

Oncology

Gene expression profile of Dclk1⁺ cells in intestinal tumors

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ABSTRACT

Background: Accumulating evidence has shown the existence of tumor stem cells with therapeutic potential. Previously, we reported that doublecortin like kinase 1 (Dclk1) marks tumor stem cells but not normal stem cells in the intestine of *Apc*^{Min/+} mice, and that Dclk1- and Lgr5-double positive tumor cells are the tumor stem cells of intestinal tumors.

Aim: To investigate molecules highly expressed in the Dclk1⁺ normal intestinal and Dclk1⁺ tumor cells in *Apc*^{Min/+} mice.

Methods: We used microarray analyses to examine the gene expression profile of Dclk1⁺ cells in both mouse normal intestinal epithelium and *Apc*^{Min/+} mouse intestinal tumors. We also performed immunofluorescence analyses.

Results: Genes related to microtubules and the actin cytoskeleton (e.g., Rac2), and members of the Src family kinases (i.e., Hck, Lyn, Csk, and Ptpn6) were highly expressed in both Dclk1⁺ normal intestinal and Dclk1⁺ tumor cells. Phosphorylated Hck and phosphorylated Lyn were expressed in Lgr5⁺ cells in the intestinal tumors of *Lgr5*^{EGFP-IRES-CreERT2/+}; *Apc*^{Min/+} mice.

Conclusion: We revealed factors that are highly expressed in Dclk1⁺ intestinal tumor cells, which may help to develop cancer stem cell-targeted therapy in future.

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1. Introduction

Colorectal cancer is the world's fourth leading cause of cancer-related deaths [1]. Current therapeutic strategies against advanced colorectal cancers, such as chemotherapy and radiation, may destroy the majority of cancer cells; however, highly resistant cancer cells survive these treatments, and hence, the survival rate of patients remains to be improved. In this context, cancer stem cell theory has emerged to enable further understanding of the mechanisms of resistance to anticancer therapy and to investigate novel therapeutic approaches. According to this theory, cancer stem cells survive chemotherapy or irradiation and cause recurrence of cancers. Therefore, strategies targeting cancer stem cells have been explored. For this purpose, a number of cancer stem cell markers have been identified [2–8]. For example, CD133 marks both normal stem cells and tumor stem cells in the intestine [2,3,9]. CD44 also marks tumor stem cells in the intestine [4], and CD44 variant is reported to protect tumor stem cells from reactive oxygen species [10].

Previously, we reported that doublecortin like kinase 1 (Dclk1) marks tumor stem cells but not normal stem cells in *Apc*^{Min/+} mouse intestines through lineage tracing experiments [11]. Dclk1 is expressed in tuft cells, which are characterized by an apical tuft of stiff microvilli that protrudes into the gut lumen [12]. Interestingly, in our previous study, we found that selective ablation of Dclk1⁺ cells in *Apc*^{Min/+} mice results in the collapse of the intestinal tumors without any apparent damage to the background normal mucosa; therefore, Dclk1⁺ tumor stem cells could serve as attractive targets for anticancer therapy [11].

To develop novel anticancer therapies targeting Dclk1⁺ tumor stem cells, it is important to find molecules suitable for drug development, such as intracellular kinases and cell surface receptors in Dclk1⁺ tumor cells. In this study, we used microarray analyses in both mouse normal intestinal epithelium and *Apc*^{Min/+} mouse intestinal tumors, and sought to examine the gene expression profile of Dclk1⁺ cells.

2. Materials and methods

2.1. Mice

Dclk1^{CreERT2-IRES-EGFP/+} mice were described previously [11]. *Lgr5*^{EGFP-IRES-CreERT2/+} and *Apc*^{Min/+} mice were obtained from the

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Table 1
The pathways up-regulated in Dcl⁺ cells compared to Dcl⁻ cells in the normal intestinal epithelium.

	MAPP name	Dcl ⁺ normal/Dcl ⁻ normal		
		Gene number	Z score	Permute P
1	Mm.Mismatch.repair.WP1257.35190	4	6.477	0
2	Mm.Glycerolipid.metabolism	12	4.086	0.001
3	Mm.Prostaglandin.and.leukotriene.metabolism	5	3.231	0.01
4	Mm.Phosphatidylinositol.signaling.system	9	3.219	0.005
5	Mm.Eicosanoid.Synthesis.WP318.35469	4	3.052	0.015
6	Mm.Calcium.Regulation.in.the.Cardiac.Cell.WP553.35443	15	2.613	0.011
7	Mm.IL-3.Signaling.Pathway.WP373.35789	11	2.612	0.014
8	Mm.Phospholipid.degradation	3	2.561	0.036
9	Mm.Cholesterol.Biosynthesis.WP103.35136	3	2.561	0.037
10	Mm.Kit.Receptor.Signaling.Pathway.WP407.35754	8	2.545	0.016
11	Mm.EPO.Receptor.Signaling.WP1249.35097	4	2.344	0.053
12	Mm.IL-4.signaling.Pathway.WP93.34389	7	2.289	0.033
13	Mm.B.Cell.Receptor.Signaling.Pathway.WP274.35701	14	2.129	0.043
14	Mm.Synthesis.and.degradation.of.ketone.bodies	1	2.077	0.165
15	Mm.Ganglioside.biosynthesis	3	2.029	0.069
16	Mm.T.Cell.Receptor.Signaling.Pathway.WP480.34406	12	2.004	0.062

Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed under specific pathogen-free conditions at the animal facilities of Kyoto University. All experiments were performed in accordance with institutional guidelines. The review board of Kyoto University granted ethical permission for this study, and the Kyoto University Animal Experimentation Committee approved the experimental protocol.

2.2. Dissociation of normal intestinal epithelium and intestinal tumors and flow cytometry analysis

From the normal intestinal epithelium, Dcl⁻ cells were harvested freshly from 1 Dcl^{CreERT2-IRES-EGFP/+} mouse at 26 weeks of age; Dcl⁺ cells, from 2 Dcl^{CreERT2-IRES-EGFP/+} mice at the same age. Dcl⁻ intestinal tumor cells were freshly isolated from 1 Dcl^{CreERT2-IRES-EGFP/+}; Apc^{Min/+} mouse at more than 16 weeks of age; Dcl⁺ intestinal tumor cells, from 7 Dcl^{CreERT2-IRES-EGFP/+}; Apc^{Min/+} mice at the same age. Dissociation of these samples was performed according to the method described in a previous report [11]. The tissues were minced and incubated with digestion buffer (Dulbecco's Modified Eagle Medium [DMEM] with 10% fetal calf serum, and 50 U/mL penicillin– 50 µg/mL streptomycin [Invitrogen, Carlsbad, CA, USA]), 1 mg/mL collagenase type XI (Sigma–Aldrich, St. Louis, MO, USA), and 0.1 mg/mL DNase I for 15 min at 37 °C. Incubated samples were filtered through a 40-µm mesh, shaken vigorously, and incubated with phosphate buffered saline containing 2 mM EDTA for 20 min at 4 °C. To obtain Dcl⁺ cells from the samples, green fluorescent protein (GFP)-expressing cells were sorted using a FACSARIA II flow cytometer (BD Biosciences, San Jose, CA, USA).

2.3. Microarray analysis

RNA was extracted from the sorted cells using the RNeasy Micro kit or RNeasy Mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany).

Microarray analysis was performed using a 3D-gene chip (Toray Industries Inc., Tokyo, Japan) once each for normal epithelium and the tumor as previously described [13]. The microarray data were registered with NCBI's Gene Expression Omnibus (GEO) database under the accession number GSE 99387.

Microarray data were applied to GenMAPP (<http://www.genmapp.org/>), using the software GenMAPP, ver.2.1 (MAPP Finder) [14,15]. The threshold of the genes of Dcl⁺ cells was more than 4 times that of the genes of Dcl⁻ cells in Tables 1 and 2. In Supplementary Table, the threshold of the genes of Dcl⁺

intestinal tumor cells was more than 4 times that of the genes of Dcl⁺ normal intestinal epithelial cells. Pathways that included the genes satisfying this criterion were selected for this study. The pathways for which the z score was more than 2 and P value was less than 0.05 were indicated. P value was generated by a t-test. "Gene number" indicates the number of genes satisfying the criterion.

2.4. Histological analysis

Immunohistochemistry was performed according to a previously published protocol [11]. Washed sections were incubated with fluorescent secondary antibody (Invitrogen, Carlsbad, CA, USA) for 1 h. The following antibodies were used: primary anti-acetylated tubulin (Sigma–Aldrich), rabbit anti-Avil (Atlas Antibody, Voltavägen, Sweden), rat anti-Camk2b (Invitrogen), rabbit anti-Ccnd1 (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-c-Jun (Abcam, Cambridge, UK), goat anti-Csk (Abcam), rabbit anti-Dcl⁺ (Abcam), mouse anti-Dcl⁺ (Abcam), mouse anti-Dcl⁺ (Abnova, Taipei, Taiwan), goat anti-Dcl⁺ (Santa Cruz, Santa-Cruz, CA, USA), goat anti-EGFP (Abcam), goat anti-Hck (Santa Cruz), rabbit anti-p-Hck (phospho Y410) (Abcam), rabbit anti-Lox (Abcam), rabbit anti-p-Lyn (phospho Y507) (Abcam), rabbit anti-Myo1b (Atlas Antibody), rabbit anti-Ptpn6 (Novus Biologicals, Littleton, CO, USA), goat anti-Rac2 (Abcam), rabbit anti-S100a1 (Abcam), and rabbit anti-Vav1 (Abcam).

3. Results

3.1. Comparison between Dcl⁺ and Dcl⁻ cells in normal intestinal epithelium

To determine the gene expression profile of Dcl⁺ cells in the normal small intestine, we performed a microarray analysis. Intestinal epithelial cells were harvested from Dcl^{CreERT2-IRES-EGFP/+} mice according to a previous report [11]. After the separation of Dcl⁺ and Dcl⁻ cells by flow cytometry, the mRNA extracted from each cellular cluster was used for microarray analysis (Supplementary Fig. 1 and Fig. 1A). The heatmap of microarray data showed the difference between the gene expression profiles comparing Dcl⁺ and Dcl⁻ cells in normal intestinal epithelium (Fig. 1B). Dcl⁺ mRNA expression was 4 times higher in Dcl⁺ cells than in Dcl⁻ cells.

First, we performed a pathway analysis (Table 1). Because tuft cells express Cox-1 and Cox-2 [16], we took note that the "eicosanoid synthesis pathway" was nominated (Table 1). Alox5ap, a gene involved in this pathway, was included in the top 200 genes

Table 2The pathways up-regulated in *Dclk1*⁺ cells compared to *Dclk1*[−] cells in the intestinal tumors of *Apc*^{Min/+} mice.

	MAPP name	Dclk1 ⁺ tumor/Dclk1 [−] tumor		
		Gene number	Z score	Permute P
1	Mm.Kit_Receptor_Signaling_Pathway_WP407_35754	9	5.84	0
2	Mm.IL-5_Signaling_Pathway_WP151_34416	8	4.807	0
3	Mm.Phospholipid.degradation	3	4.322	0.007
4	Mm.Prostaglandin_and_Leukotriene_metabolism	4	4.224	0.003
5	Mm.EPO_Receptor_Signaling_WP1249_35097	4	4.224	0.008
6	Mm.EGFR1_Signaling_Pathway_WP572_35717	13	4.205	0
7	Mm.IL-3_Signaling_Pathway_WP373_35789	9	4.189	0
8	Mm.Non-homologous_end_joining_WP1242_35403	2	3.887	0.022
9	Mm.T_Cell_Receptor_Signaling_Pathway_WP480_34406	10	3.763	0.002
10	Mm.B_Cell_Receptor_Signaling_Pathway_WP274_35701	11	3.687	0.001
11	Mm.Eicosanoid_Synthesis_WP318_35469	3	3.656	0.011
12	Mm.Ganglioside_biosynthesis	3	3.656	0.014
13	Mm.Blood_group_glycolipid_biosynthesis_neolactoseries	2	3.372	0.023
14	Mm.Globoside_metabolism	3	3.276	0.013
15	Mm.Homologous_recombination_WP1258_35588	2	3.165	0.044
16	Mm.Oxidative_Damage	4	3.119	0.018
17	Mm.Myometrial_Relaxation_and_Contraction_Pathways_WP385_35258	10	3.117	0.004
18	Mm.Keap1-Nrf2_WP1245_32699	2	2.82	0.046
19	Mm.Phosphatidylinositol_signaling_system	5	2.749	0.017
20	Mm.Calcium_Regulation_in_the_Cardiac_Cell_WP553_35443	9	2.737	0.022
21	Mm.O.Glycans_biosynthesis	2	2.673	0.054
22	Mm.Type_II_interferon_signaling_(IFNG)_WP1253_34425	3	2.469	0.053
23	Mm.Complement_Activation_Classical_Pathway_WP200_32694	2	2.417	0.065
24	Mm.Aflatoxin_B1_metabolism_WP1262_35017	1	2.383	0.096
25	Mm.IL-6_signaling_Pathway_WP387_34384	6	2.34	0.033
26	Mm.Glycerolipid_metabolism	5	2.234	0.047
27	Mm.Fatty_Acid_Omega_Oxidation_WP33_33384	1	2.107	0.152

with more than 8 times higher expression in *Dclk1*⁺ cells than in *Dclk1*[−] cells (Fig. 2A). Consistently, immunofluorescence showed that Lox was expressed in *Dclk1*⁺ cells (Fig. 2B).

A recent study reported that tuft cells are related with the type 2 immune response [17–19]. Consistently, the microarray analysis in the present study also showed that *Dclk1*⁺ cells express *IL-25* mRNA 103 times higher than *Dclk1*[−] cells do (Fig. 2A).

To further investigate other characteristics of *Dclk1*⁺ cells in the normal small intestine, we focused on studying the features of tuft cells, which express *Dclk1* [20]. Because tuft cells are unique pear-shaped cells that have an apical tuft of stiff microvilli [12], we speculated that cytoskeleton-related factors might further characterize *Dclk1*⁺ cells. *Rac2*, *S100a1*, *Avil*, and *Myo1b*, which are cytoskeleton-related genes, were included in the top 200 genes that are highly expressed in *Dclk1*⁺ cells (Fig. 2A). Double immunofluorescent staining confirmed the protein expression of these factors in *Dclk1*⁺ cells (Fig. 2B). In addition, immunofluorescence revealed that acetylated tubulin and *Vav1*, which are associated with the cytoskeleton but not listed in the top 200 genes, were also expressed in *Dclk1*⁺ cells (Fig. 2). *Dclk1* is also known to have a calcium/calmodulin-dependent protein kinase domain [21]. *Camk2b*, which is listed in the top 200 genes, is one of the calcium/calmodulin-dependent protein kinases (Fig. 2A), and immunofluorescent staining demonstrated that *Camk2b* was expressed in *Dclk1*⁺ cells (Fig. 2B). Besides these factors, we examined several other genes that had markedly high expression in *Dclk1*⁺ cells in the normal intestine (Fig. 2A). *Hck*, which belongs to the Src family kinases [22,23], was highly expressed in *Dclk1*⁺ cells (Fig. 2A). *Csk*, another Src family member [24], and *Ptpn6*, which is also related to Src family kinases [25], were included among the top 200 genes. Immunofluorescence demonstrated that *Hck*; phosphorylated *Hck* (phospho Y410), which determines the activation of *Hck* [26,27]; *Csk*; and *Ptpn6* were expressed in *Dclk1*⁺ cells in the normal intestine (Fig. 2B). Furthermore, immunofluorescent staining showed that the phosphorylated form of *Lyn* (phospho Y507), which determines the inactivation of *Lyn* [28,29], another Src fam-

ily kinase, was also expressed in *Dclk1*⁺ cells (Fig. 2B), although it is not listed in the top 200 genes.

3.2. Comparison between *Dclk1*⁺ and *Dclk1*[−] tumor cells in intestinal tumors

To investigate the characteristics of *Dclk1*⁺ intestinal tumor cells, a microarray analysis was performed in the intestinal tumors of *Dclk1*^{CreERT2-IRES-EGFP/+}; *Apc*^{Min/+} mice for genes with expressions in *Dclk1*⁺ tumor cells that were more than 8 times higher than those in *Dclk1*[−] tumor cells (Supplementary Fig. 2 and Fig. 3A). *Dclk1* mRNA expression was 7 times higher in *Dclk1*⁺ cells than in *Dclk1*[−] tumor cells. Microarray analysis revealed 141 highly upregulated genes, including *Hck*, *Alox5ap*, *S100a1*, *Rac2*, *Camk2b*, *Ptpn6*, and *Vav1* as in the case of normal intestinal epithelium (Fig. 3A). Immunofluorescent staining showed that all of these factors, along with *Avil*, *Myo1b*, acetylated tubulin, *Csk*, and phosphorylated *Lyn*, were expressed in *Dclk1*⁺ cells in the intestinal tumors of *Apc*^{Min/+} mice (Fig. 3B). Pathway analysis also nominated the “eicosanoid synthesis pathway” in *Dclk1*⁺ tumor cells (Table 2). These results revealed that *Dclk1*⁺ tumor cells retained the gene expression that was characteristic of tuft cells, including *Cox-1*, as previously described [11], as well as *Hck*, *Alox5ap*, *S100a1*, *Rac2*, *Camk2b*, *Ptpn6*, *Vav1*, *Avil*, *Myo1b*, acetylated tubulin, *Csk*, and phosphorylated *Lyn*.

3.3. Comparison of *Dclk1*⁺ tumor and *Dclk1*⁺ normal epithelial cells

Based on the similarity of gene expression profiles between *Dclk1*⁺ normal and *Dclk1*⁺ tumor cells, we directly compared their gene expression profiles. *Ccnd2* and *Ccnd1* were among the top 200 genes with expressions that were more than 8 times higher in *Dclk1*⁺ tumor cells in *Dclk1*^{CreERT2-IRES-EGFP/+}; *Apc*^{Min/+} mice than in *Dclk1*⁺ normal cells (Fig. 4A). *Ccnd2* and *Ccnd1*, which encode cyclin D2 and cyclin D1, respectively, are positively involved in cell cycle progression [30,31] and are included in the “cell cycle pathway” and “G1 to S cell cycle control pathway” (Supplementary Table). Fur-

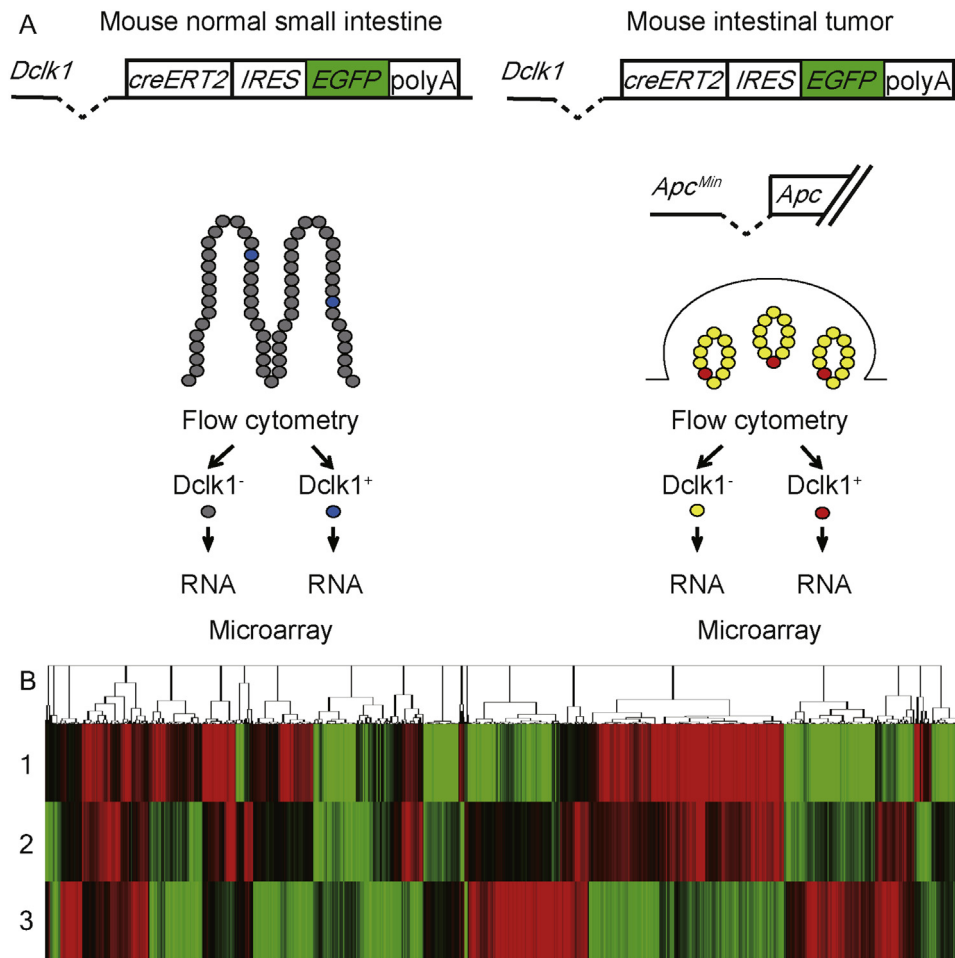


Fig. 1. Microarray analysis of *Dclk1*⁺ and *Dclk1*⁻ cells in the normal intestinal epithelium and intestinal tumors of *Apc^{Min/+}* mice.

(A) Schema showing the experimental design. *Dclk1*⁻ and *Dclk1*⁺ cells in the normal intestinal epithelium of *Dclk1^{CreERT2-IRES-EGFP/+}; Apc^{Min/+}* mice were sorted by flow cytometry using EGFP, which was integrated at the first ATG codon of *Dclk1*. mRNA was extracted from *Dclk1*⁻ and *Dclk1*⁺ cells separately and used for microarray analysis. (B) Heatmap cluster images of microarray data comparing: (1) *Dclk1*⁺ cells with *Dclk1*⁻ cells in the normal small intestine, (2) *Dclk1*⁺ tumor cells with *Dclk1*⁻ tumor cells in the intestinal tumors of *Apc^{Min/+}* mice, (3) and *Dclk1*⁺ tumor cells in the intestinal tumors of *Apc^{Min/+}* mice with *Dclk1*⁺ cells in the normal small intestine. Red and green squares indicate up-regulation and down-regulation of gene expression, respectively.

thermore, in terms of cell cycle regulation, the expression of *c-Jun* mRNA [32] in *Dclk1*⁺ tumor cells was more than 6 times higher than that in *Dclk1*⁺ normal cells. Immunofluorescent staining demonstrated that *Dclk1*⁺ tumor cells in *Apc^{Min/+}* mice expressed cyclin D1 and c-Jun, but *Dclk1*⁺ normal cells did not (Fig. 4B). Thus, the expression of these factors might endow *Dclk1*⁺ tumor cells with proliferative capacity. However, cyclin D1 and c-Jun are ubiquitously expressed not only in *Dclk1*⁺ cells but also in *Dclk1*⁻ intestinal tumors [33,34]. We previously reported that *Dclk1*⁻ and *Lgr5*-double positive (*Dclk1*⁺/*Lgr5*⁺) tumor cells are the tumor stem cells in the intestinal tumors of *Apc^{Min/+}* mice [11]. Therefore, in the intestinal tumors of *Lgr5^{EGFP-IRES-CreERT2/+}; Apc^{Min/+}* mice, where *Lgr5* expression is analyzed by EGFP immunoreactivity (Fig. 4C), we examined whether factors highly expressed in *Dclk1*⁺ tumor cells were also expressed in *Lgr5*⁺ tumor cells. Among the factors listed above, the phosphorylated forms of Hck and Lyn merged with EGFP in the intestinal tumors of *Lgr5^{EGFP-IRES-CreERT2/+}; Apc^{Min/+}* mice (Fig. 4D). Thus, these Src family kinases highly expressed in *Dclk1*⁺ cells might be associated with intestinal tumor stem cells.

4. Discussion

In the present study, we investigated molecules highly expressed in *Dclk1*⁺ normal intestinal epithelial cells (i.e., tuft cells)

and tumor cells in *Apc^{Min/+}* mice, using microarray and immunofluorescence analyses. *Dclk1*⁺ tumor stem cells retained the gene expression characteristics of tuft cells, including Cox-1, as previously described [11], as well as cytoskeleton-related factors and Src-family kinases. Meanwhile, we suggested potential molecules that may endow proliferative capacity to *Dclk1*⁺ tumor cells, as well as novel kinases, which are expressed in *Dclk1*⁺/*Lgr5*⁺ tumor stem cells.

Previous studies have shown that both tuft cells and enteroendocrine cells express *Dclk1* [12,35]. However, anti-*Dclk1* antibody stained only a subset of *Insm1*⁺ enteroendocrine cells, whereas *Dclk1* is one of the most specific tuft cell markers [35]. Another previous report showed by immunohistochemistry that *Dclk1*⁺ cells and *Insm1*⁺ cells are distinct populations [18]. In the present study, using microarray, neither *Insm1* nor *ChgA* mRNA was found to be expressed in *Dclk1*⁺ cells. Moreover, our previous report, using immunohistochemistry, showed that *Dclk1*⁺ cells did not express *ChgA* [11].

A previous study reported that tuft cells are related with the type 2 immune response [17–19]. Consistently, our microarray analysis showed that *Dclk1*⁺ normal intestinal cells highly express *IL-25* mRNA (Fig. 2A).

In this study, *Dclk1* mRNA expression was 4 times higher in *Dclk1*⁺ cells than in *Dclk1*⁻ cells in the normal intestinal epithelium.

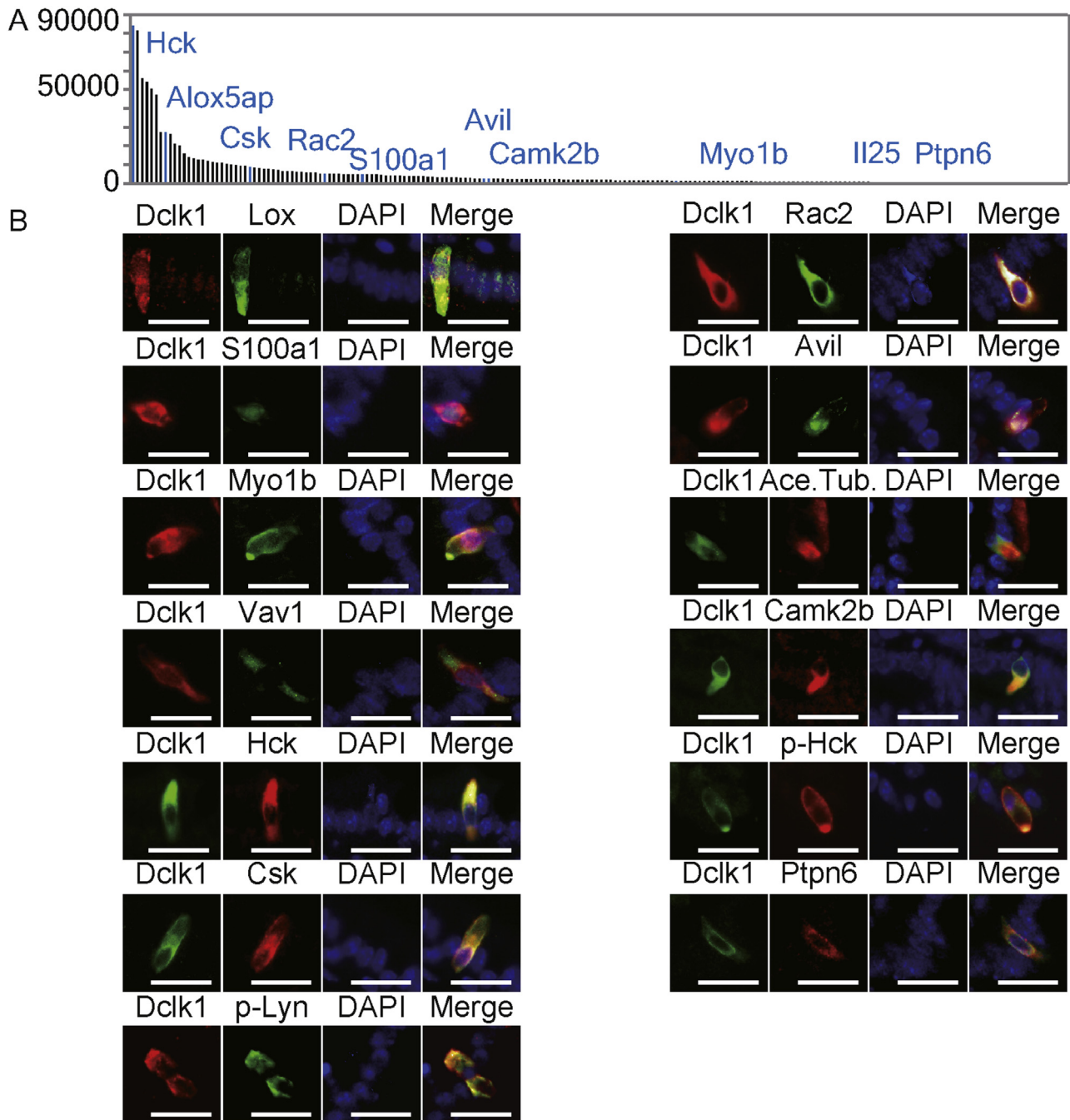


Fig. 2. Molecular characterization of *Dclk1*⁺ cells in the normal intestinal epithelium of mice.

(A) Bar graph showing the top 200 genes with expressions that were more than 8 times higher in *Dclk1*⁺ cells than in *Dclk1*⁻ cells in the normal intestinal epithelium of *Dclk1*^{CreERT2-IRES-EGFP/+} mice, as determined by microarray analysis. (B) Double immunofluorescent staining for *Dclk1* with Lox, Rac2, S100a1, Avil, Myo1b, acetylated tubulin (Ace. Tub.), Vav1, Camk2b, Hck, phosphorylated Hck (p-Hck), Csk, Ptpn6, or phosphorylated Lyn (p-Lyn) in the normal intestine of *Apc*^{Min/+} mice. Scale bar: 20 μm.

lium. Although it also appears that this was not so much higher, other factors expressed in tuft cells (e.g., Alox5ap, S100a1, Avil, Hck, and Camk2b) were also expressed more highly in *Dclk1*⁺ cells than in *Dclk1*⁻ cells. In addition, immunohistochemistry demonstrated that *Dclk1*⁺ cells expressed these factors. Therefore, it is reasonable to conclude that *Dclk1*⁺ cells were precisely sorted from *Dclk1*⁻ cells.

In terms of the similarities between *Dclk1*⁺ normal intestinal and *Dclk1*⁺ tumor cells, the pathway analysis showed that the “eicosanoid synthesis pathway” was up-regulated in both cell types (Tables 1 and 2). This was supported by the expression of Alox5ap, a member of this pathway in *Dclk1*⁺ cells (Figs. 2 and 3). In a previous

study in which tuft cells were sorted from normal intestinal epithelium by the expression of *Trpm5*, microarray analysis shows that these cells express *Dclk1* and Alox5ap [36]. The report also demonstrated that Avil, Tuba1, Tuba4, Tubb5, and Camk2b are highly expressed in tuft cells. Other reports demonstrated that tuft cells express high levels of Avil, Aldh2, Hck, Ptpn6, Vav1, Tuba1, Tuba4, Tubb5, and *Dclk1*, using single-cell mRNA sequencing [37,38]. The expression of α-tubulin and F-actin has also been confirmed previously by immunofluorescence analyses [16]. In this study, using microarray and immunofluorescence analyses, we showed that genes related to microtubules and the actin cytoskeleton, such as Rac2, S100a1, Avil1, Myo1b, acetylated tubulin, and Vav1, were

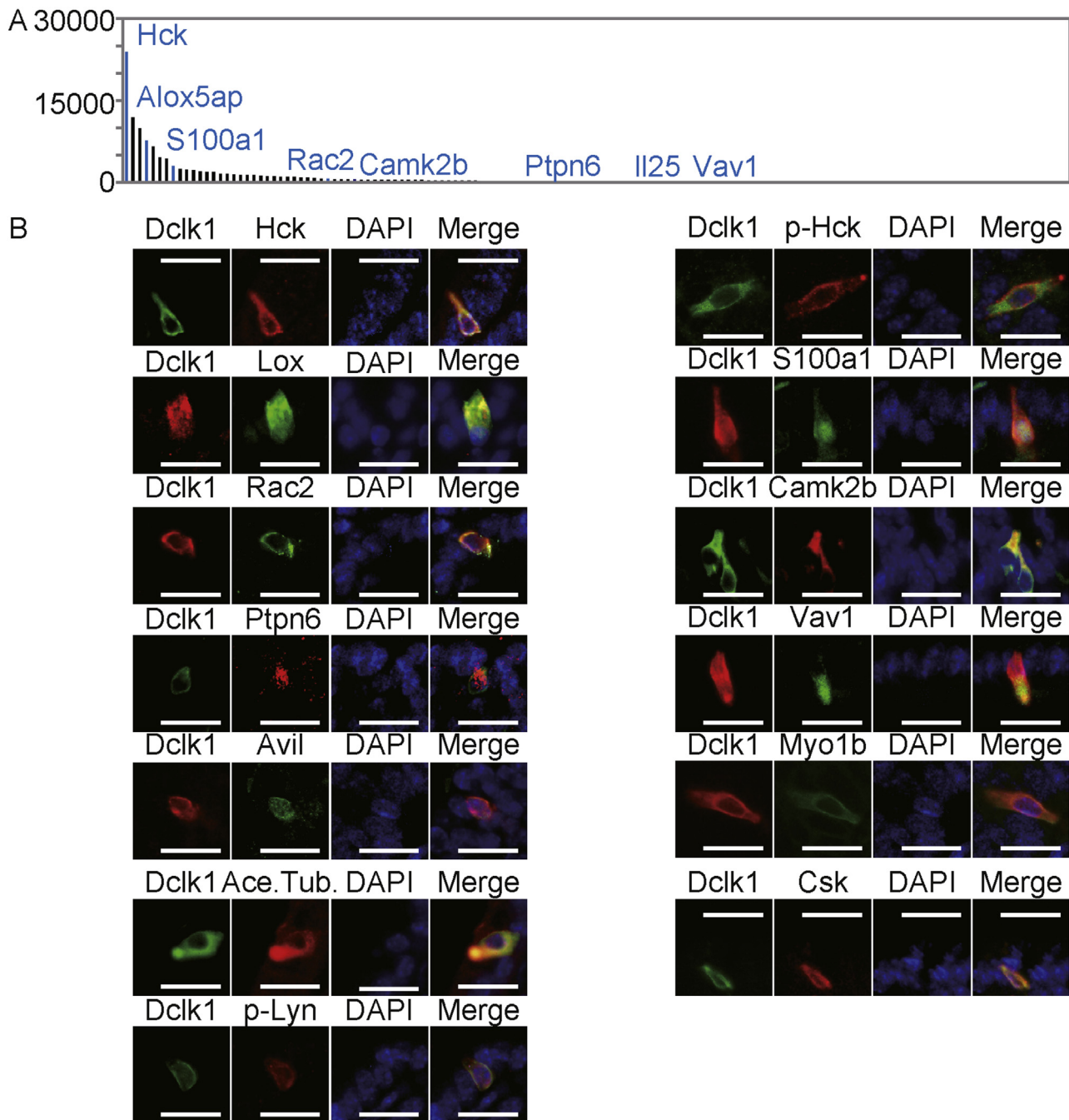


Fig. 3. Molecular characterization of Dclk1⁺ cells in the intestinal tumors of *Dclk1*^{CreERT2-IRES-EGFP/+}; *Apc*^{Min/+} mice.

(A) Bar graph showing the top 141 genes with expressions that were more than 8 times higher in Dclk1⁺ tumor cells than in Dclk1⁻ tumor cells in *Dclk1*^{CreERT2-IRES-EGFP/+}; *Apc*^{Min/+} mice, as determined by microarray analysis. (B) Double immunofluorescent staining for Dclk1 with Hck, phosphorylated Hck (p-Hck), Lox, S100a1, Rac2, Camk2b, Ptpn6, Vav1, Avil, Myo1b, acetylated tubulin (Ace. Tub.), Csk, or phosphorylated Lyn (p-Lyn) in the intestinal tumors of *Apc*^{Min/+} mice. Scale bar: 20 μ m.

expressed in Dclk1⁺ cells in both normal intestinal epithelium and intestinal tumors (Figs. 2 and 3). Among these factors, for the first time we identified a small GTP binding protein, Rac2, a critical regulator of the actin cytoskeleton [39,40], as a highly expressed molecule in Dclk1⁺ cells. Similarly, Myo1b, a motor protein that moves along actin filaments [41], was also identified. We also confirmed that Camk2b, which binds to F-actin [42], was expressed in Dclk1⁺ cells. These factors may affect the characteristic shape of Dclk1⁺ cells in normal intestinal epithelium and intestinal tumors.

In the present study, microarray and immunohistochemical analyses also demonstrated the expression of the Src family kinases (i.e., Hck, Lyn, Csk, and Ptpn6) in Dclk1⁺ cells in both normal

intestinal epithelium and intestinal tumors. Although Hck expression in Dclk1⁺ cells in organoids has been previously reported [37,38], here, we showed that both Hck and the activated form of Hck (phosphorylated Hck) were expressed in Dclk1⁺ cells in normal intestinal epithelium and intestinal tumors. Hck is over-represented in primary human acute myeloid leukemia stem cells, when compared with that in normal hematopoietic stem cells [43], and the Hck inhibitor reduced human leukemia stem cell and leukemia burden [44]. In addition, it was previously reported that Hck is a potential therapeutic target for dysplastic and leukemic cells owing to integration of the erythropoietin/PI3K pathway and regulation of erythropoiesis through the same pathway [45].

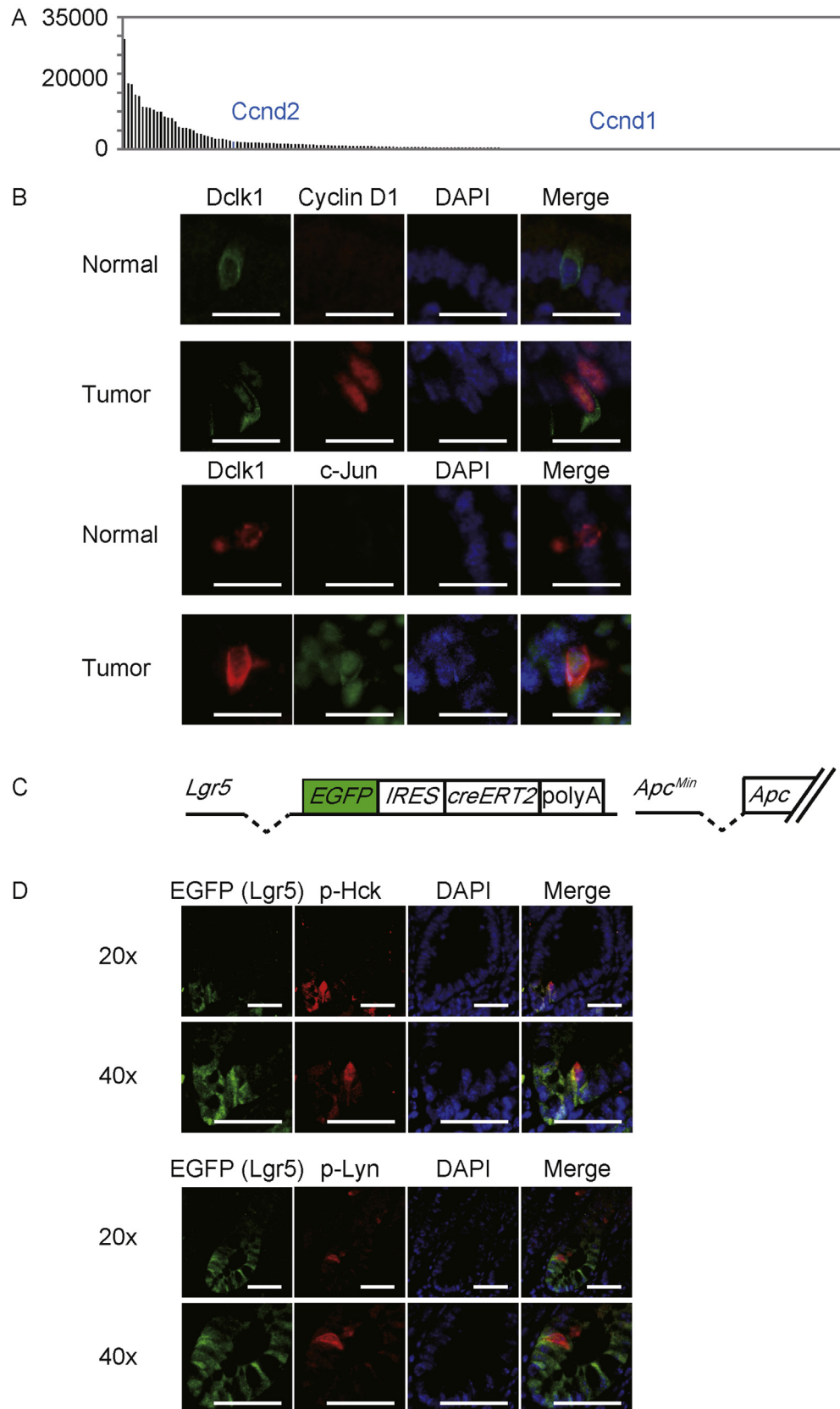


Fig. 4. Comparison between Dclk1⁺ tumor and Dclk1⁺ normal epithelial cells.

(A) Bar graph showing the top 200 genes with expressions that were more than 8 times higher in Dclk1⁺ tumor cells in the intestinal tumors of *Apc*^{Min/+} mice than in Dclk1⁺ cells in the normal intestinal epithelium. (B) Double immunofluorescent staining for Dclk1 with cyclin D1, encoded by *Ccnd1*, and c-Jun in the normal intestine and intestinal tumors of *Apc*^{Min/+} mice. Scale bar: 20 μ m. (C) Genetic scheme of *Lgr5*^{EGFP-IRES-CreERT2/+}; *Apc*^{Min/+} mice. (D) Double-immunofluorescent staining for EGFP with phosphorylated Hck (p-Hck) and phosphorylated Lyn (p-Lyn) in the intestinal tumors of *Lgr5*^{EGFP-IRES-CreERT2/+}; *Apc*^{Min/+} mice. Scale bar: 20 μ m.

These results suggest that Hck is necessary for the maintenance of leukemia stem cells, and Hck might play some roles in the maintenance of the stemness of Dcl1⁺ tumor stem cells. However, further investigation is necessary to elucidate whether Hck regulates the stemness and, if so, the mechanism by which this is achieved.

Other Src family kinases, Lyn, Csk, and Ptpn6, were identified for the first time in this study. In myeloid cells, Lyn exerts its negative role of modulation of signaling pathways with Ptpn6 [46], and Csk inactivates Lyn [29]. Although further studies are essential, as in the case of Hck in hematopoietic stem cells, these factors might contribute to the stemness of Dcl1⁺ tumor cells.

We further investigated factors that were expressed higher in Dcl1⁺ tumor cells than in Dcl1⁺ normal epithelial cells, to identify molecules specific to tumor stem cells. *Ccnd1*, which encodes cyclin D1, and *c-Jun* were highly expressed in Dcl1⁺ tumor cells (Fig. 4). They are positively involved in cell cycle progression [30,32]. Consistently, a higher number of Dcl1⁺ tumor cells in the intestinal tumors of *Apc^{Min/+}* mice uptake BrdU when compared to Dcl1⁺ normal intestinal cells [11]. However, they are expressed ubiquitously in intestinal tumors [33,34]. To identify molecules expressed in tumor stem cells, we checked the expression patterns of the above-mentioned factors in the intestinal tumors of *Lgr5^{EGFP-IRES-CreERT2/+}; Apc^{Min/+}* mice, because we have previously reported that Dcl1⁺/Lgr5⁺ tumor cells are the tumor stem cells in the intestinal tumors of *Apc^{Min/+}* mice [11]. We found that phosphorylated Hck and phosphorylated Lyn were expressed in Lgr5⁺ intestinal tumors in *Lgr5^{EGFP-IRES-CreERT2/+}; Apc^{Min/+}* mice (Fig. 4), suggesting that they might play important roles in Dcl1⁺/Lgr5⁺ tumor stem cells, as in the case of tumor stem cells in hematopoietic malignancies [43].

In summary, we identified molecules expressed in Dcl1⁺ cells in normal intestinal epithelium and intestinal tumor cells in mice. Factors such as phosphorylated Hck and phosphorylated Lyn may be potential novel therapeutic targets for colorectal cancer; however, further studies are required for the future development of cancer stem cell-based therapies.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.dld.2018.06.011>.

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