Article

Prox1 Promotes Expansion of the Colorectal Cancer Stem Cell Population to Fuel Tumor Growth and Ischemia Resistance

Graphical Abstract

Highlights
Prox1 expression is dispensable for homeostasis in the normal intestine

A subpopulation of Prox1+ cells has stem cell activity in intestinal adenomas/CRC

Loss of Prox1 decreases adenoma/CRC stem cells, tumor cell growth, and survival

Annexin A1 and filamin A mediate Prox1 effects on stem cell activity in the tumors

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In Brief
Wiener et al. now show that the Prox1 transcription factor functions as a stem cell regulator in intestinal adenomas and colorectal cancer (CRC), but not in the normal intestine. Prox1 critically contributes to tumor cell survival in hypoxia and to the expansion of the adenoma/CRC stem cell population via inhibition of the Wnt-target annexin A1 and induction of the actin-binding protein filamin A. The Prox1 pathway thus represents an attractive therapeutic target for drug development in CRC.

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**Prox1 Promotes Expansion of the Colorectal Cancer Stem Cell Population to Fuel Tumor Growth and Ischemia Resistance**

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**SUMMARY**

Colorectal cancer (CRC) initiation and growth is often attributed to stem cells, yet little is known about the regulation of these cells. We show here that a sub-population of Prox1-transcription-factor-expressing cells have stem cell activity in intestinal adenosomas, but not in the normal intestine. Using in vivo models and 3D ex vivo organoid cultures of mouse adenomas and human CRC, we found that Prox1 deletion reduced the number of stem cells and cell proliferation and decreased intestinal tumor growth via induction of annexin A1 and reduction of the actin-binding protein filamin A, which has been implicated as a prognostic marker in CRC. Loss of Prox1 also decreased autophagy and the survival of hypoxic tumor cells in tumor transplants. Thus, Prox1 is essential for the expansion of the stem cell pool in intestinal adenomas and CRC without being critical for the normal functions of the gut.

**INTRODUCTION**

Colorectal cancer (CRC) is one of the leading causes of cancer mortality in Western countries. In most CRC patients, an initial mutation occurs in the APC or CTNNB1 gene, leading to activation of the β-catenin/TCF (canonical Wnt) pathway (Fearon, 2011; Fodde and Smits, 2001). In the crypts of the normal gut, the β-catenin/TCF pathway is active in Paneth cells, in transit-amplifying (TA)/progenitor cells that have a limited proliferative capacity, and in Lgr5+ intestinal stem cells (Clevers, 2006). Lgr5+ cells are capable of efficiently initiating adenoma formation upon mutation of the Apc gene (Barker et al., 2009). In addition, progenitors of intestinal epithelial cells can convert to a stem cell-like phenotype and contribute to the initiation of CRC under inflammatory conditions (Schwitalla et al., 2013). These studies indicate that the acquisition of a stem cell-like phenotype is critical for CRC tumorigenesis.

Intestinal adenomas are highly heterogenous, containing both proliferating and differentiating cells, and they are continuously maintained by a dedicated cell population, the so-called cancer stem cells (Sampieri and Fodde, 2012; Schepers et al., 2012). Although neoplastic cells are characterized by increased cell proliferation and limited cell differentiation capacity, their detailed differentiation pathways in CRC are poorly known. Expression of the Lgr5 gene has been shown to mark a cell population with stem cell properties in mouse intestine and in intestinal adenomas (Barker et al., 2007; Schepers et al., 2012). Furthermore, the intestinal stem cell signature, including LGR5 expression, identifies CRC stem cells and predicts disease relapse also in human CRC patients (Kemper et al., 2012; Merlos-Suárez et al., 2011).

After the genetic lesion that activates the Wnt signal transduction pathway and abnormal cell proliferation, additional mutations accumulate slowly to promote adenoma progression toward CRC, tumor invasion, and metastasis (Sampieri and Fodde, 2012). Highly elevated Wnt activity after Apc deletion induces expression of the homeobox transcription factor Prox1 in intestinal tumor cells. When Prox1 was deleted in mice with an Apc mutation, adenoma growth and development of dysplasia in the tumor epithelium was inhibited (Petrova et al., 2008). Interestingly, Prox1 upregulation after loss of the tp53 tumor suppressor contributes to intestinal tumor progression in some model systems (Elyada et al., 2011).

Here, we analyzed the mechanism of Prox1-induced intestinal adenoma progression in microsatellite-stable tumor models. We found that a subpopulation of Prox1+ cells displays stem cell activity in adenomas/CRC, but not in the normal intestinal epithelium. Furthermore, Prox1 deletion reduced the size of the Lgr5+ adenoma and CRC stem cell populations, and stem cell activity and led to reduced growth and decreased tumor cell survival in an unfavorable microenvironment.
RESULTS

Prox1 Expression Is Induced in Lgr5+ Cells upon Apc Gene Deletion

To characterize Prox1-expressing cells in the pathogenesis of intestinal adenomas, we induced an acute deletion of the Apc gene throughout the whole intestinal epithelium in Apc\textsuperscript{flou/fox}, villin-Cre\textsuperscript{ER} (VApc) mice by a single tamoxifen injection (VApC\textsubscript{a}/\textsubscript{f}). As expected, Paneth cells, marking the crypt bottoms in the wild-type (WT) small intestine, were dislocated toward the lumen in most of the Apc-deleted crypts, and expansion of the cell population with an active Wnt signaling pathway was detected by EphB2 immunostaining 6 days after the tamoxifen injection (Figures S1A and S1B) (Batlle et al., 2002). Most crypts contained scattered Prox1+ cell clusters intermingled with lysozyme-positive Paneth cells (Figure S1A). Cyclin D1 marks the proliferating cell population, including the progenitor cells in WT intestine (Gregorieff and Clevers, 2005). In line with previously published results, the cyclin D1+ cell population expanded after the Apc deletion (Sansom et al., 2005) (Figures S1A and S1B). Cyclin D1\textsuperscript{high} cells were located frequently close to the Prox1+ cells; however, Prox1+ cells were not cyclin D1\textsuperscript{high} in VApC\textsubscript{a}/\textsubscript{f} mice and in tumors from Apc\textsuperscript{min+/+} mice (Figures S1C and S1D) or from CRC patients (Figure S1E). Similarly to the in vivo experiments, when we isolated intestinal organoids from the VApc mice and induced Apc gene deletion in 3D Matrigel culture by the addition of 4-hydroxy-tamoxifen (4-OH-Tam), the organoids showed emerging gene deletion in 3D Matrigel culture by the addition of 4-hydroxy-tamoxifen (4-OH-Tam) without R-Spondin1 (Figure 1A). As expected, Paneth cells, marking the crypt bottoms in the wild-type (WT) small intestine, were dislocated toward the lumen in most of the Apc-deleted crypts, and expansion of the Prox1+ cells had produced adjacent tdTomato+/Prox1+ tumor cells 1 day after the tamoxifen injection (Figure 1D), confirming that the activation of the Cre protein is specific to the Prox1-expressing tumor cells. However, 5 days after tamoxifen injection, we observed that the Prox1+ cells had produced adjacent tdTomato+/Prox1+ progeny, which occasionally stained for mucin2 of goblet cells or for lysozyme of Paneth cells, indicating that Prox1+ tumor cells can give rise to differentiated cells in the intestinal adenomas (Figures 1D and 1E).

In the Prox1-Cre\textsuperscript{ER}, Rosa26-tddTomato\textsuperscript{fox/stop/fox}, Apc\textsuperscript{min/+} mice, Prox1+ epithelial cells were very rarely labeled outside of the tumors after tamoxifen injection. In order to activate the lineage marker more effectively, we used mice harboring a Prox1-Cre\textsuperscript{ER} bacterial artificial chromosome at an ectopic genomic site (Bazigou et al., 2011). In these mice, only sporadic Prox1+ intestinal epithelial cells were positive for the red lineage marker 7 days after tamoxifen treatment (Figure 1F). Furthermore, Prox1 deletion in the intestinal epithelium of Prox1\textsuperscript{fox/fox}, villin-Cre\textsuperscript{ER} mice (VP\textsubscript{a}/\textsubscript{f}) did not result in any obvious phenotype (see Supplemental Results). These data indicate that a subpopulation of the Prox1+ cells has stem cell properties in adenomas, but not in normal intestine.

Prox1 Deletion Leads to Loss of Lgr5+ Stem Cells in Intestinal Adenomas

To analyze the significance of Prox1 specifically in adenoma stem cells, we deleted Apc and Prox1 in the Lgr5+ cells of Apc\textsuperscript{fox/fox}, Prox1\textsuperscript{fox/fox}, Lgr5-EGFP-ires-Cre\textsuperscript{ER} (LapcP) mice. In order to obtain an efficient deletion, tamoxifen was injected during 2 consecutive days and the size of the Lgr5+ cell population was analyzed 21 days thereafter. Interestingly, the majority of the adenomas contained some Prox1+ cells, suggesting an incomplete deletion of Prox1 in the crypt stem cells (Figure 2A). However, the Prox1+ adenomas contained fewer Lgr5+ cells in tamoxifen-treated LapcP (LApcP\textsubscript{a}/\textsubscript{f}) mouse intestines than in the LApcP\textsubscript{a}/\textsubscript{f} controls (Figure 2B). Furthermore, the number of stem cells was even lower in the Prox1+ crypt-like structures that contained dislocated Lgr5+ cells both in the small and large intestine, indicating that successful Prox1 deletion inhibits the expansion of Lgr5+ cells in the adenomas (Figure 2B).

To further test this hypothesis ex vivo, we used LApC\textsubscript{a}/\textsubscript{f} and LApC\textsubscript{a}/\textsubscript{f}\textsubscript{D} organoids. The Wnt-agonist R-Spondin1 is required for the survival and growth of the WT (LApc or LapcP) intestinal organoids (Sato et al., 2009). Without R-Spondin1, only organoids with Apc deletion and resulting active β-catenin/TCF pathway survive beyond 4 days (Wiener et al., 2014). Almost all the Prox1+ cells of LApC\textsubscript{a}/\textsubscript{f} organoids were Lgr5-EGFP positive 8 days after the addition of 4-OH-Tam without R-Spondin1 (Figure 2C). However, there was a marked reduction in the number of viable organoids 8 days after the simultaneous deletion of Apc

A Subpopulation of the Prox1+ Cells Has Stem Cell Activity in Intestinal Adenomas, but Not in the Normal Intestine

To study if Prox1+ cells have stem cell activity in intestinal adenomas, we produced Prox1-Cre\textsuperscript{ER} (Srinivasan et al., 2007); Rosa26-tddTomato\textsuperscript{fox/stop/fox}, Apc\textsuperscript{min/+} mice for lineage-tracing experiments. In this model, a single tamoxifen injection activated the Cre allele, resulting in expression of the tdTomato red fluorescent protein only in the Prox1+ tumor cells 1 day after the tamoxifen injection (Figure 1D), confirming that the activation of the Cre protein is specific to the Prox1-expressing tumor cells. However, 28 days after tamoxifen injection, we observed that the Prox1+ cells had produced adjacent tdTomato+/Prox1+ progeny, which occasionally stained for mucin2 of goblet cells or for lysozyme of Paneth cells, indicating that Prox1+ tumor cells can give rise to differentiated cells in the intestinal adenomas (Figures 1D and 1E).

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and Prox1 in the Lgr5+ cells (Figures 2D–2F). The viable organoids contained some Prox1+ cells at this time point (Figure 2C), suggesting that Prox1 deletion was not complete in the organoids. Since Prox1 was not expressed in the Lgr5+ stem cells before Apc was deleted, but only after 5 days of Apc deletion in vivo, these data indicate that loss of Prox1 expression does not influence the tumor initiation frequency, but instead, it inhibits the expansion of the Lgr5+ adenoma cell population.

To directly address the connection between Prox1 and stem cell number in another ex vivo organoid culture system, we isolated organoids from Apcflox/flox; villin-CreER (VApc) and Apcflox/flox; Prox1flox/flox; villin-CreER (VApcP) mice, where Apc and/or Prox1 are deleted in the whole intestinal epithelium, including the progenitor cells. Deletion of Prox1 alone from the whole intestinal epithelium derived from the Prox1flox/flox; villin-CreER (VApc) mice did not induce morphological changes in the organoids (Figure S2A). However, deletion of both Apc and Prox1 (VApcPΔ/Δ) from the organoids increased the relative expression level of the progenitor markers Nol1 and Wdr3 (Van der Flier et al., 2007) and the relative number of cyclin D1-high cells in comparison with organoids with only Apc deletion (Figures 3A–3C). As in the tamoxifen-injected VApΔ/Δ mice, the Prox1+ cells were cyclin D1-low (Figure 3B). Furthermore, RNAs encoding the Wnt-target intestinal stem cell signature genes Lgr5, Tnfrsf19, and Ascl2 were decreased upon Prox1 deletion, whereas c-Myc RNA was not changed (Figure 3C and data not shown). Microarray analysis of VApΔ/Δ and VApΔ/Δ cultures
7 days after the addition of 4-OH-Tam indicated decreased intestinal stem cell markers and changes in the expression levels of specific Wnt targets in the VApcPΔ/Δ organoids (Figure S2B). These data suggested that Prox1 deletion decreases the number of adenoma stem cells, resulting in a skewed enrichment of the progenitor population in the organoids 7 days after Apc deletion. Indeed, the Prox1-deleted organoids were less efficient in forming new organoid subcultures when they were dissociated to small clusters containing approximately four to seven cells (Figure 3D).

**PROX1 Silencing Decreases Stem Cells in Human CRC Organoids**

To model the effect of PROX1 on stem cell activity in CRC progression, we chose to use the human SW1222 cell line (mutant CRC genes in this cell line are listed in Table S1).
is enriched for stem cells that can self-renew and differentiate into multiple lineages (Yeung et al., 2010). While the “small colonies” in SW1222 cell-derived 3D cultures have a limited growth potential and lack lumens, the large glandular “megacol- onies” produce crypt-like structures consisting of polarized cells surrounding a central lumen (Yeung et al., 2010). Lumens are stem cell-dependent structures present in well-differentiated tumors and likewise in ex vivo and in vitro 3D cultures from human and mouse adenomas and stem cell-containing CRC cell lines (Ashley et al., 2013).

Since the SW1222 cells have heterogenous PROX1 and nuclear β-CATENIN expression levels (Figure S2C), we isolated subclones with a low (PROX1low) or a high (PROX1high) percentage of PROX1+ cells (Figure S2D). We then silenced PROX1 in the PROX1high clone by two different lentiviral short hairpin RNAs (shRNAs) (Figure S2E and data not shown). Interestingly, the PROX1-silenced SW1222-PROX1high cells formed a strikingly reduced number of glandular colonies (Figure 3E), suggesting that PROX1 silencing has a profound effect on CRC stem cell activity. Furthermore, PROX1 silencing decreased the number of lumens in the glandular colonies derived from SW1222-PROX1high cells (Figure 3F). After 12 days of 3D culture, the LGR5 and TNFRSF19 RNAs were decreased, whereas no changes were observed in the expression of other WNT-target genes, such as MYC (Figure 3G), PROX1 silencing in CRC patient-derived organoids also resulted in decreased frequency of new lumen-containing organoids (Figure 3H). Notably, the shPROX1 organoids formed small colonies without lumens (Figure 3H).

Similarly to the SW1222 cell line, PROX1 silencing in CRC patient-derived organoids resulted in a marked reduction of LGR5 and TNFRSF19 RNA levels (Figure 3I), even in the presence of a KRAS mutation (see Supplemental Experimental Procedures). LGR5 in situ hybridization further indicated that PROX1 silencing decreases the number of CRC stem cells (Figures 3J and 3K). Notably, there was no difference in the LGR5 RNA in PROX1-silenced SW1222 cells in 2D culture (data not shown), thus ruling out the possibility that LGR5 is a direct target of PROX1. These results suggest that loss of PROX1 decreases the CRC stem cells also in human CRC, and that this is independent of the presence of KRAS mutations.

**PROX1+ Tumor Cells Proliferate Less Than Prox1+ Tumor Cells, yet PROX1 Deletion Leads to Decreased Cell Proliferation in the Tumors In Vivo**

To test the in vivo significance of our findings, we implanted SW1222-PROX1high cells and their PROX1-silenced counterparts subcutaneously into immunocompromised (NOD scid gamma [NSG]) mice and monitored tumor growth. Similarly to the Prox1-deleted intestinal adenomas (Petrova et al., 2008), the PROX1-silenced tumors grew slower than controls transduced with scrambled shRNA (Scr) at this ectopic implantation site (Figure 4A). Notably, we detected 60% fewer tumor nests in the PROX1-silenced samples as compared to Scr controls (200.4 ± 46.8 and 70.9 ± 11.8 tumor nests, respectively; p < 0.01) when they were excised 21 days after the implantation. Unlike the scrambled shRNA-transduced tumors, the PROX1-silenced tumors contained tumor nests with only few glandular structures (Figure 4B). Similarly to the PROX1-silenced SW1222 cells, subcutaneously implanted Prox1-deleted mouse VApcPΔ/Δ organoids grew slower and contained fewer glands than VApcΔ/Δ organoids (Figures 4C and 4D). Prox1 deletion decreased Lgr5 and Tnfrsf19 RNAs, but not of Muc RNA in the tumors, confirming our ex vivo results (Figure 4E).

Intestinal epithelial progenitor cells proliferate rapidly, but only for a limited number of cell cycles (Gregorieff and Clevers, 2005). Careful analysis of the Prox1+ and Prox1− cells in control SW1222-PROX1high or in VApcΔ/Δ organoid-derived tumors indicated that the Ki67+ proliferating cells were enriched in the Prox1− cell population (Figures S3A and S3B), suggesting that most of the Prox1+ cells are nonproliferative or slowly proliferating. Interestingly, the Prox1+/Lgr5+ cell population had a higher frequency of Ki67+ cells than the Prox1+/Lgr5− population in the LAp/Δ/Δ intestine (Figure 4F), indicating that the rapidly proliferating Prox1+ cells are enriched in the Lgr5+ population. Similarly, bromodeoxyuridine labeling of two highly proliferating organoid cultures (VApcΔ/Δ and VCK1Δ/Δ) showed that only a small proportion of the Prox1+ cells proliferated (Figure S3C). VCK1Δ/Δ organoids are deleted of tp53 and Csnk1a1, encoding casine kinase Iz (Ckiiz), which phosphorylates β-catenin, targeting it to ubiquitin-mediated destruction; thus, the VCK1Δ/Δ organoids display activated Wnt signaling pathway (Elyada et al., 2011). Whereas Prox1+ cells appeared to proliferate slowly, we observed a decreased overall frequency of Ki67+ tumor cells in subcutaneously growing PROX1-silenced SW1222-PROX1high tumors, in Prox1-deleted VApcPΔ/Δ organoid tumors in NSG mice, and in the Lgr5+ cells of LAp/Δ/Δ intestinal tumors (Figure 4G; Figure S3D). A possible explanation for these apparently contradictory data is that Prox1 deletion results in decreased proliferation in the stem cell population, which leads to exhaustion of the CRC stem cell pool and consequently to a decrease of the overall ratio of proliferating cells. Indeed, a slower proliferation rate of the PROX1-silenced SW1222-PROX1high cell-derived organoids was observed only after a 14-day culturing period, when the PROX1− organoids already had an extensive lumen forming activity, but not at 6 days (Figure S3E). This indicates a delayed effect of PROX1 silencing on the overall cell proliferation in the organoids, in line with the idea that PROX1 influences tumor growth by regulating the size of the proliferating stem cell pool.

The PROX1+ (adherent) cells of the SW480 CRC cell line (SW480A) are unable to initiate subcutaneous tumors in NSG mice, whereas the PROX1− cells (SW480R) are round, form cell clusters, and are tumorigenic (Petrova et al., 2008). To test if PROX1 silencing in already established CRC xenografts regulates stem cells, we implanted cells from the PROX1− stem cell-like SW480R subclone, expressing a doxycycline-inducible PROX1 shRNA construct (SW480R-sh) (Petrova et al., 2008), into NSG mice. Doxycycline treatment was started 8 days after subcutaneous injection, when the tumors were already visible. We observed reduced growth of the PROX1-silenced tumors after day 16 (Figures 4H and 4I), at a time point when the LGR5 RNA level had already markedly decreased in the doxycycline-treated tumors (Figure 4J). These data suggest that PROX1 regulates stem cells also in established tumors.
Figure 3. Prox1 Deletion Leads to Reduced Stem Cell Activity in Ex Vivo Organoids

(A) The schematic outline of the mouse organoid experiments. GF, growth factors.

(B) Prox1 and cyclin D1 immunostaining of sections from VApcΔ/Δ and VApcΔΔ organoids 7 days after the deletion and 5 days after removing R-Spondin1 from the culture medium.

(C) RNA levels of progenitor markers (Nol1, cyclin D1, and Wdr3), stem cell markers (Lgr5 and Tnfrsf19), and Myc in VApcΔ/Δ and VApcΔΔ organoids analyzed by real-time quantitative PCR (qRT-PCR).

(D) The organoid initiating frequency of VApcΔ/Δ and VApcΔΔ organoids.

(E) The proportion of glandular colonies derived from 1,000 SW1222-PROX1 high cells (transduced with Scr, sh1, or sh2 PROX1 shRNA lentivirus).

(F) The number of lumens in the glandular colonies derived from Scr or shPROX1-transduced (sh1, sh2) SW1222-PROX1 high cells at day 14 (n = 10 for sh1 and n = 12 for sh2). The lumens (asterisks) were detected by phalloidin staining and counted in optical sections of confocal microscopic images. The boxplot indicates the minimum, the first quartile, median, third quartile, and maximum.

(G) Real-time qPCR of the indicated RNAs from SW1222-PROX1 high cell-derived organoids, transduced with shPROX1 or Scr lentivirus and grown in Matrigel for 14 days.

(legend continued on next page)
PROX1 Silencing Increases Annexin A1 Expression in Multiple CRC Models

To determine which genes are responsible for the effect of PROX1 on the adenoma/CRC stem cells, we tested PROX1-regulated candidate genes based on the microarray data derived from the SW480R subclone (Petrova et al., 2008). Based on our initial results, we focused further on the calcium-dependent phospholipid binding protein Annexin A1 (ANXA1), which has been shown to inhibit breast cancer metastasis (Maschler et al., 2010). PROX1 suppressed ANXA1 RNA (4.21 ± 0.08-fold, mean ± SD) and protein expression in SW480R-sh cells (Figure S4 A). Anxa1 was increased after 4-OH-Tam addition to Vapc organoids and even further elevated when also Prox1 was deleted (Figure 5 A; Figures S4 B and S4C), suggesting that Prox1 suppresses Anxa1 expression. Furthermore, the Prox1−

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cells showed more intense Anxa1 staining than Prox1+ cells in tumor sections from Apcmin/+ mice (Figure S4D).

We observed a mutually exclusive staining for Anxa1 and Prox1 in tumors from VCKΔ/Δ mice and in human CRC samples (Figures S6B and S5C). Furthermore, the Lgr5+ intestinal stem cells in the LApc mice showed very little or no epithelial Anxa1 expression before Apc deletion (Figure S4E). They contained a low level of Anxa1 after tamoxifen injection, whereas the Anxa1high cells were negative for Lgr5 in the resulting adenomas (Figure S4F). Importantly, transfection of a dominant-negative TCF4 (transcription factor 7-like 2 [TCF7L2]) construct, a Wnt pathway suppressor to the PROX1– subclone of the SW480 CRC cell line (SW480A) (Petrova et al., 2008), led to suppression of ANXA1 expression (Figure 5D). These data indicate that Anxa1 is minimally expressed in the normal intestinal epithelium, where the activation of the Wnt pathway after Apc deletion increases Anxa1 expression, which is then suppressed by the induction of Prox1 in the tumor cells.

ANXA1 Silencing Mimics the Effects of PROX1 in CRC

We next tested the possibility that the strong ANXA1 suppression mediates the effects of PROX1, such as the associated rearrangement of the actin cytoskeleton, changes in cell shape, and adherence to the culture plates (Petrova et al., 2008). Indeed, ANXA1 overexpression in the SW480R subclone induced an elongated cell shape and increased the number of tightly adherent cells, whereas ANXA1 suppression resulted in rearrangement of the actin cytoskeleton, rounded cell shape, and decreased adherence (Figures S5A–S5C). Interestingly, Anxa1 silencing in VAPcΔ/Δ organoids increased the Lgr5 and Tnfrsf19 stem cell marker RNAs, without affecting Myc (Figure 5E). Although ANXA1 silencing had no effect on PROX1 expression in SW1222-PROX1low or SW480 cells in 2D culture (data not shown), it resulted in increased lumen formation (Figure 5F), upregulation of Tnfrsf19 and Lgr5 RNAs, and increase of LGR5+ cells in 3D organoids derived from shANXA1-transduced cells (Figures 5G and S5H). In contrast, ANXA1 overexpression in SW1222-PROX1high cells decreased the proportion of glandular colonies, the number of organoid lumens, and LGR5 and Tnfrsf19 RNA levels when compared to the controls (Figures S5D–S5F). Furthermore, the ANXA1 silenced SW1222-PROX1low cell-derived tumors grew faster than control tumors, contained more Ki67+ cells, and were composed of larger nest structures (Figure 5I; Figure S5G). Similar to the effect of PROX1, ANXA1 silencing in SW1222 cell-derived organoids was associated with enhanced cell proliferation only after 16 days, when the organoids showed extensive outpocketing and lumen formation, but not at day 6 (Figure S5H). These data indicate that ANXA1 silencing mimics the effects of PROX1 expression, leading to an expansion of the stem cell pool via increased proliferation.

Silencing the Actin-Binding Protein FILAMIN A Decreases Stem Cells in CRC

Reanalysis of the microarray data derived from the SW480R subclone showed that focal adhesion and regulation of actin cytoskeleton were the top Kyoto Encyclopedia of Genes and Genomes pathway categories affected when PROX1 was silenced (data not shown). Thus, we searched for PROX1-regulated genes directly affecting the cytoskeleton among published CRC stem cell gene sets (de Sousa E Melo et al., 2011) and prognosis markers in CRC subgroups (Sadandam et al., 2013). We found the gene encoding the actin-binding protein filamin A (FLNA) in both gene sets. FLNA connects actin filaments to transmembrane receptors, including β1 integrin, modulates cell migration, and functions as a central mechanotransduction element of the cytoskeleton (Ehrlicher et al., 2011; Zhou et al., 2010). Interestingly, we found highly increased Flna expression in the Prox1+ and Lgr5+ tumor cells (Figure 6A and data not shown) and a correlation between PROX1 and FLNA levels in the SW1222-PROX1high cell-derived organoids (Figure 5B). FLNA was also upregulated in the ANXA1 silenced organoids (Figures 6B and 6C). Furthermore, PROX1 silencing in the SW1222-PROX1high and CRC patient-derived organoids resulted in markedly decreased FLNA RNA (Figure 6D) and protein (Figure 6E).

Because both PROX1 and ANXA1 regulated FLNA expression in intestinal adenomas and in CRC 3D organoids, we studied FLNA function by silencing its expression in VAPcΔ/Δ organoids and in SW1222-PROX1high cells. This led to reduced Lgr5 and Tnfrsf19 RNAs (Figure 6F), to a decrease of SW1222 cell-derived glandular colonies and lumens (Figure 6G), and to a decreased number of Ki67+ cells in the organoids (Figure 6H). Surprisingly, FLNA silencing did not affect the growth of the cells in 2D culture conditions (data not shown). Overall, these results suggest a model where the loss of Prox1 leads to an elevated Anxa1 level and a reduction in Flna, which limit the expansion of stem cells.
of the adenoma/CRC stem cell population via decreasing cell proliferation.

**PROX1 Silencing Reduces Cell Survival in Hypoxic Tumor Xenografts**

Interestingly, when grown to a large size in Matrigel culture (>150 μm diameter, >5 days after subculture) or injected subcutaneously into NSG mice, Prox1-deleted VApcΔ/Δ organoids contained more apoptotic cells than VApcΔ/Δ organoids (Figure 7A). Also, we detected an intense staining for the apoptosis marker active CASPASE-3 inside the PROX1-silenced SW1222-PROX1high cell-derived subcutaneously growing tumors that were positive for the hypoxia marker carbonic anhydrase IX, but not at the tumor margin (Figure 7B and data not shown), suggesting reduced tumor cell survival in the ischemic/hypoxic tumor interior. Interestingly, we found no difference in the endomucin+ blood vessel density between Scr and shPROX1 tumors, even when vascular endothelial growth factor (VEGF) was overexpressed in the tumor cells (Figures S6A and S6B). However, the shPROX1 tumors were smaller than the Scr tumors, even when VEGF was overexpressed in the tumor cells and VEGF failed to rescue the large necrotic areas inside the tumors.
PROX1-silenced tumors (Figures S6A and S6B). Thus, the presence of the large necrotic areas in the shPROX1 tumors could not be explained by the lack of angiogenic factors.

Although ANXA1 silencing mimicked several of the effects of PROX1 in the intestinal adenoma and CRC models, the apoptosis rate in the ANXA1-silenced subcutaneously growing tumors was not significantly affected when analyzed 9 or 24 days after implantation (data not shown). This raised the possibility that in unfavorable conditions also alternative pathways contribute to the apoptosis in the shPROX1 tumors. Autophagy is an essential cellular process for the survival of cells under hypoxia or nutrient deprivation (Sato et al., 2007). Interestingly, PROX1 silencing in the SW480R cells resulted in a decrease of the autophagy-associated LC3-II protein both in normal medium and in starvation conditions (Figures 7C and 7D). Furthermore, lack of PROX1 prevented the accumulation of the LC3-containing early autophagosomes in the presence of the lysosomal inhibitors bafilomycin A or chloroquine, which inhibit the fusion of autophagosomes with the lysosomes (Figure 7E). Importantly, the inhibition of chloroquine or bafilomycin at a low dose to the LApcΔ/Δ organoid cultures reduced the proportion of viable organoids to the same level as Prox1 deletion in the LApcΔ/Δ organoid cultures, thus highlighting the important role of autophagy in promoting tumor cell survival specifically in the Prox1+ cells (Figure 7H).

**DISCUSSION**

Prox1 is critical for the fate of several types of stem and progenitor cells (Elisir et al., 2012). Here, we show that Prox1 is not expressed in the Lgr5+ stem cells of normal intestinal crypts, but is induced soon after the initiating mutation in intestinal tumorigenesis. We show that the Prox1+ cells give rise to more differentiated Prox1− cells in the adenomas, but not in the normal intestine, indicating that a subpopulation of the Prox1+ cells has cancer stem cell activity. Upon Prox1 deletion, the number of stem cells declined and this was reflected later on as decreased overall tumor cell proliferation and tumor growth.

Stem-like cells provide an important drug target in cancer, as they are able to persist in tumors as a distinct population, self-renew, and differentiate, and they are associated with tumor relapse (Sampieri and Fodde, 2012). Therefore, development of specific therapies targeted at cancer stem cells may improve survival. In elegant studies, Schepers et al. demonstrated that the Lgr5+ cells in intestinal adenomas have stem cell properties (Schepers et al., 2012). Interestingly, recent studies indicated that intestinal adenomas contain fewer stem cells than Lgr5+ cells, suggesting that only a subpopulation of the Lgr5+ cells function as stem cells (Kozar et al., 2013). Furthermore, Myant et al. have shown that the Rac1 GTPase is an important regulator of the proliferation of the Lgr5+ cell population in intestinal adenomas (Myant et al., 2013). Although the Lgr5+ cells may be dispensable for homeostasis of the normal intestine and for increased proliferation of intestinal epithelium after Apc deletion (Metcalfe et al., 2014), the expansion of cells derived from the Lgr5+ cells in adenomas after Apc deletion clearly indicates that they contribute to tumor growth (Schepers et al., 2012). Importantly, Prox1 deletion not only decreased the number of Lgr5+ cells in intestinal adenomas in vivo, but it also reduced their proliferation, the organoid-initiating frequency and the number of megacolonies and lumens in the glandular CRC organoids, which all represent indicators of cancer stem cells (Ashley et al., 2013). Furthermore, PROX1 silencing after the tumor establishment resulted in a decrease of the LGR5+ tumor cell marker before a decline in the overall tumor growth rate.

Mechanistically, we show that the phospholipid-binding protein ANXa1 is increased by the Wnt pathway activation after Apc deletion, while Prox1 suppresses its expression. ANXA1 and PROX1 showed also mutually exclusive expression patterns in human CRC samples. Strikingly, the silencing of ANXA1 in the PROX1+ cells mimicked the effects of PROX1 in the CRC cells. Among its other effects, ANXA1 inhibits the proinflammatory phospholipase A2, which has been shown to stimulate the proliferation of CRC cells by producing various lipid mediators and which regulates intercellular junctions and the actin cytoskeleton (Cristante et al., 2013; Parente and Solito, 2004; Surrel et al., 2009). Both ANXA1 silencing and PROX1 expression increased stem cell markers and the number of LGR5+ cells in tumor organoids and enhanced lumen formation, proliferation of tumor cells, and tumor growth in vivo. Furthermore, ANXA1 silencing and PROX1 expression increased FLNA, which stabilizes the cortical 3D actin networks, links them to the transmembrane receptor β1 integrin, and functions as a central mechanotransduction element of the cytoskeleton (Ehrlicher et al., 2011; Zhou et al., 2010). The role of the actin cytoskeleton in the expansion of the adenoma stem cell population was supported by our FLNA results, showing that silencing this actin-binding protein, which was expressed in the Lgr5+ stem cells of intestinal adenomas, dramatically inhibited the activity and proliferation of the adenoma/CRC stem cells in 3D, but not in 2D, culture conditions.

FLNA is one of the markers of a CRC subtype characterized by poor disease-free survival (Sadanandam et al., 2013). FLNA expression is increased in a number of cancers, and it has been recently shown to boost the hypoxia response and tumor progression (Zheng et al., 2014). Consistent with this, the FLNA-expressing PROX1+ tumor cells were more resistant to apoptosis than the FLNA low PROX1− tumor cells both in vivo and in the organoids. There was a striking increase in apoptosis, particularly in the central parts of the PROX1-silenced tumors growing subcutaneously in mice. However, the sensitivity of the PROX1+ tumor cells to apoptosis was not due to insufficient expression of angiogenic growth factors, as shown by the inability of VEGF overexpression to rescue the difference. Instead, the analysis of autophagy markers and use of lysosomal inhibitors of autophagy indicated that PROX1 expression sustains autophagy, which is known to be essential for the survival of CRC cells (Sato et al., 2007). This finding is particularly
Figure 7. Loss of PROX1 Reduces CRC Stem Cell Survival under Unfavorable Conditions

(A) Percentage of caspase-3+ apoptotic cells among the E-cadherin+ epithelial cells in VApcDAΔ (black columns) and Prox1-deleted (VApcPAΔ; red columns) organoids 8 days after the addition of 4-OH-Tam and 6 days after the removal of R-Spondin1 (right panel, n = 14) or growing subcutaneously in NSG mice (left panel, n = 22).

(B) Active caspase-3 staining of subcutaneously growing Scr and shPROX1-transduced SW1222-PROX1hsh tumors in NSG mice. Note that the shPROX1-transduced tumor interior is highly apoptotic (asterisk).

(C and D) Immunoblotting of SW480R-sh cells for the indicated proteins. Cells were either cultured in complete medium (C) or in starvation medium lacking amino acids 8 hr before protein isolation (D) in the absence or presence of 100 nM bafilomycin A or 30 μM chloroquine.

(E) The number of LC3+ granules in the indicated cell lines transduced with Scr or shPROX1 lentivirus and cultured in starvation medium containing 100 nM bafilomycin in hypoxia for 8 hr (n = 50).

(F) Flow cytometric analysis of the Lgr5-EGFP+ cells in LApcDAΔ organoids, 9 days after the Apc deletion and 3 days after the addition of 15 μM chloroquine or 0.2 μM bafilomycin A.

(G) The plating efficiency and survival percentage of VApcDAΔ organoids in the presence or absence of chloroquine or bafilomycin A. Note that for organoid survival chloroquine or bafilomycin were added 2 days after organoid splitting for 3 days.

(legend continued on next page)
interesting considering that hypoxia-induced autophagy can promote tumor cell survival and adaptation to antiangiogenic treatment, which is used in CRC therapy (Hu et al., 2012). Our results are in agreement with the findings of Ragusa et al., who show that PROX1 promotes the metabolic adaptation of CRC cells in unfavorable microenvironments, and thus critically contributes to the metastatic outgrowth of CRCs (Ragusa et al., 2014).

In summary, we show here that PROX1 is induced in intestinal stem cells in adenoma/CRC soon after the activation of the Wnt pathway. While the APC mutation induces both PROX1 and ANXA1 expression in the epithelium, PROX1 restricts ANXA1 levels and induces FLNA, which stimulates cell proliferation and promotes stem cell activity in the adenomas, and may counteract stem cell exhaustion during tumor growth. The net effect is the expansion of the adenoma/CRC stem cell population and increased tumor growth. In the hypoxic parts of tumor transplants, PROX1 promotes tumor cell survival by increasing autophagy. Based on this study, PROX1 regulates the number of adenoma/CRC stem cells without affecting the homeostasis of the normal intestine, thus providing an attractive therapeutic target pathway for drug development in CRC.

EXPERIMENTAL PROCEDURES

A detailed description of the experimental procedures is provided in Supplemental Experimental Procedures.

Intestinal Crypt/Organoid Cultures

The National Board for Animal Experiments at the Provincial State Office of Southern Finland approved all experiments performed with mice. Intestinal crypts from APC<sup>flox/flox</sup>, villin-<sup>Cre</sup>ER<sup>+</sup>, APC<sup>flox/flox</sup>, Prox1<sup>flox/flox</sup> (Harvey et al., 2005); villin-<sup>Cre</sup>ER<sup>+</sup>, Csknl1<sup>−/−</sup>, villin-<sup>Cre</sup>ER<sup>+</sup> (Elyada et al., 2011), APC<sup>flox/flox</sup>, Lgr5-EGFP-<sup>Cre</sup>ER<sup>+</sup> (Barker et al., 2007) and APC<sup>flox/flox</sup>, Prox1<sup>−/−</sup>/Lgr5-EGFP-<sup>Cre</sup>ER<sup>+</sup> mice were isolated and cultured as described previously (Sato et al., 2009, 2011). To activate the endogenous β-catenin/TCF pathway in mouse organoids, cultures were treated with 300 nM 4-hydroxy-tamoxifen (4-OH-Tam) for 48 hr. Organoids with the endogenously active β-catenin/TCF pathway were then selected and cultured in growth factor-deficient medium.

Human Organoid Cultures

For 3D culture, SW1222 or patient-derived CRC cells were extensively trypsinized, embedded into Matrigel (500–2,000 cells/50 μl Matrigel/well), and grown for 3–16 days. The ethics committee of the Department of Surgery at Helsinki University Hospital approved all experiments involving patient samples, and informed consent was obtained from the patients. Tissue samples isolated from CRC patients were processed according to a previously published method (Sato et al., 2011).

In Vivo Experiments

Mice were injected with 2 mg tamoxifen (Sigma) dissolved in 200 μl sunflower oil (Sigma) at the age of 8–9 weeks. The mice were euthanized at the indicated time points. The mice were on the C57Bl/6 background. In all experiments littermate controls were used.

Statistical Analysis

Statistical comparison of two groups was done by two-tailed unpaired or paired t test using the SPSS software unless otherwise indicated. For nonparametric tests, the Mann-Whitney U test was used, and the data are presented as boxplots, showing the five statistics (minimum, first quartile, median, third quartile, maximum). The statistical significance is marked by *p < 0.05, **p < 0.01, and ***p < 0.005.

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(H) Relative survival rate of LApcaΔ/Δ or LApccΔ/Δ organoids 8 days after 4-OH-Tam and 6 days in R-Spondin1-free medium. Chloroquine (15 μM) or bafilomycin A (0.2 μM) were added 4 days after 4-OH-Tam for 4 days.

The boxplots in (A) and (E) indicate the minimum, the first quartile, median, third quartile, and maximum. Mean ± SD are shown for (F)–(H). Scale bars represent 100 μm. See also Figure S6.
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