestinal Wnt-driven tumor initiation at least by promoting Sox9 expression (Fig. 6 E).

Intriguingly, Sox9 mRNA levels remained unchanged upon Elp3 deficiency in the Apc+/min model, as shown by quantitative real-time PCR and in situ hybridization (Fig. 6 C and not depicted, respectively). Moreover, Sox9 transcription was properly induced upon Wnt 3a stimulation in RKO cells (unpublished data), suggesting that Elp3 regulates Sox9 expression at the posttranscriptional level. Of note, mRNA levels of various proteins known to regulate the acetylation or sumoylation of Sox9 (Tip60, Ubc9, Pias1, Pias4, and Senp2, respectively; Hattori et al., 2006, 2008) were properly expressed upon Elp3 deficiency in Apc+/min mice (unpublished data), suggesting that Sox9 post-translational modifications known to interfere with its stability are not responsible for the decreased protein levels seen upon Elp3 deficiency. In yeast, Elp3 promotes translational efficiency through rRNA modification (Huang et al., 2005). To understand if Sox9 decrease upon Elp3 deficiency is due to translational defects, we expressed a Sox9 cDNA in wild-type and elp3-deleted fission yeast cells (Fig. 6 F). Strikingly, the absence of Elp3 impeded Sox9 expression, which was restored upon overexpression of the unmodified rRNAAlbAAA, a critical target of Elongator. As Elp3 promotes the translation of AAA-enriched polypeptides in yeast (Bauer et al., 2012), we next generated a Sox9 expression construct in which all three AAA codons (K167, 242, and 249) found in the Sox9 murin sequence were mutated into their cognate Elp3-insensitive AAG codons (Sox9ΔAAA). We then assessed Sox9ΔAAA expression in control versus Elp3 mutant yeast strain. Interestingly, Sox9ΔAAA but not wild-type Sox9 was properly expressed in yeast Elp3 mutant strain (Fig. 6 G). Therefore, Elp3-mediated rRNA modification is critical for Sox9 proper translation.

Elp3 expression is required to maintain a pool of Lgr5+Dclk1+/Sox9+ cells in the intestine

To better understand how the Elp3-dependent translation of Sox9 expression impacts on the number of Dclk1+ cells, we assessed Elp3, Sox9 and Dclk1 levels in ex vivo organoid cultures generated from ApcΔCBC mice and depleted for Elp3 or Sox9. As expected, Elp3 deficiency decreased both Dclk1 and Sox9 protein levels (Fig. 7 A). In agreement with our previous data, Sox9 mRNA levels did not decrease in Elp3-depleted organoids (unpublished data). Yet, both Dclk1 and G61B, but not Cox1, mRNA levels were decreased upon Elp3 depletion, supporting the notion that Elp3 controls the maintenance of a subpopulation of Dclk1+ cells which differ from Tuft cells upon constitutive Wnt activation (Fig. 7 A).

Importantly, Sox9 depletion in ex vivo organoids also largely decreased Dclk1 mRNA and protein levels, suggesting that a pool of Dclk1+ cells relies on both Sox9 and Elp3 expression for its maintenance. Finally, Dclk1 depletion in Apc+/min ex vivo organoids, did not affect Sox9 or Elp3 mRNA and protein levels, but decreased the number of spheroid structures as seen for Sox9 or Elp3 depletion (Fig. 5 H), strengthening the notion that both Elp3 and Sox9 are required to maintain Dclk1+ cells.

Having established a link between Sox9 expression and Dclk1+ cells, we next explored whether a Dclk1+/Sox9+ cell population can be detected in Lgr5-expressing cells and if, so, whether Elp3 plays any role in its maintenance. Lgr5+/Dclk1+/Sox9+ cells were indeed identified by IF in ex vivo organoids from ApcΔCRC mice (Fig. 7 B). To address the role of Elp3 in their maintenance, we sorted EGFP-Lgr5+ cells from single-cell suspension of digested organoids generated from ApcΔCRC or ApcΔCBC Elp3ΔCRC mice by FACS analysis (unpublished data). Although Lgr5+ cell number did not change upon Elp3 deficiency, the amount of Dclk1+/Sox9+ cells in the Lgr5+ population strongly decreased upon Elp3 deficiency in ex vivo organoids from ApcΔCBC mice (unpublished data and Fig. 7 C, respectively). Therefore, Elp3 promotes Wnt-driven tumor initiation, at least in part, by maintaining the pool of Lgr5+/Dclk1+/Sox9+ cells in the intestine.

Intestinal crypt regeneration upon irradiation requires Elp3

Because Wnt signaling critically promotes intestinal regeneration upon DNA damage (Ashton et al., 2010), and because Dclk1 expression has been shown to be required for survival after radiation injury (May et al., 2014), we next explored whether Elp3 deficiency had any consequence on radiation-induced intestinal regeneration. Elp3Δcontrol or Elp3ΔIEC mice were subjected to 14Gy irradiation and cell proliferation was assessed in intestinal crypts. The number of regenerating crypts dramatically decreased upon Elp3 deficiency, as assessed by Ki67 staining performed 3 d after irradiation (Fig. 8 A). Sox9 protein, but not mRNA, levels severely decreased upon Elp3 deficiency in regenerating crypts from irradiated mice (Fig. 8 B). Importantly, mRNA and protein levels of Dclk1 were also dramatically impaired in IECs of irradiated Elp3-deficient mice (Fig. 8 C). To further explore whether Elp3 promotes radiation-induced intestinal regeneration through Sox9 expression, we expressed the Elp3 insensitive Sox9ΔAAA mutant into control or Elp3-deficient RKO cells and assessed cell proliferation post-irradiation (Fig. 8 D). Importantly, Elp3-deficient RKO cells in which Sox9 expression was expectedly defective failed to efficiently proliferate post-irradiation. On the other hand, Elp3-deficient RKO cells in which the Sox9ΔAAA mutant was ectopically ex-
pressed proliferated similarly to control RKO cells and showed normalized levels of Dclk1 (Fig. 8 D). Collectively, our data demonstrate that Elp3 mediates regeneration after irradiation by maintaining Sox9 expression.

**DISCUSSION**

We defined here Elp3 as a target of Wnt signaling dispensable for intestinal homeostasis but essential for Tuft cell differentiation, Wnt-driven tumor initiation, and radiation-induced regeneration in the intestine. Although nuclear levels of β-catenin and constitutive Wnt-dependent gene transcription do not require Elp3 in the intestine, our data demonstrate that the translation of Sox9, a protein induced by Wnt, is controlled by Elp3 to maintain a pool of Lgr5+/Dclk1+/Sox9+ cancer stem cells.

Tuft cells derive from Lgr5+ crypt columnar stem cells but their differentiation and biological roles remain poorly understood.
understood (Gerbe et al., 2011; Bjerknes et al., 2012). We found here that Elp3 is critical for Tuft cell differentiation, as its absence leads to dramatic reduction of Dclk1+ Tuft cells in the intestinal epithelium. The reduction of differentiated Tuft cells in Elp3-deficient mice did not lead to any detectable functional defect or to significant change in intestinal architecture, indicating that Tuft cells are dispensable for global intestinal homeostasis. Mechanistically, it is unclear how Elp3 promotes Tuft cell differentiation. Levels of the Atoh1/Math1 transcription factor, which appear to be critical for secretory cell lineage commitment and for Tuft cell differentiation, are normally expressed in Elp3-deficient intestinal crypts (Shroyer et al., 2007; Gerbe et al., 2011). Therefore, Elp3 expression may control a late step of the epithelial cell lineage commitment. A gene network between Atoh1, Hes1, and Gfi1B was previously demonstrated to be critical for epithelial lineage commitment in the intestine (Bjerknes et al., 2012). High concentrations of Gfi1B in daughter cells of short-lived progenitors commit them to Tuft cell differentiation. Our data support this model, as Gfi1B mRNA levels are severely
Figure 6. Elp3 drives Sox9 expression. (A; left) Anti-Sox9 IHC analyses were conducted with intestinal crypts of 120-d-old mice of the indicated genotypes. (right) The number of Sox9+ cells per intestinal crypt was quantified in each genotype (mean values ± SD; Student’s t test; *, P < 0.05; ***, P < 0.001; n ≥ 4). (B) Protein extracts from IECs of 120-d-old mice of the indicated genotypes were subjected to anti-Sox9 and -Hsp90 (loading control) WB analyses. (C) IECs were isolated from 120-d-old mice of the indicated genotypes and the resulting total mRNAs were subjected to quantitative real-time PCR analysis to assess Sox9 and Igfbp4 mRNA levels (mean values ± SD; Student’s t test; **, P < 0.01; n = 5). (D) RKO cells were untreated or stimulated with Wnt 3a for the indicated periods of time and the resulting cell extracts were subjected to WB analyses using the indicated antibodies. (E) ApcΔπCBC organoids were infected with the indicated shRNA construct and their growth was assessed. (right) the number of organoids per well was quantified. The histogram shows the data from three different mice (mean values ± SD; Student’s t test; *, P < 0.05; **, P < 0.01). (F) Total protein extracts from fission yeast cells of the indicated genotypes expressing Sox9 and containing either a plasmid overexpressing the tRNAlysUUU (K) or the corresponding empty vector (l) were subjected to anti-Sox9 and anti-α-tubulin (loading control) WB analyses. (G) A construct generating wild-type Sox9 or a Sox9 mutant in which all lysine residues (K) were mutated into arginines (R; Sox9ΔAAA) was expressed in yeast cells of the indicated genotypes and the resulting cell extracts were subjected to WB analyses.
decreased in Elp3-deficient intestinal crypts. Therefore, Elp3 may promote Tuft cell differentiation through Gfi1B.

Cell migration of multiple primary and transformed cells requires Elongator (Close et al., 2006; Johansen et al., 2008; Crepe et al., 2009; Lee et al., 2009). Cell invasion of glioblastoma-, neuroblastoma-, and melanoma-derived cells also relies on Elp3 (Close et al., 2006, 2012). Further, our results show that colonosphere formation from colon cancer–derived cells was greatly dependent on Elp3. It remains to be seen whether the inactivation of Elp3 in ex vivo organoid cultures generated from human cases of intestinal cancer also interferes with their growth. We also show here that Elp3 is essential in initial steps of tumor development in vivo using a mouse model of intestinal Wnt-dependent tumor initiation. Our data demonstrate that Elp3 expression increased upon constitutive Wnt activation. Therefore, we highlight a positive correlation between Elp3 expression and the activation of a major oncogenic pathway. This suggests that Elp3 may also be critically involved in the development of other epithelial malignancies harboring deregulated Wnt signaling (Michaelson and Leder, 2001; Ayyanan et al., 2006). Future studies will be dedicated to the identification of all oncogenic pathways that regulate Elp3 expression.

Dclk1+ cells are differentiated Tuft cells in normal intestinal epithelium but they were also defined as cancer stem cells upon Apc loss (Nakanishi et al., 2013). A subpopulation of long-lived and quiescent Dclk1+ cells acting as tumor-initiating cells was also identified (Westphalen et al., 2014). Surprisingly, loss of Apc in these Dclk1+ cells did not cause nuclear translocation of β-catenin and, consequently, did not trigger adenoma formation. These quiescent Dclk1+ cells could nevertheless be reactivated and act as tumor-initiating cells only when Apc loss was combined with an inflammatory stimulus such as dextran sodium sulfate (DSS) colitis (Westphalen et al., 2014). We show that Elp3 deficiency in Apc-mutated Lgr5+ intestinal stem cells blocks tumor initiation by limiting the number of Lgr5+/Dclk1+/Sox9+ cancer stem cells. Because a bidirectional conversion between tumor initiating and differentiated cells that relies on inflammatory signals has been established (Schwitalla et al., 2013), it remains to be tested whether fully differentiated Dclk1+ Tuft cells can undergo dedifferentiation to convert into so-called Dclk1+ tumor initiating cells.

Our data defined Elp3 as a critical protein for Sox9 translation in both normal and Apc-mutated intestinal crypts.
Sox9 is expressed in several differentiated epithelial lineages, such as Tuft and Paneth cells. Moreover, two distinct Sox9-expressing stem cell populations were also defined using EGFP-expressing transgenic mice, namely Sox9-EGFP\textsuperscript{high} and Sox9-EGFP\textsuperscript{low} populations (Van Landeghem et al., 2012). Sox9-EGFP\textsuperscript{low} cells are Lgr5-enriched stem cells, whereas Sox9-EGFP\textsuperscript{high} cells express enteroendocrine and +4 stem cell markers. The decreased Sox9 expression seen upon Elp3 deficiency does not result from a lower number of Paneth cells, as they appear to be functional, at least based on intact lysozyme levels seen in Elp3-deficient intestinal crypts. Similarly, the enteroendocrine and +4 stem cells populations are also intact upon Elp3 deficiency. A study concluded that Sox9 is dispensable for Tuft cell differentiation (Gerbe et al., 2011).

Whether Elp3 promotes Tuft cell differentiation through Sox9 regulation remains unclear, and it is likely that other candidates whose Wnt-dependent protein translation relies on Elp3 contribute to Tuft cell differentiation.

The role of Sox9 in Wnt-driven tumor development in vivo remains controversial. Indeed, Sox9 inactivation appears...
to increase tumor burden in the Apc<sup>+/min</sup> model, which appears to be consistent with the fact that Sox9 acts as a Wnt inhibitor (Akiyama et al., 2004; Shi et al., 2013). On the other hand, Insulin Receptor Substrate-1 (IRS-1) deficiency also inhibits protein translation of those polypeptides necessary for cell proliferation in Apc-mutated intestinal crypts (Van Landeghem et al., 2007). Whereas our data disclose a role of Elp3 in the transcription of Myc, both Elp3 and Myc may control the same pool of cancer stem cells to drive tumor development in the intestine. Interestingly, the genetic inactivation of Raptor, an essential component of mTORC1, also interferes with Wnt-dependent tumor growth but not with normal gut homeostasis (Faller et al., 2014). mTORC1 is required for cell proliferation in Apc-mutated intestinal crypts through S6K activation by increasing the rate of translational elongation of yet to be defined polypeptides. Therefore, it is tempting to speculate that Elp3 may act in this pathway by promoting protein translation of those polypeptides through tRNA modifications.

As cancer stem cells critically rely on Elp3 to promote Wnt-dependent cancer development, and given the fact that Elp3 deficiency in normal intestine did not interfere with intestinal homeostasis, targeting Elp3 activity may represent a promising therapeutic approach to treat colon cancer. Future studies in human samples of intestinal cancers will be performed to generalize our conclusions and to validate the hypothesis that Elp3 is a suitable target to treat intestinal cancers showing constitutive Wnt signaling.

**MATERIALS AND METHODS**

**Cell lines and reagents.** HT29 and RKO cells were cultured in McCoy’s 5A medium supplemented with 10% FBS, 1% glutamine, and 1% penicillin/streptomycin. HEK 293 Lentivax cells were cultured in DMEM medium supplemented with 10% FBS, 1% glutamine, and 1% penicillin/streptomycin.

Anti–β-catenin, –Elp1, and –Hsp90 antibodies used in Western blot analyses were obtained from Santa Cruz Biotechnology, Inc. The anti-Elp3 antibody was provided by Cell Signaling Technology. Anti–α-tubulin and –acetyl-α-tubulin antibodies were purchased from Sigma-Aldrich. Anti-Histone
Generating the conditional Elp3 KO mouse. The Elp3 gene (Ensembl gene ID, mouse ENSMUSG00000022031) is located on chromosome 14. Elp3 exon 1 harbors the translation initiation codon. The targeting strategy was designed to generate the conditional KO (cKO), as well as the constitutive KO alleles of the Elp3 gene (Taconic; Fig. 2 A). The targeting vector was generated using BAC clones from the C57BL/6J RPCIB-731 BAC library (clones RP23-233H18, RP23-220M13, RP23-236C3, RP23-435F17, and RP23-450B15) and transfected into the C57BL/6NTac ES cell line (Taconic). Exon 2 was flanked by loxP sites, and the positive selection marker was flanked by F3 (Puroycin resistance [PuroR]) sites and inserted into intron 1. Homologous recombinant clones were isolated using positive (PuroR) selection. Mutant Elp3 mice were generated by Taconic. In brief, E3.5 blastocysts from superovulated BALB/c females were injected with targeted C57BL/6 N.tac ES cells and transferred to pseudo-pregnant NMR1 females. Chimerism was determined according to the black/white coat color which reflects the contribution of ES cells to the BALB/c host. Highly chimeric mice were bred to C57BL/6 females and germline transmission was identified by C57BL/6 (black) in offspring was performed by Southern blot analysis. Crossing of chimeric mice with Flp Deleter mice for in vivo selection marker deletion was performed to generate mice heterozygous for the conditional KO allele. The constitutive KO allele was obtained after Cre-mediated recombination. Deletion of exon 2 results in the loss of function of Elp3 by generating a frameshift to all downstream exons. For genotyping analysis, the positive selection marker was flanked by F3 (Puromycin resistance [PuroR]) on request. To assess radiation-induced intestinal regeneration, 8–10-wk-old mice were exposed to 14Gy from a 137Cs source, as previously described (Ashton et al., 2010). Mice were given buprenorphine twice a day and were sacrificed 72 h after radiation.

Intestinal epithelial cells extraction and ex vivo organoid cultures. Intestines and colons were extracted from mice and cecum was removed. Intestine was divided into three parts of the same size and defined as the proximal, middle, and distal intestine. Bowels were subsequently cut in half longitudinally. Half of the tissue was rolled and used for histological purposes and the other half was used for intestinal epithelial cells extraction. In brief, bowels were washed for 10 min at 37°C in a PBS-DTT (1 mM) buffer, and then incubated for 15 min at 37°C in a HBSS-EDTA buffer (30 mM). Cells were harvested, washed twice in PBS, and flash frozen.

Ex vivo organoid cultures were performed as previously described (Sato et al., 2009). In brief, small pieces of intestine were incubated in 2 mM EDTA-PBS for 30 min at 4°C. Crypts were extracted, washed twice in PBS and cultured at same density in Matrigel (BD). DMEM/F12 supplemented with EGF (20 ng/ml), Noggin (100 ng/ml), and R-Spondin (500 ng/ml) was added every 2 d. Apc-mutated organoids were cultured in DMEM/F12 supplemented with EGF (20 ng/ml) and Noggin (100 ng/ml) without R-Spondin. For the generation of organoids from ApcΔCΔC and ApcΔCΔC Elp3ΔCΔC mice, animals were treated with tamoxifen.

Limiting dilution assay. Crypts from ApcΔCΔC and ApcΔCΔC Elp3ΔCΔC mice treated with tamoxifen were extracted as described above. To generate a unicellular suspension of the extracted crypts, we digested them for 30 min at 37°C with 10× Trypsin and centrifuged them at 150 rcf for 5 min. The pellet was resuspended in 1%BSA/1× PBS and filtered in a 70-μm cell strainer to be centrifuged at 150 rcf for 5 min. Finally, the pellet was resuspended in 1 ml 1%BSA/1× PBS and EGFP-Lgr5+ cells were sorted using the FACSAria (BD). EGFP-Lgr5+ sorted cells were counted and seeded in Matrigel as indicated in the figure. DMEM/F12 supplemented with EGF (20 ng/ml) and Noggin (100 ng/ml) was added every 2 d. Wells positive for organoid growth were quantified after 7 d. H3, -H2AX, and -Dclk1 antibodies were obtained from Abcam, and anti-Sox9 and Ephrin B2 antibodies were purchased from EMD Millipore and R&D Systems, respectively.

Transgenic mouse models, tamoxifen injections, and exposures to radiation. The ApcΔCΔC strain (C57BL/6J-ApcMin/J), as well as the Lgr5–EGFP-RES-creERΔ12 (B6.129P2-Lgr5tm1(cre/ERSR1)Cle/J) heterozygote knock-in mouse were purchased from The Jackson Laboratory, whereas the Villin Cre/+ strain (ApcΔCΔC) as control or to mice resulting from the breeding between Lgr5–EGFP-RES-creERΔ12 mice and the Elp3ΔCΔC strain to generate the Lgr5–EGFP-Elp3ΔCΔC strain. For the simultaneous inactivation of Elp3 and Apc in Lgr5Δ cells, four daily injections of 2 mg tamoxifen in sunflower oil were performed intraperitoneally to the stem cell–specific Lgr5–EGFP-RES-creERΔ12 knock-in mice as control (Lgr5–EGFP-Elp3ΔCΔC) or to mice resulting from the breeding between Lgr5–EGFP-RES-creERΔ12 mice and the Elp3ΔCΔC strain to generate the Lgr5–EGFP-Elp3ΔCΔC strain. DMEM/F12 supplemented with EGF (20 ng/ml) and Noggin (100 ng/ml) was added every 2 d. Wells positive for organoid growth were quantified after 7 d.

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Statistical analysis was performed by as previously described (Hu and Smyth, 2009).

**Flow cytometry.** Crypts were extracted after 30 min of incubation in 2 mM EDTA-PBS at 4°C, and then digested into single cells using the trypLE express solution (Life Technologies) containing 200 U of DNase for 30 min at 37°C. Cells were washed in 1% BSA/PBS. Resuspended cells were sorted on the BD FACSAria. For phenotyping, organoids were collected, washed in PBS, and digested for 20 min using the trypLE express solution. The resulting single-cell solution was fixed with 4% paraformaldehyde solution, permeabilized with Perm-Wash solution (BD), and stained with anti-Sox9 and -Dclk1 antibodies (Abcam) for 30 min at 4°C. Corresponding secondary antibodies (Life Technologies) were incubated for 30 min at 4°C. The FlowJo software was used to analyze data.

**Total RNA extraction and real-time PCR.** Total RNA from cell lines was extracted using the total RNA kit (Omega Biotek) according to manufacturer’s instructions. For mice samples, total RNA was extracted using the TriPure Isolation Reagent (Roche). All samples were subsequently treated with the Recombinant DNase (Roche). cDNAs were synthesized using the Revert Aid H Minus First Strand cDNA Synthesis kit (Fermentas). Subsequent PCRs were performed using the Power SYBR Green PCR Master kit (Takara Bio Inc.) on the LightCycler 480 (Roche). The mean value of mRNA levels in control mice was set to 100% and levels in mutant mice were expressed relative to that after normalization with glyceraldehyde-3-phosphate dehydrogenase GAPDH. Data are plotted as mean values ± SD. All primer sequences used are available upon request.

**Lentiviral infections.** The control or shRNAs targeting Dclk1, ELP3, β-catenin, or Sox9 were purchased from Sigma-Aldrich. Lentiviral infections in RKO or HT29 cells were performed as previously described (Creppe et al., 2009). Lentiviral infections in ex vivo organoids were performed as previously described (Koo et al., 2012). Lentiviral infections in RKO or HT29 cells were performed by incubating the sections in citrate buffer at pH 6.0 or EDTA at pH 9.0 for 20 min. Primary antibodies used were as follows: Dclk1 (1:50; pH 6.0; Abcam), Sox9 (1:50; pH 6.0; Millipore), KIF7 (1:50; pH 6.0; Cell Marque), corresponding secondary antibody detection kits for reduced background on murine tissue were used (Histofine Simple Stain Mouse MAX PO; Medac) and stained on an automated stainer (LabVision Autostainer 480S; Thermo Fisher Scientific). All slides were scanned with a Panoramic 250 slide scanner (3D Histech.com).

For immunofluorescence, frozen sections were incubated in the target retrieval solution (Dako) for 7 min. Primary antibodies are listed in the Cell lines and reagents section. Slides were analyzed using a sp5 confocal microscope (Leica).

**Expression of Sox9 in fission yeast and tRNA overexpression.** The pLVX-Sox9-EGFP expression construct, a gift from S.T. Magness (University of North Carolina at Chapel Hill, Chapel Hill, NC), was used as template to generate the Sox9ΔAAA mutant by site-directed mutagenesis, using the protocol provided by the manufacturer (Agilent Technologies). Wild-type Sox9 and the Sox9ΔAAA cDNA mutant were amplified by PCR and cloned in the fission yeast ura4-based pREP4 that allows expression from the nmt1 promoter, which is repressed by thiamine. Wild-type and elp3-deleted strains were transformed with the pREP4-Sox9 plasmid and selected in EMM medium in the absence of uracil. The tRNAlysUUU cloned in a leu2-based vector as previously described (Bauer et al., 2012), or an empty vector, were also transformed and co-selected with the pREP4-sox9 on medium lacking both leucine and uracil. The expression of Sox9 was induced by growing cells for 22 h in the absence of thiamine. Cells were harvested at OD 0.5 (10 ml) and lysed in the presence of zirconium beads, as previously described (Fersht et al., 2007). Total protein extracts were subjected to anti-Sox9 and α-tubulin (loading control) Western blot analyses.

**Statistical analysis.** Student’s t test was performed to test statistical relevance, unless otherwise specified. For quantification of ELP3 immunohistochemistry of human biopsies, total H-DAB positive areas of each slide were calculated using the ImageJ software. Specifically, H-DAB positivity was quantified from the transformed images by ImageJ IHC Toolbox. Measurements were calculated in pixels, and the analysis was done blindly. Data are expressed as percentage of H-DAB-positive pixels on total pixels per slide.
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