Digestive and Liver Disease 50 (2018) 1353–1361

Contents lists available at ScienceDirect

Digestive and Liver Disease

journal homepage: www.elsevier.com/locate/dld

Oncology

Gene expression profile of Dclk1$^+$ cells in intestinal tumors

Yuichi Yamaga, Akihisa Fukuda, Yuki Nakanishi, Norihiro Goto, Yoshihide Matsumoto, Takuto Yoshioka, Takahisa Maruno, Tsutomu Chiba, Hiroshi Seno*

Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto, Japan

ARTICLE INFO

Article history:
Received 1 September 2017
Received in revised form 12 June 2018
Accepted 13 June 2018
Available online 22 June 2018

Keywords:
Colorectal cancer
Microarray analysis
Tumor stem cells

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*$^{EGFP\text{-}IRES\text{-}CreERT2/+}$; *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.

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

* Corresponding author at: Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawahara-cho, Sakyo-Ku, Kyoto 606-8507, Japan.
E-mail address: seno@kuhp.kyoto-u.ac.jp (H. Seno).

https://doi.org/10.1016/j.dld.2018.06.011
1590-8658/

Table 1
The pathways up-regulated in Dclk1$^+$ cells compared to Dclk1$^-$ cells in the normal intestinal epithelium.

	MAPP name	Dclk1$^+$ normal/Dclk1$^-$ 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, Dclk1$^-$ cells were harvested freshly from 1 *Dclk1*$^{CreERT2\text{-}IRES\text{-}EGFP/+}$ mouse at 26 weeks of age; Dclk1$^+$ cells, from 2 *Dclk1*$^{CreERT2\text{-}IRES\text{-}EGFP/+}$ mice at the same age. Dclk1$^-$ intestinal tumor cells were freshly isolated from 1 *Dclk1*$^{CreERT2\text{-}IRES\text{-}EGFP/+}$; *Apc*$^{Min/+}$ mouse at more than 16 weeks of age; Dclk1$^+$ intestinal tumor cells, from 7 *Dclk1*$^{CreERT2\text{-}IRES\text{-}EGFP/+}$; *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 Dclk1$^+$ 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 Dclk1$^+$ cells was more than 4 times that of the genes of Dclk1$^-$ cells in Tables 1 and 2. In Supplementary Table, the threshold of the genes of Dclk1$^+$ intestinal tumor cells was more than 4 times that of the genes of Dclk1$^+$ 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-Dclk1 (Abcam), mouse anti-Dclk1 (Abcam), mouse anti-Dclk1 (Abnova, Taipei, Taiwan), goat anti-Dclk1 (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 Dclk1$^+$ and Dclk1$^-$ cells in normal intestinal epithelium

To determine the gene expression profile of Dclk1$^+$ cells in the normal small intestine, we performed a microarray analysis. Intestinal epithelial cells were harvested from *Dclk1*$^{CreERT2\text{-}IRES\text{-}EGFP/+}$ mice according to a previous report [11]. After the separation of Dclk1$^+$ and Dclk1$^-$ 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 Dclk1$^+$ and Dclk1$^-$ cells in normal intestinal epithelium (Fig. 1B). *Dclk1* mRNA expression was 4 times higher in Dclk1$^+$ cells than in Dclk1$^-$ 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 2
The 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 family 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*$^{CreERT2\text{-}IRES\text{-}EGFP/+}$; *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*$^{CreERT2\text{-}IRES\text{-}EGFP/+}$; *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-

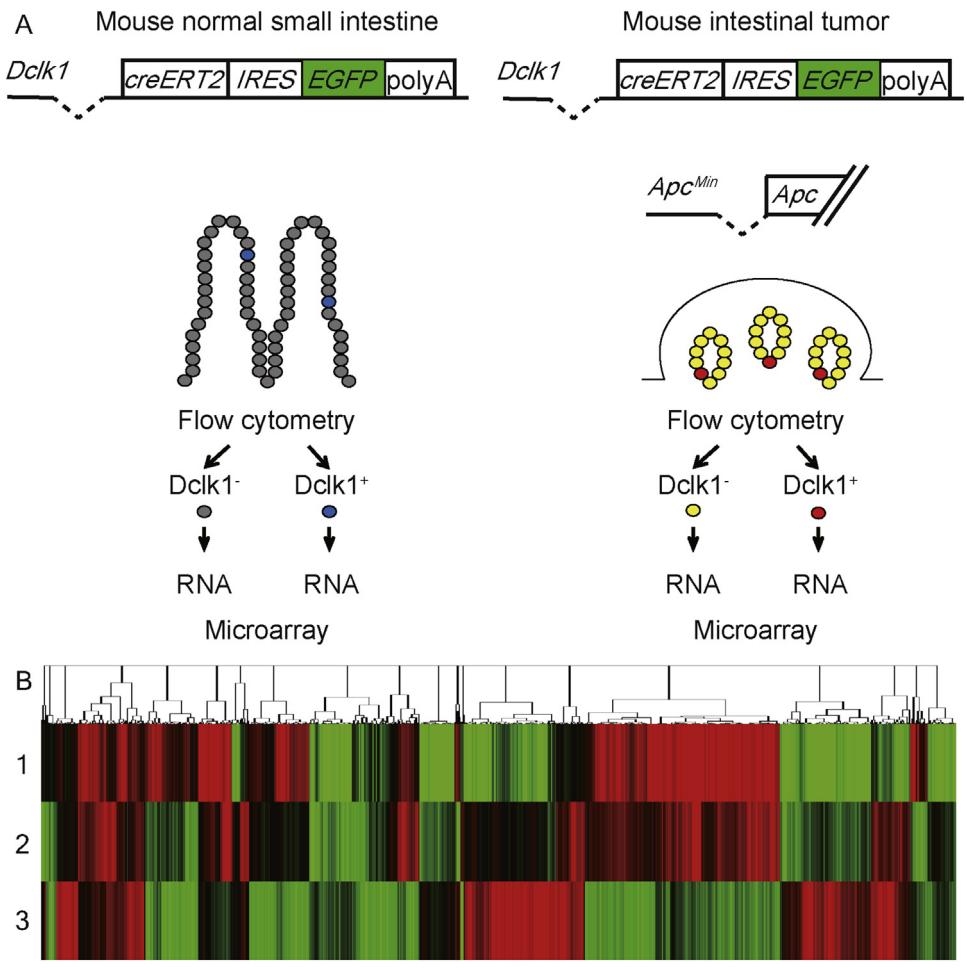

Fig. 1. Microarray analysis of Dclk1$^+$ and Dclk1$^-$ cells in the normal intestinal epithelium and intestinal tumors of *Apc*$^{Min/+}$ mice.
(A) Schema showing the experimental design. Dclk1$^-$ and Dclk1$^+$ cells in the normal intestinal epithelium of *Dclk1*$^{CreERT2\text{-}IRES\text{-}EGFP/+}$ mice and in the intestinal tumors of *Dclk1*$^{CreERT2\text{-}IRES\text{-}EGFP/+}$; *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*$^{EGFP\text{-}IRES\text{-}CreERT2/+}$; *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*$^{EGFP\text{-}IRES\text{-}CreERT2/+}$; *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 epithe-

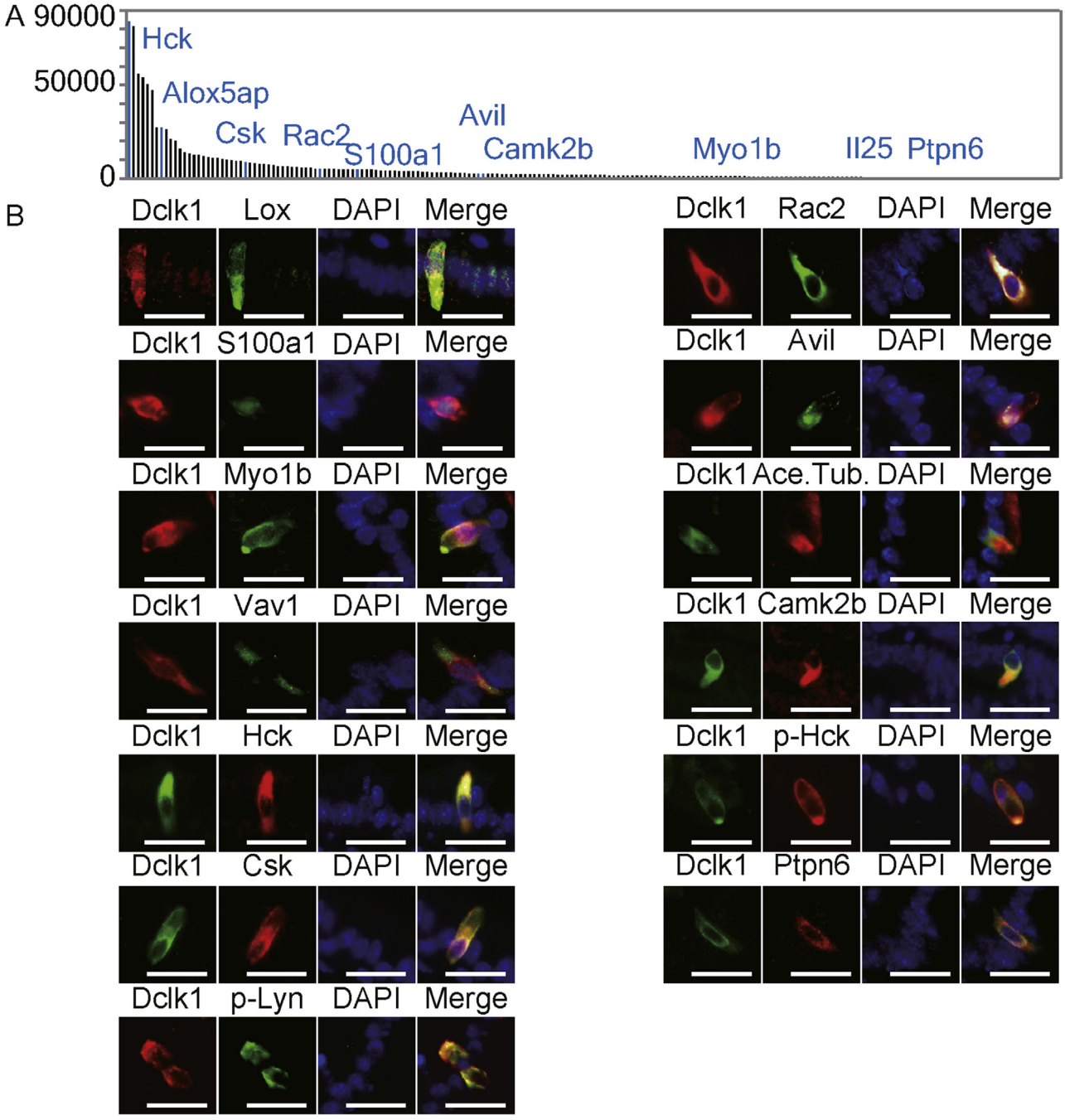

Fig. 2. Molecular characterization of Dclk1$^+$ cells in the normal intestinal epithelium of mice.
(A) Bar graph showing the top 200 genes with expressions that were more than 8 times higher in Dclk1$^+$ cells than in Dclk1$^-$ cells in the normal intestinal epithelium of *Dclk1*$^{CreERT2\text{-}IRES\text{-}EGFP/+}$ 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

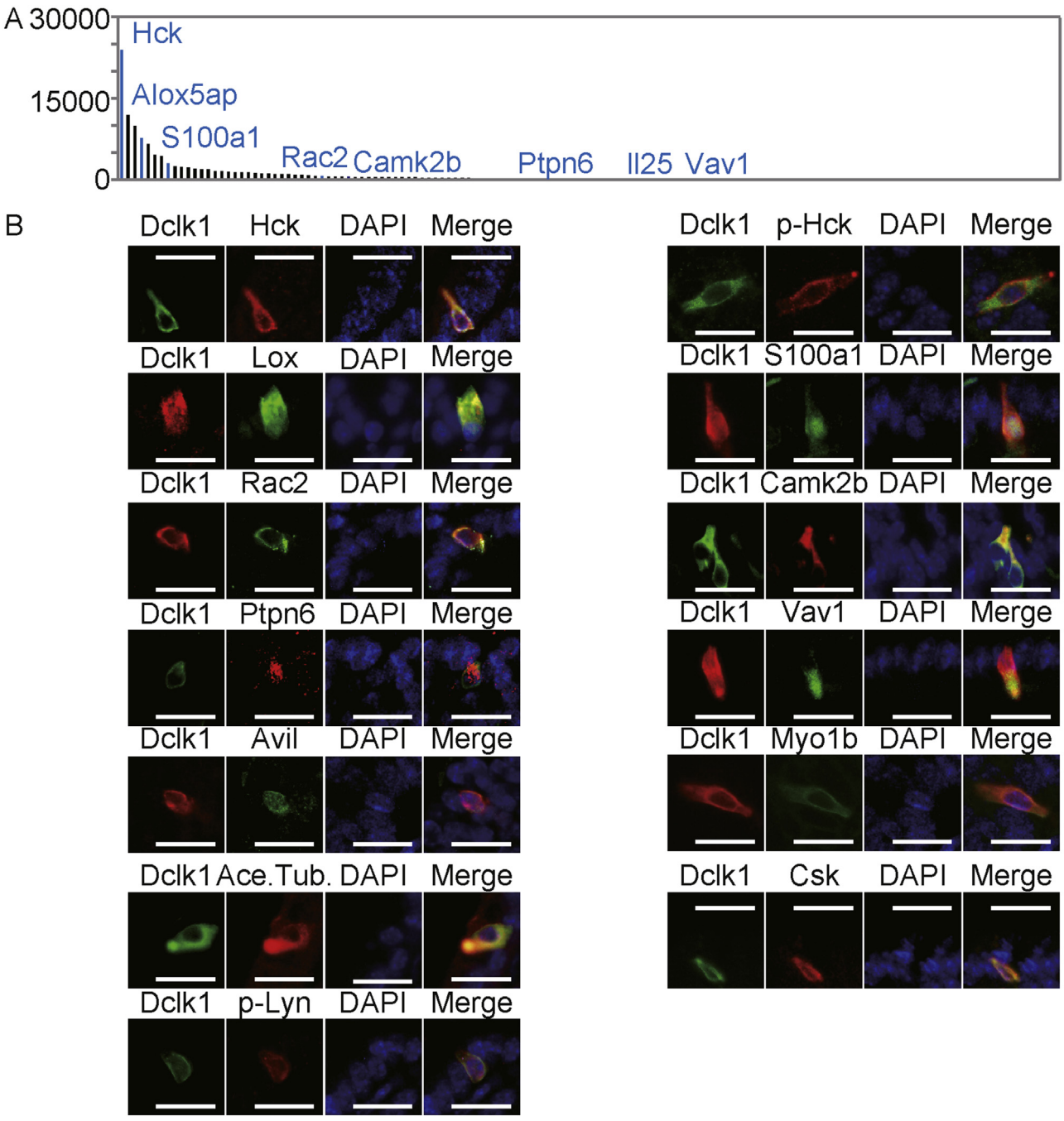

Fig. 3. Molecular characterization of Dclk1$^+$ cells in the intestinal tumors of *Dclk1*$^{CreERT2\text{-}IRES\text{-}EGFP/+}$; *Apc*$^{Min/+}$ mice.
(A) Bar graph showing the top 141 genes with expressions that were more than 8 times higher in Dclk1$^+$ tumor cells than in Dclk1$^-$ tumor cells in *Dclk1*$^{CreERT2\text{-}IRES\text{-}EGFP/+}$; *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 μm.

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 overrepresented 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].

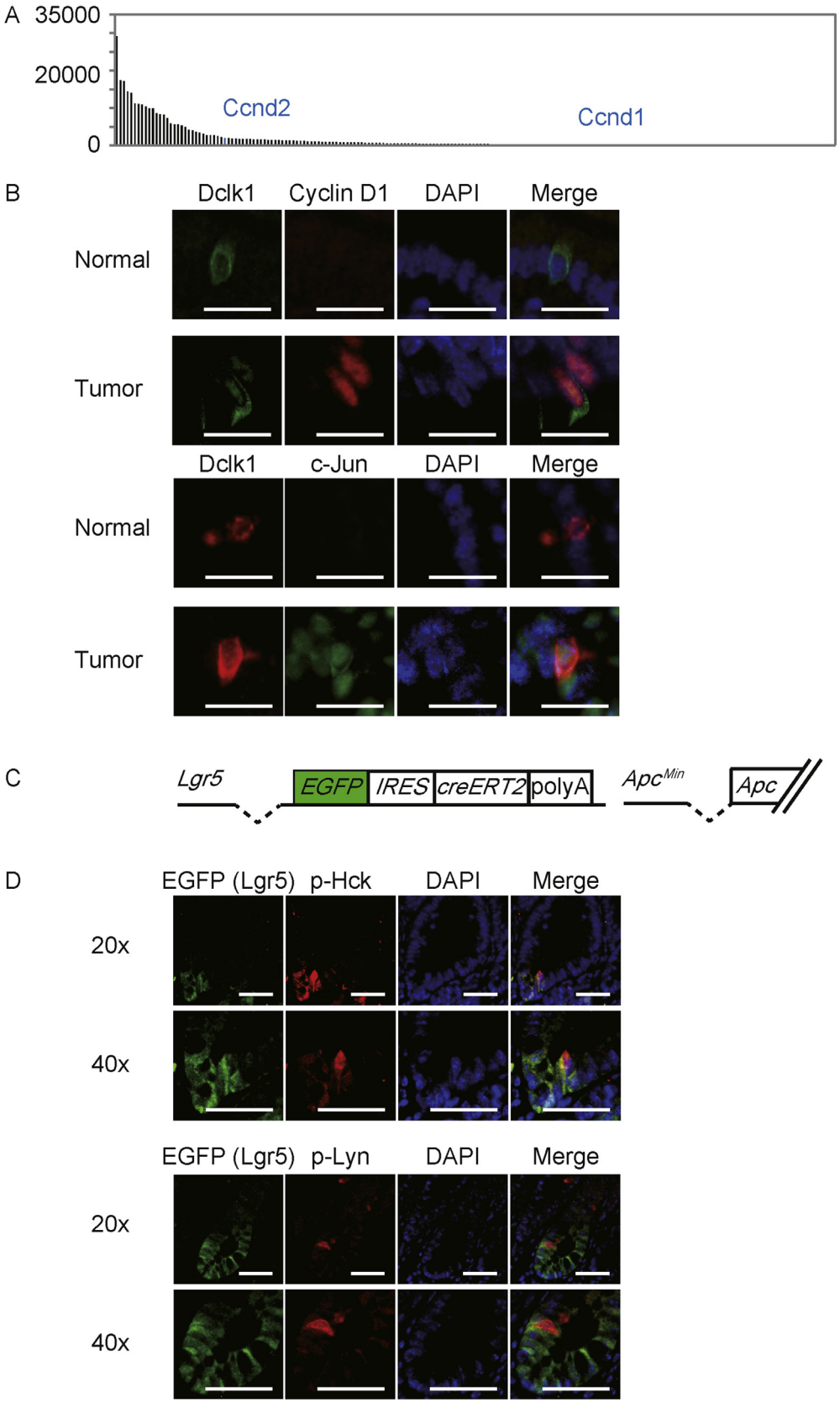

Fig. 4. Comparison between Dclk1^{+} tumor and Dclk1^{+} normal epithelial cells.
(A) Bar graph showing the top 200 genes with expressions that were more than 8 times higher in Dclk1^{+} tumor cells in the intestinal tumors of *Apc*$^{Min/+}$ mice than in Dclk1^{+} cells in the normal intestinal epithelium. (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 μm. (C) Genetic scheme of *Lgr5*$^{EGFP\text{-}IRES\text{-}CreERT2/+}$; *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*$^{EGFP\text{-}IRES\text{-}CreERT2/+}$; *Apc*$^{Min/+}$ mice. Scale bar: 20 μm.

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 Dclk1$^+$ 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 Dclk1$^+$ tumor cells.

We further investigated factors that were expressed higher in Dclk1$^+$ tumor cells than in Dclk1$^+$ normal epithelial cells, to identify molecules specific to tumor stem cells. *Ccnd1*, which encodes cyclin D1, and *c-Jun* were highly expressed in Dclk1$^+$ tumor cells (Fig. 4). They are positively involved in cell cycle progression [30,32]. Consistently, a higher number of Dclk1$^+$ tumor cells in the intestinal tumors of $Apc^{Min/+}$ mice uptake BrdU when compared to Dclk1$^+$ 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 $Lgr5^{EGFP\text{-}IRES\text{-}CreERT2/+}$; $Apc^{Min/+}$ mice, because we have previously reported that Dclk1$^+$/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 $Lgr5^{EGFP\text{-}IRES\text{-}CreERT2/+}$; $Apc^{Min/+}$ mice (Fig. 4), suggesting that they might play important roles in Dclk1$^+$/Lgr5$^+$ tumor stem cells, as in the case of tumor stem cells in hematopoietic malignancies [43].

In summary, we identified molecules expressed in Dclk1$^+$ 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.

Acknowledgements

This work was supported partly by Grants-in-Aid KAKENHI (15J03212, 25112707, 26293173, 16K09394, 16K15427, and 17H04157). This work was also supported by the Kobayashi Foundation for Cancer Research, Naito Foundation, Princess Takamatsu Cancer Research Fund13-24514, Mitsubishi Foundation, Mochida Foundation, and Japanese Society of Gastroenterology.

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.

References

[1] Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, et al. GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Available from: http://globocan.iarc.fr. [Accessed 3 April 2017].
[2] O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 2007;445:106–10.
[3] Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, et al. Identification and expansion of human colon-cancer-initiating cells. Nature 2007;445:111–5.
[4] Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, et al. Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci U S A 2007;104:10158–63.
[5] Vermeulen L, Todaro M, de Sousa Mello F, Sprick MR, Kemper K, Perez Alea M, et al. Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. Proc Natl Acad Sci U S A 2008;105:13427–32.
[6] Merlos-Suárez A, Barriga FM, Jung P, Iglesias M, Céspedes MV, Rossell D, et al. The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse. Cell Stem Cell 2011;8:511–24.
[7] Schepers AG, Snippert HJ, Stange DE, van den Born M, van Es JH, van de Wetering M, et al. Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. Science 2012;337:730–5.
[8] Yu T, Chen X, Zhang W, Colon D, Shi J, Napier D, et al. Regulation of the potential marker for intestinal cells, Bmi1, by beta-catenin and the zinc finger protein KLF4: implications for colon cancer. J Biol Chem 2012;287:3760–8.
[9] Zhu L, Gibson P, Currle DS, Tong Y, Richardson RJ, Bayazitov IT, et al. Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. Nature 2009;457:603–7.
[10] Ishimoto T, Nagano O, Yae T, Tamada M, Motohara T, Oshima H, et al. CD44 variant regulates redox status in cancer cells by stabilizing the xCT subunit of system xc(−) and thereby promotes tumor growth. Cancer Cell 2011;19:387–400.
[11] Nakanishi Y, Seno H, Fukuoka A, Ueo T, Yamaga Y, Maruno T, et al. Dclk1 distinguishes between tumor and normal stem cells in the intestine. Nat Genet 2013;45:98–103.
[12] Sato A. Tuft cells. Anat Sci Int 2007;82:187–99.
[13] Horio M, Sato M, Takeyama Y, Elshazley M, Yamashita R, Hase T, et al. Transient but not stable ZEB1 knockdown dramatically inhibits growth of malignant pleural mesothelioma cells. Ann Surg Oncol 2012;19(Suppl. 3):S634–45.
[14] Dahlquist KD, Salomonis N, Vranizan K, Lawlor SC, Conklin BR. GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. Nat Genet 2002;31:19–20.
[15] Doniger SW, Salomonis N, Dahlquist KD, Vranizan K, Lawlor SC, Conklin BR. MAPPFinder: using Gene Ontology and GenMAPP to create a global gene-expression profile from microarray data. Genome Biol 2003;4:R7.
[16] Gerbe F, van Es JH, Makrini L, Brulin B, Mellitzer G, Robine S, et al. Distinct ATOH1 and Neurog3 requirements define tuft cells as a new secretory cell type in the intestinal epithelium. J Cell Biol 2011;192:767–80.
[17] von Moltke J, Ji M, Liang HE, Locksley RM. Tuft-cell-derived IL-25 regulates an intestinal ILC2-epithelial response circuit. Nature 2016;529:221–5.
[18] Gerbe F, Sidot E, Smyth DJ, Ohmoto M, Matsumoto I, Dardalhon V, et al. Intestinal epithelial tuft cells initiate type 2 mucosal immunity to helminth parasites. Nature 2016;529:226–30.
[19] Howitt MR, Lavoie S, Michaud M, Blum AM, Tran SV, Weinstock JV, et al. Tuft cells, taste-chemosensory cells, orchestrate parasite type 2 immunity in the gut. Science 2016;351:1329–33.
[20] Gerbe F, Brulin B, Makrini L, Legraverend C, Jay P. DCAMKL-1 expression identifies Tuft cells rather than stem cells in the adult mouse intestinal epithelium. Gastroenterology 2009;137:2179–80, author reply 80–1.
[21] Omori Y, Suzuki M, Ozaki K, Harada Y, Nakamura Y, Takahashi E, et al. Expression and chromosomal localization of KIAA0369, a putative kinase structurally related to Doublecortin. J Hum Genet 1998;43:169–77.
[22] Quintrell N, Lebo R, Varmus H, Bishop JM, Pettenati MJ, Le Beau MM, et al. Identification of a human gene (HCK) that encodes a protein-tyrosine kinase and is expressed in hemopoietic cells. Mol Cell Biol 1987;7:2267–75.
[23] Ziegler SF, Marth JD, Lewis DB, Perlmutter RM. Novel protein-tyrosine kinase gene (hck) preferentially expressed in cells of hematopoietic origin. Mol Cell Biol 1987;7:2276–85.
[24] Okada M, Nada S, Yamanashi Y, Yamamoto T, Nakagawa H. CSK: a protein-tyrosine kinase involved in regulation of src family kinases. J Biol Chem 1991;266:24249–52.
[25] Cornall RJ, Cyster JG, Hibbs ML, Dunn AR, Otipoby KL, Clark EA, et al. Polygenic autoimmune traits: Lyn, CD22, and SHP-1 are limiting elements of a biochemical pathway regulating BCR signaling and selection. Immunity 1998;8:497–508.
[26] Sicheri F, Moarefi I, Kuriyan J. Crystal structure of the Src family tyrosine kinase Hck. Nature 1997;385:602–9.
[27] Poh AR, O'Donoghue RJ, Ernst M. Hematopoietic cell kinase (HCK) as a therapeutic target in immune and cancer cells. Oncotarget 2015;6:15752–71.
[28] Hata A, Sabe H, Kurosaki T, Takata M, Hanafusa H. Functional analysis of Csk in signal transduction through the B-cell antigen receptor. Mol Cell Biol 1994;14:7306–13.
[29] Ingley E. Src family kinases: regulation of their activities, levels and identification of new pathways. Biochim Biophys Acta 2008;1784:56–65.
[30] Baldin V, Lukas J, Marcote MJ, Pagano M, Draetta G. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes Dev 1993;7:812–21.
[31] Ando K, Ajchenbaum-Cymbalista F, Griffin JD. Regulation of G1/S transition by cyclins D2 and D3 in hematopoietic cells. Proc Natl Acad Sci U S A 1993;90:9571–5.
[32] Kovary K, Bravo R. The jun and fos protein families are both required for cell cycle progression in fibroblasts. Mol Cell Biol 1991;11:4466–72.
[33] Nateri AS, Spencer-Dene B, Behrens A. Interaction of phosphorylated c-Jun with TCF4 regulates intestinal cancer development. Nature 2005;437:281–5.
[34] Colnot S, Niwa-Kawakita M, Hamard G, Godard C, Le Plenier S, Houbron C, et al. Colorectal cancers in a new mouse model of familial adenoma-

tous polyposis: influence of genetic and environmental modifiers. Lab Invest 2004;84:1619–30.
[35] Bjerknes M, Khandanpour C, Möröy T, Fujiyama T, Hoshino M, Klisch TJ, et al. Origin of the brush cell lineage in the mouse intestinal epithelium. Dev Biol 2012;362:194–218.
[36] Bezençon C, Fürholz A, Raymond F, Mansourian R, Métairon S, Le Coutre J, et al. Murine intestinal cells expressing Trpm5 are mostly brush cells and express markers of neuronal and inflammatory cells. J Comp Neurol 2008;509:514–25.
[37] Grün D, Lyubimova A, Kester L, Wiebrands K, Basak O, Sasaki N, et al. Single-cell messenger RNA sequencing reveals rare intestinal cell types. Nature 2015;525:251–5.
[38] Basak O, Beumer J, Wiebrands K, Seno H, van Oudenaarden A, Clevers H. Induced quiescence of Lgr5+ stem cells in intestinal organoids enables differentiation of hormone-producing enteroendocrine cells. Cell Stem Cell 2017;20, 177–90.e4.
[39] Didsbury J, Weber RF, Bokoch GM, Evans T, Snyderman R. rac, a novel ras-related family of proteins that are botulinum toxin substrates. J Biol Chem 1989;264:16378–82.
[40] Polakis PG, Weber RF, Nevins B, Didsbury JR, Evans T, Snyderman R. Identification of the ral and rac1 gene products, low molecular mass GTP-binding proteins from human platelets. J Biol Chem 1989;264:16383–9.
[41] Coluccio LM. Differential calmodulin binding to three myosin-1 isoforms from liver. J Cell Sci 1994;107(Pt. 8):2279–84.
[42] Shen K, Teruel MN, Subramanian K, Meyer T. CaMKIIbeta functions as an F-actin targeting module that localizes CaMKIIalpha/beta heterooligomers to dendritic spines. Neuron 1998;21:593–606.
[43] Saito Y, Kitamura H, Hijikata A, Tomizawa-Murasawa M, Tanaka S, Takagi S, et al. Identification of therapeutic targets for quiescent, chemotherapy-resistant human leukemia stem cells. Sci Transl Med 2010;2, 17ra9.
[44] Saito Y, Yuki H, Kuratani M, Hashizume Y, Takagi S, Honma T, et al. A pyrrolopyrimidine derivative targets human primary AML stem cells in vivo. Sci Transl Med 2013;5, 181ra52.
[45] Roversi FM, Pericole FV, Machado-Neto JA, da Silva Santos Duarte A, Longhini AL, Corrocher FA, et al. Hematopoietic cell kinase (HCK) is a potential therapeutic target for dysplastic and leukemic cells due to integration of erythropoietin/PI3K pathway and regulation of erythropoiesis: HCK in erythropoietin/PI3K pathway. Biochim Biophys Acta 2017;1863:450–61.
[46] Scapini P, Pereira S, Zhang H, Lowell CA. Multiple roles of Lyn kinase in myeloid cell signaling and function. Immunol Rev 2009;228:23–40.