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Runx1 is a tumor suppressor gene in the mouse gastrointestinal tract

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The *Runx1* transcription factor plays an important role in tissue homeostasis through its effects on stem/progenitor cell populations and differentiation. The effect of *Runx1* on epithelial differentiation of the secretory cell lineage of the colon was recently demonstrated. This study aimed to examine the role of *Runx1* in tumor development in epithelial cells of the gastrointestinal tract. Conditional knockout mice that lacked *Runx1* expression in epithelial cells of the GI tract were generated. These mice were crossed onto the Apc^{Min} background, killed and their intestinal tumor phenotypes were compared with Apc^{Min} *Runx1* wild-type control mice. *Apc*-wild-type *Runx1*-mutant mice were also examined for tumor development. Colons from *Runx1* knockout and wild-type mice were used for genome-wide mRNA expression analyses followed by gene-specific quantitative RT-PCR of whole colon and colon epithelium to identify *Runx1* target genes. *Runx1* deficiency in intestinal epithelial cells significantly enhanced tumorigenesis in Apc^{Min} mice. Notably, epithelial *Runx1* deficiency in *Apc*-wild-type mice was sufficient to cause tumor development. Absence of *Runx1* was associated with global changes in the expression of genes involved in inflammation and intestinal metabolism, and with gene sets indicative of a metastatic phenotype and poor prognosis. Gene-specific analysis of *Runx1*-deficient colon epithelium revealed increased expression of genes linked to an expansion of the stem/progenitor cell population. These results identify *Runx1* as a novel tumor suppressor gene for gastrointestinal tumors and support a role for *Runx1* in maintaining the balance between the intestinal stem/progenitor cell population and epithelial differentiation of the GI tract. (*Cancer Sci* 2012; 103: 593–599)

The human and mouse family of *RUNX* transcription factors are composed of three members, *RUNX1, RUNX2* and *RUNX3*. They function as either transcriptional activators or repressors depending on the target gene, cell type and the presence of co-factors. *RUNX1* has been implicated in homeostasis of the hematopoietic stem cell number, as well as in developmental lineage specification, thereby being a critical factor for hematopoiesis and hematopoietic function.[1,2] In mice, *Runx1* is essential for mammalian development as germline knockouts (KO) are embryonic lethal due to a complete failure of hematopoiesis.[3] In humans, polymorphisms that reduce the binding of *RUNX1* to specific target genes have been associated with susceptibility to the autoimmune diseases psoriasis, systemic lupus erythematosus (SLE) and rheumatoid arthritis.[4] Moreover, *RUNX1* is frequently mutated in a common subtype of human acute myeloid leukemia (AML), where often the rate-limiting step is formation of mutant fusion genes/proteins that combine the N-terminal DNA-binding domain of *RUNX1* (RHD) with the full-length activation domains of oncogenes.[5] An example is the eight twenty-one (ETO) t(8;21) translocation that is present in approximately 40% of human AML. Notably, these fusion proteins can function as dominant negative mutants that silence *RUNX1* target genes leading to a loss of function of *RUNX1* in AML.[6,7] An example is derepression of vascular endothelial growth factor alpha (VEGFA) (an independent prognostic factor for relapse-free survival in AML) expression in human AML caused by t(8;21) translocations.[8] Knockin of a *Runx1*-ETO fusion transgene causes embryonic lethality in mice similar to the germline *Runx1* KO, further consistent with the fusion protein having a dominant negative effect on *Runx1*.[9] Small intragenic mutations in *RUNX1* have also been reported to exert a dominant negative effect on *RUNX1* function in AML,[10] in myelodysplasia and familial platelet disorders that can lead to AML[11–14] in chronic myelomonocytic leukemia that predisposes to AML[15] and in T-lymphoblastic lymphomas.[10]

Less is known about the role of *RUNX1* in epithelial cancers. *RUNX1* has been reported to be overexpressed in human endometrial cancer and in a mouse skin cancer model.[16] In contrast, *RUNX1* expression has been reported to be downregulated in some human pituitary cancers,[17] recurrent deletions of *RUNX1* were found in human pancreatic adenocarcinomas[18] and homozygous deletions in *RUNX1* have been observed in esophageal adenocarcinoma cell lines and xenografts.[19] *RUNX1* downregulation is associated with an increase in FOXO1 expression and hyperproliferation of breast cancer cells, and has been reported in hormone-negative breast cancers lacking *HER2* amplification.[20,21] In the gastrointestinal tract, *RUNX1* has been reported to be downregulated in human gastric cancer cells,[22] and very recently, *Runx1* was identified as an intestinal cancer susceptibility gene in a *Sleeping Beauty* transposon-mediated mutagenesis screen in mice.[23]

The role of another family member, *RUNX3*, is better characterized in epithelial cancers as it has been reported to act as either an oncogene or tumor suppressor, with a predominance of reports describing its function as a tumor suppressor gene in several human epithelial cancers, such as colorectal, gastric, esophageal, lung, liver and melanomas.[24–30] In the intestinal tract, dysregulation of *RUNX3* is linked to both inflammation and cancer. Targeted *Runx3* deficiency in mouse leukocytes leads to inflammatory bowel disease (IBD),[31,32] while hypermethylation of the *RUNX3* promoter in human intestinal epithelial cells has been observed in ulcerative colitis.[33,34] Silencing of *RUNX3* by hypermethylation has also been reported in human microsatellite instable/CpG island methylator phenotype (MSI/CIMP) colorectal cancer (CRC).[35–37] Further, in the gastrointestinal (GI) tract, both *RUNX1* and *RUNX3* are directly involved in the transforming growth factor beta (TGFβ) signaling pathway and act to promote expression of TGFβ and its pathway genes such as *BMPs* and *SMADs*.[38] Thus, there is evidence that *RUNX1* and *RUNX3* expression, function and role in cancer overlap in some tissues, an observation that has been further supported by the recent report of single nucleotide

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polymorphisms (SNP) in both *RUNX1* and *RUNX3* that confer susceptibility to CRC.[39] Finally, in contrast, a third *RUNX* family member, *RUNX2,* is proposed to largely function as an oncogene in bone and other cancers.[5,40]

Recently, inducible Mx-Cre-mediated conditional deletion of *Runx1* in mouse colon resulted in decreased expression of *Klf4* and goblet cell depletion, demonstrating a role for *Runx1* in regulation of intestinal epithelial differentiation of the secretory cell lineage.[41] Several lines of evidence indicate that *RUNX1* can both positively and negatively interact with Notch signaling,[42–44] which is important for the proper differentiation and organization of cells in the GI tract via crosstalk with Wnt/β-catenin and other canonical signaling pathways.[45,46] Upregulation of Notch signaling is implicated in both gastric and colorectal cancers, where it represses goblet cell differentiation.[45,47,48] Inhibition of Notch activity converts proliferative enterocytes into goblet cells[45] possibly via activation of *KLF4* gene expression.[49]

Because of the reported role of *RUNX1* in *KLF4* signaling and goblet cell differentiation and its interaction with Notch signaling activity, here we investigated the role of epithelial expression of *Runx1* in intestinal tumorigenesis. We found that deficiency of *Runx1* significantly enhanced tumorigenesis in Apc^{Min} mice, thus providing the first direct evidence that *Runx1* is a tumor suppressor gene in the mammalian GI tract. Notably, *Apc*-wild-type *Runx1* knockout mice also developed intestinal tumors when aged >10 months. Loss of epithelial expression of *Runx1* was associated with increased expression in the whole colon of genes involved in inflammation and intestinal metabolism, and with gene sets indicative of a metastatic phenotype and poor prognosis. Gene-specific analysis in *Runx1*-deficient colonic epithelial cells revealed increased expression of several genes implicated in stem cells and *Notch* signaling.

Materials and Methods

Mice. C57BL/6J (later referred to as wildtype [WT]) and C57BL/6J-Apc^{Min} mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6-*Runx1* floxed mice[3] were obtained from Dr Daniel Littman (New York University, New York, NY, USA). *Villin*-Cre transgenic mice that are fully congenic on the C57BL/6 genetic background were obtained from a colony maintained in our laboratory. The *Runx1* floxed allele was introgressed into an Apc^{Min} strain of mice that carried a hemizygous copy of the *Villin*-Cre transgene. For details of mouse husbandry see the Data S1.

Genotyping. DNA was isolated from tail snips using a Qiagen DNA Easy kit (Qiagen, Valencia, CA, USA). The genotype of the *Apc, Villin-Cre* and *Runx1* loci was determined by PCR assays as previously described.[3,50,51] The PCR was performed on an Applied Biosystems GeneAmp 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA).

Oligonucleotide microarray and data analysis. Two-color microarray-based gene expression analysis was carried out using Agilent 4x44K whole mouse genome microarrays (G4122F; Agilent Technologies, Wilmington, DE, USA). This array includes more than 41 000 mouse genes and transcripts. Per sample, 500 ng of RNA was spiked, labeled, amplified and hybridized using Agilent reagents, according to Agilent Gene Expression oligo microarrays protocol (version 5.0.1). For each group (Villin-Cre transgenic and non-transgenic), half of the samples were labeled with Cy3 and half of the samples with Cy5 to prevent bias of the data due to labeling with these fluorescent dyes. Arrays were scanned using an Agilent Microarray Scanner, and the intensity of fluorescent images was quantified using Agilent Feature Extraction software (version 9.5.1). Preprocessing of data, quality checks and significance analysis were all performed as previously described.[52] Differential expression of genes was quantified by the moderated t-statistic. Gene Set Enrichment Analysis (GSEA) was used as a computational method to identify sets of genes with coordinate differences in gene expression (GSEA v2.0, http://www.broad.mit.edu/gsea/).[53,54] Gene sets analyzed were obtained from the Molecular Signatures Database (MSigDB_v2.5, http://www.broad.mit.edu/gsea/msigdb/index.jsp) using settings as previously described.[55] Gene expression data has been submitted to the Gene Expression Omnibus. Accession number GSE34292.

Statistical analysis. Two-sided *P*-values for tumor counts were determined using the Wilcoxon rank-sum test comparing gender and age-matched classes produced in the same genetic crosses.

Histopathology, immunohistochemistry, RNA extraction, isolation of intestinal epithelium, qRT-PCR and tumor analysis (See Data S1, Supplemental Methods).

Results

Phenotype of intestinal-specific *Runx1*-deficient mice. Homozygous *Runx1* floxed mice expressing the intestinal-specific *Villin-Cre* transgene were compared with *Villin-Cre*-negative littermates. We observed no differences in survival, gender ratio, growth, adult size or weight. Formalin-fixed intestinal tissues from all regions were examined using histopathological analysis and no differences were discernable. Tissues were further tested via immunohistochemistry for Ki-67 (cell proliferation), lysozyme (Paneth cells), Muc2 and Alcian blue (goblet cells) and no differences were observed (Fig. S1). Knockout of Runx1 expression was confirmed using immunohistochemistry (Fig. S2) and qRT-PCR (Fig. S3).

***Runx1* deficiency results in enhanced tumorigenesis in Apc^{Min} mice.** We tested the role of *Runx1* in intestinal tumorigenesis by creating Apc^{Min} mice that were homozygous for a *Runx1* floxed allele and were either positive or negative for the intestinal-specific *Villin-Cre* transgene. Direct comparison of littermate test (*Villin-Cre* positive, *Runx1* KO) and control mice (*Villin-Cre* negative, *Runx1* wild type) provided strong evidence that *Runx1* is a tumor suppressor in the mouse intestine. Intestinal tumor multiplicity in the *Runx1* KO mice was threefold higher in the colon ($P < 0.05$), greater than fourfold higher in the cecum ($P < 0.05$) and almost twofold higher in the small intestine ($P < 0.05$). More than one-third of the *Runx1* KO mice developed more than 200 tumors, with the highest tumor count at 321 tumors. In contrast, the majority of *Runx1* wild-type mice developed <100 tumors and <5% developed more than 200 tumors (Table 1). We also measured tumor sizes and found that tumors in the small intestine of *Runx1* KO mice were larger than *Runx1* wild-type mice, with the difference strongest in the ileum (Fig. S4). All of these phenotypic differences were observed in both male and female mice. All tumors examined using

Table 1. Deficiency for *Runx1* enhances tumorigenesis in Apc^{Min} mice

Runx1 genotype	Gender	*n*	Colon tumors	Colon tumor incidence (%)	Cecal tumors	Small intestine tumors
Wild type	F	30	1.3 ± 1.9	78	0.2 ± 0.6	90 ± 59
Knockout	F	31	4.0 ± 2.5*	100	1.0 ± 1.6*	155 ± 63*
Wild type	M	36	1.5 ± 1.7	78	0.3 ± 0.6	66 ± 37
Knockout	M	38	4.5 ± 3.6*	100	1.2 ± 1.2*	135 ± 60*

Apc^{Min} *Runx1* KO mice and littermate Apc^{Min} *Runx1* wild-type mice were killed at 100 days of age. Tumors were isolated and scored as described in the Materials and Methods section. Two-sided *P*-values for tumor counts were determined using the Wilcoxon rank-sum test comparing gender and age-matched classes produced in the same genetic crosses. **P*-value: <0.05.

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histopathology were adenomas, and thus resemble the same types of tumors normally observed in Apc^{Min} mice. Moreover, in contrast to Apc^{Min} mice that do not normally develop gastric tumors, approximately 10% of Apc^{Min} *Runx1* KO mice developed 1, 2, 3 or 4 small tumors in the pyloric canal (Fig. S5A). Histological analysis revealed the tumors to be small, polypoid adenomas in the terminal pylorus or at the junction of the pylorus with the duodenum (Fig. S5B).

***Runx1* deficiency results in tumorigenesis in $Apc^{+/+}$ mice.** We aged a group of approximately 20 $Apc^{+/+}$ *Runx1* KO and *Runx1* wild-type mice to approximately 12 months to evaluate the intestinal phenotype of *Runx1* deficiency alone. We found that approximately 33% of *Runx1* KO mice developed 1, 2 or 3 tumors in the duodenum, while no tumors were observed in *Runx1* wild-type control mice (Table 2). All tumors examined using histopathology were adenomas. Four tumors were examined using light microscopy; all were relatively small, approximately 3–4 mm diameter. All tumors were classified as sessile or flat adenomas, with one tumor showing a tendency towards pedunculation (fibrovascular expansion of the lamina propria) at one margin. Three tumors overlay, wholly or in part, the underlying Brunner's glands, and in places the epithelial component appeared to be contiguous with the Brunner's glands (Fig. S6A). No evidence of local invasion or metastasis was observed. Immunohistochemical analysis indicated that more than half of these duodenal tumors demonstrated upregulation of β-catenin protein (Fig. S6B).

Gene expression analysis of epithelial *Runx1* KO and *Runx1* WT colon. To identify epithelial *Runx1*-mediated alterations in gene expression in the mouse GI tract, RNA was isolated from the medial colon of *Runx1* KO mice and from gender and age-matched *Runx1* wild-type littermate control mice. Gene expression analysis was then carried out using Agilent 4x44K whole mouse genome microarrays, followed by quantitative RT-PCR of select target genes. Examination of the 300 most significant genes in the array study identified several functional clusters of genes, with the largest group consisting of genes involved in inflammation, immune responses and the hematopoietic system. This group included *Duoxa2, C4bp, Hemt1, Slc7a11, Cxcl14, Crisp3, Cd55, Clca3, Tnip3, Indo, Pou2af1, Runx2, Mst1, Immunoglobulins* (many), *Cd300lf, Rorc, Cd40, Ehf, Mmp9, Cd2, Cebpd, Retnlg, Il1rl1, Ccl8, Icos, Nfkbiz, Ccr10, Il1rl2, Gpr17, Cxcr4* and *Tnfrsf13b*. The second largest group of *Runx1* target genes was comprised of genes implicated in intestinal metabolism, including several lipases and genes associated with goblet cell function. This group included *Mptx, Pnliprp2, Retnlb, Pnliprp1, Clps, Es1, Clca3, Tgn, Slc1a1, Itlna, Spink4, Scin, Ero1l, Pax6, Mmp9, Hsp110 and Retnlg*. Figure S7 lists the most significant differentially expressed genes. Differential expression was confirmed by qRT-PCR analysis (Fig. 1). Notably, for all six genes out of the top seven genes (ranked by *P*-value) for which reliable primers could be generated, differential expression was confirmed, thereby validating the array data.

Gene set enrichment analysis. Changes in biological processes are accompanied by coordinate variation in expression of sets of genes. Gene Set Enrichment Analysis (GSEA) was applied to identify associations with gene sets differentially expressed between *Runx1* KO and *Runx1* WT colon (Table 3). Interestingly, a significant association was found with the gene set that distinguishes pediatric acute myeloid leukemia (AML) subtype inv^{16}[*CBFβ-MYH11*] (gene set #2)[56] thereby illustrating that genes expressed on mutation of CBFβ in human AML overlap significantly with the genes expressed on epithelial *Runx1* deficiency in mouse colon. Because Runx1 binds CBFβ to form a functional heterodimeric transcription factor,[9] these data support the validity of our approach. The significant association with the 'tryptophan metabolism' gene set (gene set #9) is in accordance with alteration of expression of genes involved in inflammation and immune responses that might lead to IBD and increased CRC risk.[57] The association with the 'peroxisome proliferator-activated receptor (PPAR) signaling pathway' (gene set #11) is in accordance with the alteration of expression of genes involved in intestinal metabolism,[58] and also predisposes to increased CRC risk[50] Moreover, two gene sets indicate that

Table 2. Deficiency for *Runx1* causes duodenal tumors in $Apc^{+/+}$ mice

Genotype	*n*	Mean age	Incidence (%)	Tumor multiplicity
Runx1 wild type	10	12 months	0	0
Runx1 knockout	9	12 months	33	0.7

Runx1 knockout mice and littermate wild-type mice were aged to approximately 12 months, killed and the tissues were formalin fixed. The entire intestinal tract was examined for tumors as described in the Materials and Methods section.

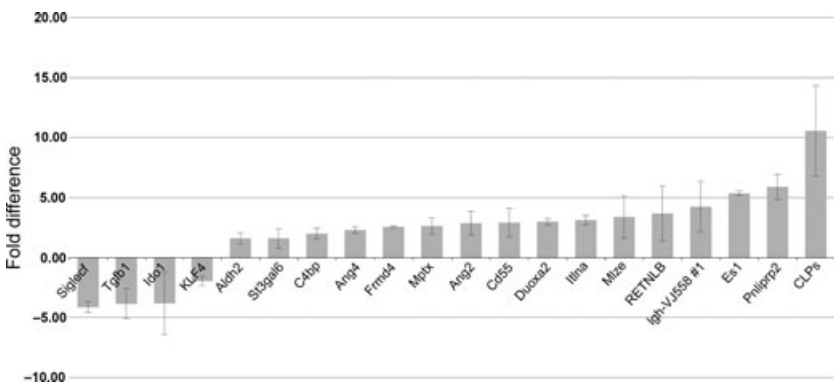

Fig. 1. Change in gene expression in *Runx1* knockout (KO) whole colon. List of 20 *Runx1* target genes examined using qRT-PCR. Details of the qRT-PCR assay are described in the Materials and Methods section. Each bar represents the mean and standard error of multiple experiments that measured fold differences in the mRNA expression of specific target genes in whole colon tissue from gender and aged matched pairs of $Apc^{+/+}$ *Runx1* KO and *Runx1* wild-type mice. All tissues were from adult mice generally approximately 100 days of age, and the mRNA were isolated from 1-cm sections of colon from the same region of the distal colon. All mRNA values were normalized to 18S values. Four replicates of each assay were performed for each matched pair of mRNA (KO versus wild type) and these sets of assays were repeated at least twice for each pair of mRNA). At least two matched pairs of mRNA were tested for each gene, with most genes tested in at least three matched pairs of mRNA. To be included in this figure, genes met the flowing criteria: (i) the mean fold difference was at least 1.5 (and almost all of the 20 genes showed at least a twofold difference); and (ii) each gene showed a change in gene expression in the same direction in each matched pair of mRNA (i.e. all upregulated or all downregulated in each matched pair of mRNA).

Table 3. Gene Set Enrichment Analysis (GSEA) gene sets 'UP or DOWN' in *Runx1* knockout compared with *Runx1* wild type (FWER < 0.05)

#	Collection†	Name of Gene Set†	Size (number of genes)	ES	NES	FDR *q*-value	FWER *P*-value
Gene sets 'UP' in *Runx1* knockout compared with *Runx1* wild type (FWER < 0.05)							
1	Curated (c2)	BOQUEST_CD31PLUS_VS_CD31MINUS_DN	155	0.55	2.40	0	0
2	Curated (c2)	ROSS_CBF_MYH	32	0.68	2.18	0.009	0.012
3	Curated (c2)	BRCA_PROGNOSIS_NEG	68	0.58	2.17	0.009	0.017
4	Curated (c2)	EMT_UP	43	0.62	2.16	0.007	0.018
5	Curated (c2)	AGEING_KIDNEY_UP	203	0.48	2.13	0.009	0.028
6	Curated (c2)	AGEING_KIDNEY_SPECIFIC_UP	103	0.53	2.13	0.007	0.029
7	Curated (c2)	BRUNO_IL3_DN	44	0.61	2.11	0.009	0.039
8	Curated (c2)	PRMT5_KD_UP	126	0.51	2.10	0.008	0.041
Gene sets 'DOWN' in *Runx1* knockout compared with *Runx1* wild type (FWER < 0.05)							
9	Curated (c2)	TRYPTOPHAN_METABOLISM	24	−0.72	−2.16	0.004	0.010
10	Curated (c2)	HCC_SURVIVAL_GOOD_VS_POOR_UP	89	−0.52	−2.08	0.006	0.029
11	Curated (c2)	HSA03320_PPAR_SIGNALING_PATHWAY	51	−0.57	−2.05	0.006	0.044

†Obtained from the Molecular Signatures Database (MSigDB_v2.5, http://www.broad.mit.edu/gsea/msigdb/index.jsp). ES, GSEA enrichment score; FDR, false discovery rate; FWER, family wise error rate; NES, GSEA normalized enrichment score.

Runx1 deficiency is associated with expansion of a pluripotent (stem) cell population at the expense of more differentiated cells (gene set #1[59] and #7[60]) while other gene sets indicate that *Runx1* deficiency is associated with more metastases (gene set #4[61]) and poor prognosis in breast cancer (gene set #3[62]) and hepatocellular cancer (gene set #10). Taken together, these data imply that tumor-free colons from *Runx1*-deficient mice express gene profiles related to expanded stem/pluripotent progenitor cell populations compared with colon from *Runx1* wild-type mice.

Gene-specific expression analysis of *Runx1* KO and *Runx1* WT colon epithelium. Considering the reported interactions between *Runx1* and *Notch* signaling activity combined with the whole colon GSEA results indicating putative effects of *Runx1* on regulation of the size of the stem/progenitor cell compartment, we decided to examine expression of individual genes associated with intestinal stem/progenitor cells and intestinal differentiation. Colon epithelium was isolated from *Runx1* KO and *Runx1* WT colon and gene expression was analyzed using qRT-PCR for genes analyzed in the whole colon plus the putative stem cell marker *Aldh1a1*, and the Notch pathway genes *Notch1*, *Jag1* and *Hes1* (Fig. 2). Our data from the analysis of *Runx1*-deficient colon epithelium confirmed the expression patterns for many of the whole colon genes, but also revealed increased expression of *Aldh1a1*, *Notch1*, *Jag1* and *Hes1*, thereby supporting a role for *Runx1* in direct regulation of the expression of Notch signaling pathway genes.

Discussion

Here we showed that *Runx1* is a novel tumor suppressor gene in the GI tract. Conditional ablation of *Runx1* expression in Apc^{Min} mice caused a significant increase in the number of tumors, in all regions of the intestine, and in both male and female mice. At a low frequency we also observed small tumors in the pyloric canal leading from the stomach to the duodenum, a type of tumor that is not normally seen in Apc^{Min} mice at 100 days.[63] It is very possible that these gastric tumors arose from the combination of *Apc* deficiency and excision of *Runx1* in a rare population of progenitor cells that express villin in the antral glands of the pylorus.[64,65] Moreover, *Runx1* deficiency also caused the development of duodenal tumors in *Apc* wild-type mice that were aged to approximately 12 months. To examine the effects of *Runx1* epithelial deficiency on gut homeostasis preceding colon tumor development, genome-wide mRNA expression profiles of colon from *Runx1* KO mice were compared with *Runx1* WT mice. The largest clusters of differentially expressed genes were implicated in: (i) inflammation, immune responses and hematopoeitic processes; and (ii) intestinal metabolism, such as lipases, goblet cell proteins and transporters. Virtually all of the most significantly regulated genes identified (ranked by *P*-values) were confirmed using qRT-PCR, lending confidence to the array data (Fig. 1). Analysis of the differential expression of gene sets using GSEA revealed a significant association with genes expressed on mutation of CBFβ, a key partner of RUNX1 in transcriptional activation, further supporting the validity of our approach. Other gene sets confirmed the effects of *Runx1* deficiency on inflammation and immune responses and on intestinal metabolism (Table 3).

Two of the top genes implicated in inflammation and immune cell responses are involved in complement activity: *C4bp* and *Cd55*,[66] which inhibit complement by differing mechanisms.[67,68] As both *C4bp* and *Cd55* are upregulated in *Runx1* KO colon, these changes suggest that Runx1 promotes complement recruitment and activity. *C4bp* has been reported to be increased in the plasma of colon cancer patients,[55] while *Cd55* expression has been reported to be increased in the intestine of Apc^{Min} mice[69] and in human colorectal cancer[70] where its expression is associated with tumor recurrence, metastasis and a poor prognosis.[71] Thus, *Runx1* inhibition of *C4bp* and *Cd55* expression in the colon is consistent with its tumor-suppressive role in the GI tract.

A second large cluster of *Runx1* target genes in mouse colon are involved in intestinal cell metabolism, in particular genes expressed in goblet and Paneth cells. An example is *Retnlb*, resistin-like beta, which is expressed in intestinal goblet cells where it is involved in lipid metabolism, host defense and immune responses.[72] *Retnlb* expression is increased in inflammatory bowel disease, gastric cancer and colorectal cancer, where it is associated with upregulation of interferon (IFN) gamma activity.[73,74] *Retnlb* was also reported to be highly upregulated in tumors isolated from Apc^{Min} mice.[75] Our gene expression studies indicated that *Retnlb* expression is inhibited by *Runx1* in mouse colon (Figs 1,2). Two other genes involved in intestinal metabolism that are upregulated in *Runx1* KO colon are *Ang4* (angiogenin 4) and *Spink4* (serine peptidase inhibitor, Kazal type 4). Both of these genes have been described as transcriptional targets of β-catenin signaling in Paneth cells whose expression is upregulated in mouse $Apc^{-/-}$ cells,[76,77] thereby connecting *Runx1* to Wnt/β-catenin signaling.

In addition to the putative link between *Runx1* and Wnt/β-catenin signaling, *Runx1* has also been connected to *Notch* signaling. Recently, *Runx1* was reported to be implicated in regulation and differentiation of the intestinal secretory cell lineage,

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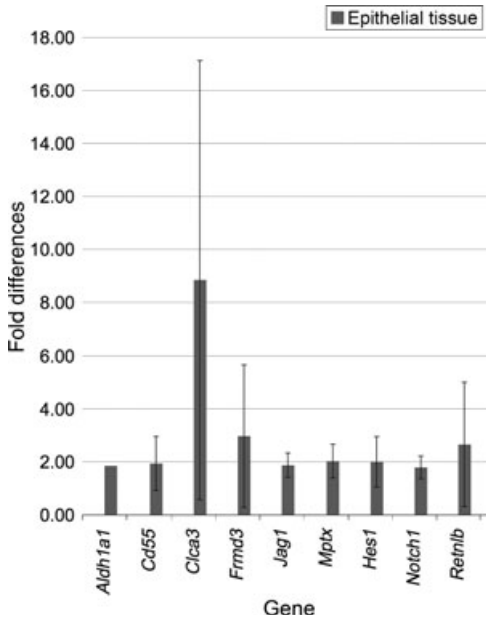

Fig. 2. Changes in gene expression in *Runx1* knockout (KO) colon epithelial cells. List of nine *Runx1* target genes examined using qRT-PCR. Details of the qRT-PCR assay are described in the Materials and Methods section. Each bar represents the mean and standard error of multiple experiments that measured fold differences in the mRNA expression of specific target genes in colon epithelial tissue from gender and age-matched pairs of $Apc^{+/+}$ *Runx1* KO and *Runx1* wild-type mice. All tissues were from adult mice generally approximately 100 days of age, and the mRNA were isolated from 1-cm sections of colon from the same region of the distal colon. All mRNA values were normalized to 18S values. Four replicates of each assay were performed for each matched pair of mRNA (KO versus wild type) and these sets of assays were repeated at least twice for each pair of mRNA). At least two matched pairs of mRNA were tested for each gene with most genes tested in at least three matched pairs of mRNA. To be included in this figure, genes met the flowing criteria: (i) the mean fold difference was at least 1.5 (and almost all of the nine genes showed at least a twofold difference); and (ii) each gene showed a change in gene expression in the same direction in each matched pair of mRNA (i.e. all upregulated or all downregulated in each matched pair of mRNA).

identifying the *Kruppel like factor 4* (*Klf4*) gene as a target of *Runx1*.[40] Klf4 is a zinc-finger transcription factor that is strongly expressed in terminally differentiated intestinal epithelial cells. *Klf4* has also been associated with differentiation of the secretory cell lineage and with inhibition of intestinal cell proliferation via blocking progression of the cell cycle.[78,79] Haploinsufficiency for *Klf4* enhanced tumorigenesis in the Apc^{Min} mouse[80] and *KLF4* has been reported to be downregulated in human colorectal cancer.[81] In the GI tract, expression of *KLF4* is inhibited by Notch signaling and inhibition of Notch signaling results in a shift of intestinal crypt cells in favor of a goblet cell fate and away from an absorptive cell fate.[48,80] Moreover, it is known that *Hes1*, a key Notch pathway gene, represses expression of *Math1*, a gene required for goblet cell differentiation.[82] In the present study we confirmed using qRT-PCR that *Runx1* positively regulated expression of *Klf4* in mouse colon (Fig. 1) and found that *Runx1* appeared to regulate expression of Notch pathway genes (*Notch1*, *Jag1* and *Hes1*) in colon epithelial cells (Fig. 2). Taken together, these data suggest that positive regulation of *Klf4* by *Runx1* might result from inhibition of Notch signaling and thus de-repression of *Klf4* by Notch. Of interest, *Runx3* has been reported to directly interact with *Notch1* and suppress *Notch* signaling in hepatocellullar cancer cells,[83,84] hence it is possible that *Runx3* and *Runx1* exert similar suppressive effects on *Notch* signaling in the intestinal tract.

Both *Wnt* and *Notch* signaling pathways are involved in regulation of the stem-/pluripotent progenitor cell compartment.[45] Moreover, Runx1 itself has been described as a regulator of stem cell proliferation, with isoform-specific positive and negative effects on the cell cycle.[85] Therefore, one mechanism through which *Runx1* might exert its suppressive effects on tumor development is by limiting expansion of the stem-/pluripotent progenitor cell compartment. *Aldh1a1* (*Aldh1*) was recently implicated as a putative biomarker for colon cancer stem cells.[86] In the present study, *Runx1* negatively regulated expression of *Aldh1a1* in colonic epithelial cells (Fig. 2). This finding is consistent with a putative role for *Runx1* in regulation of the stem/progenitor population, further supported by *Runx1* negative regulation of *Notch1*, *Jag1* and *Hes1* in colonic epithelial cells (Fig. 2).

Moreover, GSEA revealed significant associations of *Runx1* deficiency with gene sets representing an increased metastatic phenotype (Table 3, gene set #4) and poor prognosis in breast and hepatocellular cancer (Table 3, gene sets #3 and #10), phenomena that might be related to (cancer) stem cell characteristics. Considered together, these data imply that colon from *Runx1* KO mice expresses genes involved in expanded (cancer) stem/pluripotent progenitor cell populations compared with *Runx1* wild-type mice.

In summary, we have provided the first evidence that *Runx1* is a tumor-suppressor gene in the mammalian GI tract. Deficiency for *Runx1* in intestinal epithelial cells significantly enhanced tumorigenesis in Apc^{Min} mice, while *Runx1* deficiency alone was sufficient to cause tumorigenesis in *Apc*-wild-type mice. We have identified a set of *Runx1* target genes in mouse colon indicative of changes in gut homeostasis, that is, genes involved in inflammation and intestinal metabolism, which are known to increase the risk of tumor development. Moreover, the results of the present study support a role for *Runx1* in suppressing *Notch* pathway signaling, a finding that is consistent with *Runx1* regulating the stem cell/progenitor cell compartment in intestinal crypts and consequent Notch-mediated effects on intestinal cell differentiation and proliferation. Further studies are required to explore the genetic and epigenetic status and role of *Runx1* in human colorectal cancers.

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Disclosure Statement

All authors declare that they do not have any competing financial interest in relation to the work described.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Analysis of intestinal biomarkers in *Runx1*-deficient intestinal tissues.

Fig. S2. Immunohistochemistry for Runx1.

Fig. S3. qRT-PCR *Runx1*/18S.

Fig. S4. Tumor size analysis.

Fig. S5. Deficiency for *Runx1* causes gastric tumors in $Apc^{Min/+}$ mice.

Fig. S6. Deficiency for *Runx1* causes intestinal tumorigenesis in $Apc^{+/+}$ mice.

Fig. S7. Most significant *Runx1* target genes identified in the microarray analysis.

Data S1. Including: mice; tumor analysis; RNA extraction and quality control; isolation of intestinal epithelium; qRT-PCR; qRT-PCR primers; histopathology; and immunohistochemistry.

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