Table 3 Ct-OATP1B3 and Lt-OATP1B3 mRNA levels in colon tissues and pooled human hepatocytes

mRNA name	Tissue (n)[a]	Median value (25-75th percentile) (×10^3 copies/ng total RNA)
Ct-OATP1B3	Cancer (34)	19.5 (5.3-50.2)
	Normal (1)	N/A[b]
	Pooled hepatocytes	N/A[c]
Lt-OATP1B3	Cancer (21)	1.6 (1.2-2.4)
	Normal (20)	1.4 (1.2-1.7)
	Pooled hepatocytes	56.9 ± 2.5[d]

[a]The number of Ct-OATP1B3 or Lt-OATP1B3 mRNA positive patients is shown in parentheses.
[b]The Ct-OATP1B3 mRNA copy number in the normal tissue is 4.3×10^3 copies/ng total RNA.
[c]The Ct-OATP1B3 mRNA expression level in pooled hepatocytes is under the quantification limit.
[d]The Lt-OATP1B3 mRNA expression level in pooled hepatocytes is expressed as the mean value ± SD.

began with an examination of the association of Ct-OATP1B3 mRNA levels and expression frequencies with cancer stages. As the results show, the Ct-OATP1B3 mRNA expression frequencies in early (0, I, and II) and advanced (III and IV) cancer stages were 88.9% (16/18) and 93.8% (15/16), respectively (Table 4). The median Ct-OATP1B3 mRNA level tended to be higher in the early stages than that in advanced stages (22.9×10^3 vs. 11.6×10^3 copies/ng total RNA, Table 4), although this difference was not statistically significant (Mann–Whitney U test, $P = 0.3$).

Next, to test the association of Ct-OATP1B3 mRNA expression with the differentiation status of colon cancer, the Ct-OATP1B3 mRNA levels between well-differentiated and moderately differentiated cancer tissues were compared (poorly differentiated and undifferentiated cancer tissues could not be obtained for use in this study). The median value of Ct-OATP1B3 mRNA levels in well-differentiated cancer tissues was 5.2-fold higher than that in moderately differentiated cancer tissues (58.1×10^3 vs. 11.2×10^3 copies/ng total RNA, Mann–Whitney U test, $P = 0.004$) (Figure 3). It should be also noted that three out of five cancer tissue specimens that did not express Ct-OATP1B3 mRNA were moderately differentiated cancer (the degree of differentiation in the rest two specimens was unavailable) (data not shown).

Table 4 Ct-OATP1B3 mRNA levels in early and advanced stages of colon cancer tissues

Cancer stage	Gene expression frequency	Gene expression levels[a]
	% (n, positive/total)	Median value (25-75th percentile)
Early (0, I, and II)	88.9 (1/1, 2/2, and 13/15)	22.9 (11.1-62.2)
Advanced (III and IV)	93.8 (12/12 and 3/4)	11.6 (4.8-32.7)

[a]The value is expressed as $\times 10^3$ copies/ng total RNA.

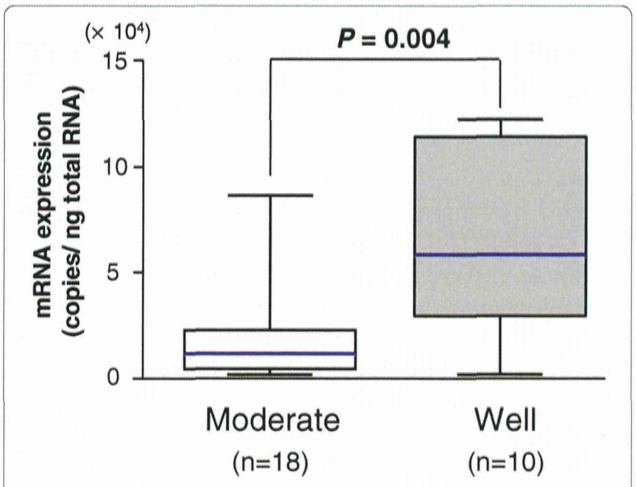

Figure 3 Comparison of the Ct-OATP1B3 mRNA expression levels between well and moderately differentiated colon cancer tissues. The colon cancer specimens with positive Ct-OATP1B3 mRNA expression were classified into two groups by the degree of differentiation, well-differentiated (n = 10), and moderately differentiated (n = 18). Data are shown as the box-and-whisker plot. The statistical significance of the median difference between two groups was determined using the Mann–Whitney U test.

Although the association of the Ct-OATP1B3 mRNA levels with other factors (age, sex and tumor location) was also examined, no statistically significant correlation could be found (data not shown).

Expression profile of each OATP1B3 mRNA isoform in human lung cancer

Ct- and Lt-OATP1B3 mRNA in human lung cancer was also separately quantified by qPCR. Compared with the results obtained from colon cancer tissues, the Ct-OATP1B3 mRNA expression frequency in lung cancer was relatively low (28.6%, 8/28) (Figure 4). Nevertheless, this frequency was statistically high compared with that obtained from normal lung tissues (7.1%, 2/28) (Fisher's exact test, $P = 0.039$) (Figure 4), and the mRNA level in each cancer tissue was higher than that in the matched normal tissue (Additional file 1: Figure S1B).

Regarding Lt-OATP1B3 mRNA expression, its positive frequencies in cancer and normal lung tissues were 7.1% (2/28) and 3.6% (1/28) (Figure 4), and the Lt-OATP1B3 mRNA level was lower than the Ct-OATP1B3 mRNA level in each matched pair (Additional file 2: Figure S2B).

Again, the similar Ct- and Lt-OATP1B3 mRNA expression results were obtained using a ΔΔCt-method with the GAPDH mRNA level as a normalization control (data not shown).

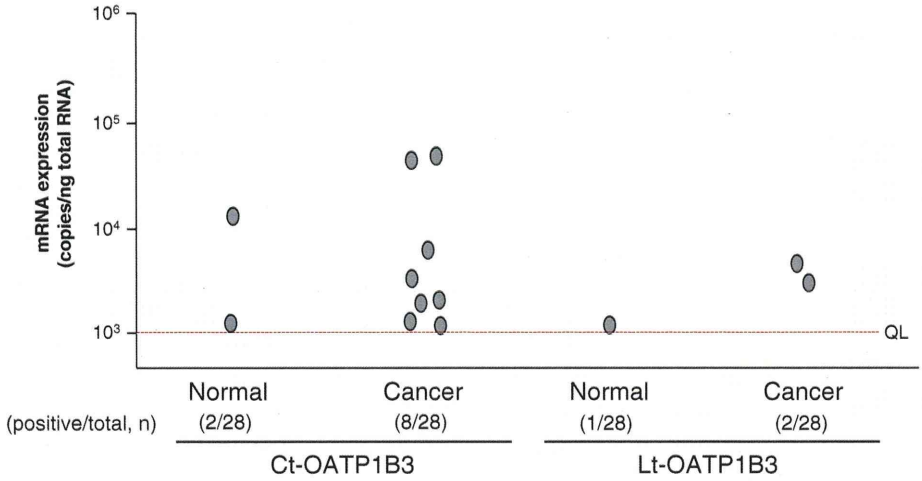

Figure 4 Expression profiles of Ct- and Lt-OATP1B3 mRNA in human lung cancer patients. Using the same quantification and calculation methods as described in the legend of Figure 2, the copy numbers of two OATP1B3 mRNA isoforms in each sample were separately determined. Each dot represents the mean of Ct- or Lt-OATP1B3 mRNA expression levels (copies/ng total RNA), which was obtained from three independent determinations, each performed in duplicate. The red line indicates the QL value. The total number of specimens, together with the number of positive expression specimens, in each group is shown in parentheses.

Comparison of Ct-OATP1B3 mRNA levels between colon and lung cancer

When Ct-OATP1B3 mRNA levels between colon and lung cancer tissues were compared, it was found that the median value of Ct-OATP1B3 mRNA levels in colon cancer tissues was 8.1-fold higher than that in lung cancer tissues (19.5×10^3 vs. 2.4×10^3 copies/ng total RNA, Mann–Whitney U test, $P = 0.037$) (Figure 5).

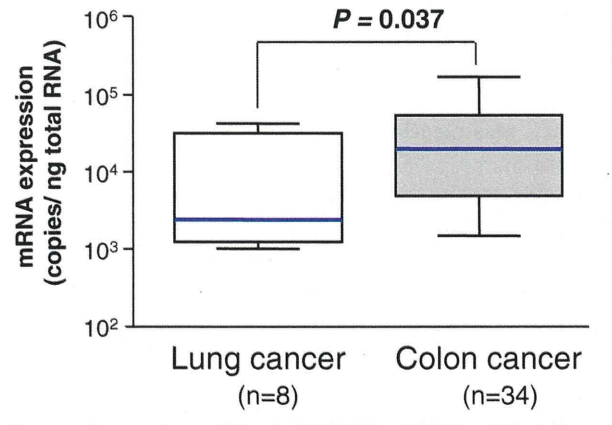

Figure 5 Comparison of the Ct-OATP1B3 mRNA levels between lung and colon cancer tissues. The Ct-OATP1B3 mRNA expression levels in lung (n = 8) and colon (n = 34) cancer tissues are shown as the box-and-whisker plot. Horizontal solid lines denote the median mRNA levels in each group. The statistically significant difference of the median Ct-OATP1B3 mRNA levels between two groups was examined using the Mann–Whitney U test.

Functional expression analysis of endogenous Ct-OATP1B3 in colon cancer cell lines

The high Ct-OATP1B3 mRNA expression in cancer tissues highlights the possibility that it plays certain roles in cancer biology. Thus, the functional expression analysis of Ct-OATP1B3 was performed using human LS180 and HCT116 cells (colon cancer). The results of mRNA quantification showed that, similar to those observed in colon cancer tissues, Ct-OATP1B3 mRNA was highly expressed in LS180 and HCT116 cells, and that the mRNA level in LS180 cells was comparable with that of Lt-OATP1B3 in pooled hepatocytes (Table 3, Figure 6A).

Subsequently, endogenous Ct-OATP1B3 protein expression and functional analysis were performed by Western blotting and transport assays. Despite high Ct-OATP1B3 mRNA expression levels in colon cancer cells, its protein expression was not detected in the soluble membrane fraction of such cells when using either anti-OATP1B3 antibodies (which was obtained from Sigma) or anti-OATP1B3 rabbit serum (which was developed in this study) (Figure 6 and Additional file 3: Figure S3). Na^+/K^+ ATPase, which is a plasma membrane marker protein, was detected in all samples. Considering the possibility that Ct-OATP1B3 could be localized somewhere within intracellular fractions, the same analysis was also performed using whole cell lysates. However, no Ct-OATP1B3 protein expression was detected in any of those cells (Additional file 4: Figure S4). Consistently, CCK-8 transport activity was not detected in colon cancer cells, nor was this activity inhibited by BSP, which is

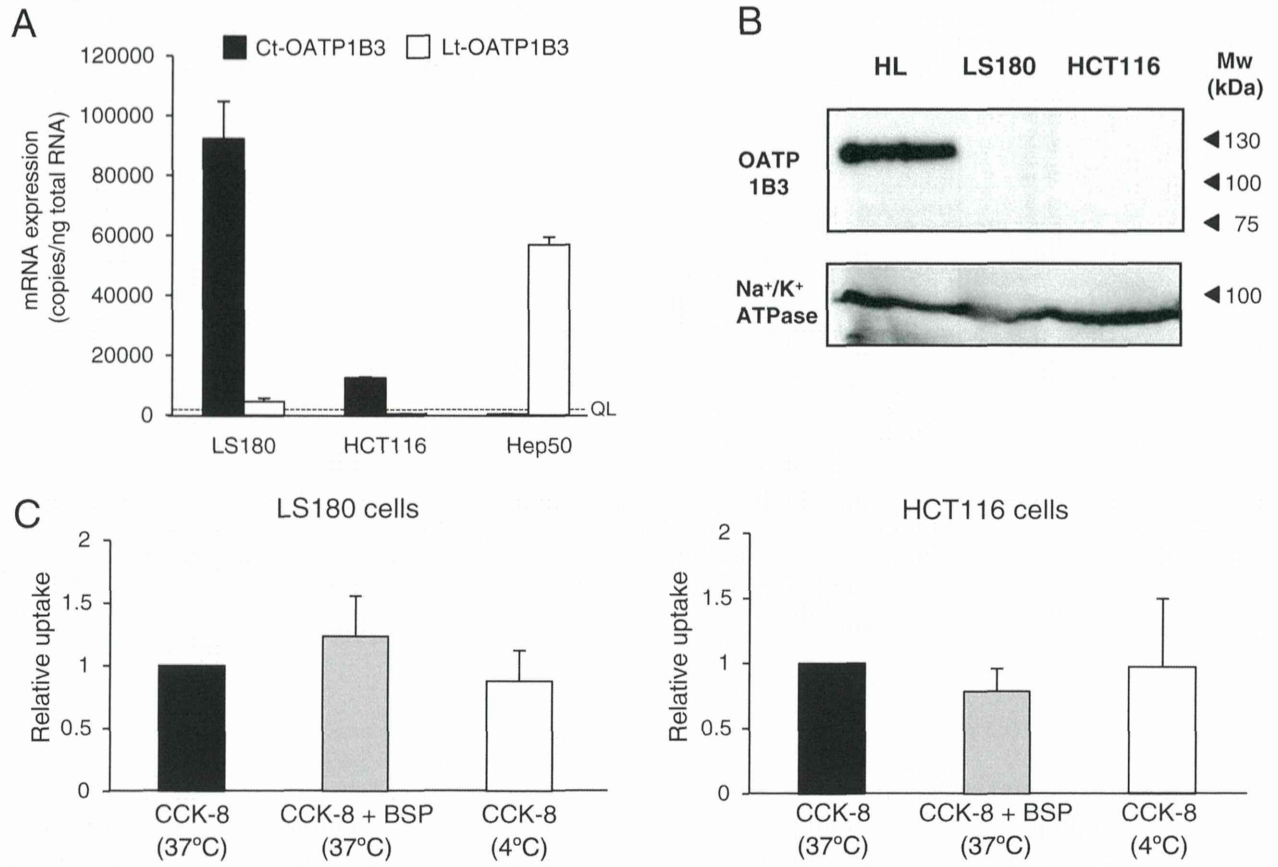

Figure 6 Protein expression and functional analysis of Ct-OATP1B3 in colon cancer cell lines. (A) Quantification of Ct- and Lt-OATP1B3 mRNA expression levels in human pooled hepatocytes (Hep50) and colon cancer cells (LS180 and HCT116) was separately performed by qPCR. The black and white bars indicate Ct- and Lt-OATP1B3 mRNA levels, respectively. The values are shown as means ± S.D. of three independent experiments, each performed in duplicate. The dashed line indicates the QL value. **(B)** The membrane protein expression of Ct-OATP1B3 in colon cancer cells was examined by Western blotting using rabbit anti-OATP1B3 antibodies that recognize the C-terminal peptide, which was supposed to be the common region among both isoforms. The soluble membrane fraction of human liver tissue (HL) was used as a positive control. Na^+/K^+ ATPase was used for a plasma membrane marker. **(C)** The CCK-8 uptake was examined by transport analysis using LS180 and HCT116 cells. The CCK-8 uptake (1 μM) level was determined in the absence (black bar) or presence (gray bar) of BSP (100 μM). The CCK-8 uptake in cancer cells is expressed as the relative ratio against the uptake of CCK-8 without BSP at 37°C. The white bar indicates transporter-independent CCK-8 accumulation level, which was determined at 4°C. This experiment was performed three times in duplicate.

a known OATP1B3 inhibitor (Figure 6C). Similar results were obtained from transport assay using E_2G as a substrate (data not shown).

Ct-OATP1B3 functional expression analysis in cells transfected with each Ct-OATP1B3 isoform expression plasmid

We further examined the functional expression of Ct-OATP1B3 using HCT116 cells transiently expressing each OATP1B3 isoform. The immunocytochemistry and transport assay results did not show any Ct-OATP1B3 protein expression or CCK-8 uptake activity in HCT116 cells transiently transfected with each Ct-OATP1B3 isoform (Additional file 5: Figure S5). However, Lt-OATP1B3 protein expression, as well as its transport activity, was clearly detected in the same experimental condition.

Similar results were also obtained from HEK293 cells stably expressing an OATP1B3 isoform (Additional file 6: Figure S6). As expected, Lt-OATP1B3 expression and function were clearly detected in HEK293 cells stably expressing Lt-OATP1B3. However, despite comparable mRNA levels among the cells, the results of Western blotting and transport assays failed to show functional Ct-OATP1B3 expression in any HEK293 cells expressing either Ct-OATP1B3-C or CT-OATP1B3-v1.

Discussion

In agreement with our previous study, the results of the present study have shown that, even when using a larger number of the matched-pair tissue specimens, the Ct-

OATP1B3 mRNA expression level in individual cancer tissue is always higher than the level in matched normal tissues, and is always predominant over the Lt-OATP1B3 mRNA level in each cancer tissue. These findings can be regarded as supporting evidence that Ct-OATP1B3 is the *bona fide* OATP1B3 mRNA isoform expressed in human colon and lung cancer. Thus, special attention should be paid to Ct-OATP1B3 expression (rather than Lt-OATP1B3) in future studies on cancer-associated OATP1B3. Furthermore, due to the high mRNA sequence similarities between the two isoforms, an elaborative experimental design is strongly recommended for such studies.

Cancer-specific RNA, DNA modification, or secreted molecules have been used as cancer biomarkers, such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19–9 (CA19-9) in colon cancer [19-21]. In addition, numerous studies have been conducted to identify new clinically useful cancer biomarkers, as exemplified by Kallikrein-related peptidase 10 (KLK10) mRNA, which shows a cancer-specific profile [22]. Accordingly, the high positive rate (87.2%) of Ct-OATP1B3 mRNA expression in colon cancer tissues, along with its high T/N ratio, motivates us to evaluate it as a possible colon cancer biomarker candidate. Cancer biomarkers should possess sufficient and appropriate sensitivity and specificity that allows them to fulfill their roles in a given cancer therapy (cancer detection, metastasis or recurrence risk assessment, or response prediction). Therefore, in order to evaluate those properties, the area under the receiver operating characteristic curve analysis has been widely used (higher score is preferable with the maximum value = 1) [23]. A preliminary examination shows that the value of Ct-OATP1B3 mRNA is 0.93 for colon cancer diagnosis (Additional file 7: Figure S7). This value is comparable to that of serum CEA (0.86) or KLK10 mRNA (0.89) and higher than that of CA19-9 (0.58) [19,22], suggesting that Ct-OATP1B3 mRNA may possess the clinically useful level of diagnostic power necessary to discriminate between cancer and normal colon tissues. In addition, the newly-identified clinico-pathological Ct-OATP1B3 mRNA expression features, which are its high rate of detection in early stages of colon cancer as well as its association with the well-differentiated cancer status, are considered noteworthy.

Based on the above considerations, it is reasonable to expect that Ct-OATP1B3 mRNA may be a highly promising candidate of colon cancer biomarker. However, we should reiterate the limitations of this study, which underscore the necessity of further investigations using an expanded cohort, various populations, and multicenter analyses in order to produce a comprehensive evaluation of its usefulness and restrictions. Elucidation of associations between Ct-OATP1B3 mRNA expression and prognostic values is another key issue that needs to be addressed (we were unable to pursue in this study due to use of recently obtained tissue specimens). Furthermore, it will be important to explore whether Ct-OATP1B3 mRNA can be detected in blood, because it has become evident that serum exosomes secreted from cancer cells contain cancer cell–derived molecules [24], such as the tumor-specific mRNA splicing variant detected in serum exosomes obtained from several glioblastoma patients [25].

Taken together, although extensive research efforts will be necessary before Ct-OATP1B3 mRNA can be established as a colon cancer biomarker, it is considered likely that such research will be worthwhile to promote in view of the urgent need for diagnostic tools in colon cancer, including the need for the development of a more reliable and less-invasive detection marker for patients in early stages of the disease. It has been acknowledged that examination of serum CEA and CA19-9 does not have sufficient capability for early identification of colon cancer due to their low abnormality rates in stage I (12-19% and 8%, respectively) and stage II patients (47-49% and 17%, respectively) [20,21]. Therefore, once established, it is speculated that Ct-OATP1B3 mRNA, together with other markers and image diagnostic methods such as colonoscopy, computerized tomography, and magnetic resonance imaging, may improve the accuracy and sensitivity of current colon cancer screening, and may provide other important clinico-pathological information that ultimately contribute to reduction in the incidence of morbidity and mortality of the disease.

In addition to colon cancer, Ct-OATP1B3 mRNAs were also detected in about one third of lung cancer patients as well. This finding suggests that Ct-OATP1B3 mRNA may also be an indicator of lung cancer occurrence, although to a lesser degree. In line with the observations that Ct-OATP1B3 mRNA has been detected in other cancer types [6,7], it will be necessary to clarify Ct-OATP1B3 mRNA expression preference in various cancer types to expand its clinical potential.

Functions of genes that are overexpressed in a cancer-specific manner are often involved in oncogenic processes [26-28]. Therefore, it is rational to assume that association of Ct-OATP1B3 mRNA expression with well-differentiation cancer status, as well as differential expression levels between lung and colon cancer, have some links with certain cancer cell biological processes.

Based on the predicted transporter-like structure of Ct-OATP1B3 translation products, which are Ct-OATP1B3-C and Ct-OATP1B3-v1 (Figure 1), one of the plausible functions of Ct-OATP1B3 is hormone uptake into cancer cells, as has been reported with the OATP1A2 function in prostate cancer [29]. The previous results provided by a cell-based exogenous Ct-OATP1B3-v1 transient expression system might also support this possibility [6,7]. However, our results did not identify any functional expression of

endogenous Ct-OATP1B3 in colon cancer cells, nor were we able to identify exogenous Ct-OATP1B3 function in HEK293 cells stably expressing Ct-OATP1B3-C or Ct-OATP1B3-v1. Thus, these results show apparent inconsistencies regarding the functional expression of Ct-OATP1B3. Because the experimental transport assay procedures employed in those studies are quite similar, the reason for the above-mentioned controversy is currently unclear. Therefore, further research aimed at providing convincing experimental evidence showing whether or not the Ct-OATP1B3 protein exists will be necessary to solve the above-mentioned arguments. However, in such future studies, we suggest taking into consideration the possibility that the translation efficiency of Ct-OATP1B3-v1 or Ct-OATP1B3-C might be very low. This is because the genomic DNA sequence around the Ct-OATP1B3-v1 start codon shows much less homology to the Kozak sequence (Figure 1B) [30], and because Ct-OATP1B3-C has a long 5'-untranslated region.

Nevertheless, we believe it is important to publicize our unexpected results pointing out that the transport function may not be the major role of endogenous Ct-OATP1B3 in cancer cells. This is because the results provide, rather than exclude, additional possibilities relating to how Ct-OATP1B3 plays a functional role in cancer cell biology. Recently, it has been shown that long non-coding RNA can directly interact with proteins to promote cancer metastasis [31], while another report has shown that a short cell-penetrating peptide derived from the Wilm's tumor protein 1 has inhibitory effects on cancer proliferation and clonogenic activity [32]. In our previous report, the existence of Ct-OATP1B3-derived short peptides was suggested [5]. Therefore, while it is already clear that Ct-OATP1B3 plays far-reaching roles in cancer cells, numerous *in vivo* as well as *in vitro* experiments still remain to be conducted. The results of these experiments can be expected to provide important clues that will help identifying the functions of Ct-OATP1B3 protein, peptides, or the mRNA itself in cancer cell biology.

Conclusion

Our results not only provide further evidence of the primary Ct-OATP1B3 mRNA expression profile in human colon and lung cancer, but also identify new clinicopathological features of the Ct-OATP1B3 mRNA expression. Even though these results should be interpreted with caution due to several study limitations, it can nevertheless be said that Ct-OATP1B3 mRNA has the potential to become a promising biomarker candidate for colon (and lung) cancer diagnosis. On the other hand, our data suggests that, although existence of a transporter-like Ct-OATP1B3 protein cannot be fully excluded, it may not functionally active at detectable levels. This highlights the need to give serious consideration to any molecules potentially originating from the Ct-OATP1B3 gene in further investigations aimed at obtaining a more precise understanding of the roles played by Ct-OATP1B3 in cancer cells. We believe that such functional studies on Ct-OATP1B3 will provide new insights into cancer biology, while simultaneously enhancing translational research into its role as a cancer biomarker candidate.

Additional files

Additional file 1: Figure S1. The Ct-OATP1B3 mRNA expression T/N ratio in each pair of the colon and lung tissues. The T/N ratio of Ct-OATP1B3 mRNA was calculated in individual colon cancer (A) and lung cancer (B) patients who showed its positive expression, where the Ct-OATP1B3 mRNA expression value of the normal tissue was set to the baseline. Ct-OATP1B3 mRNA levels in normal tissues were tentatively corrected as 10^3 copies/ng total RNA (identical to the QL value) if the mRNA level was the QL. The values obtained from each matched pair were connected by a line. Gray lines indicate the T/N ratios that were calculated using the corrected values, while the blue lines indicate the T/N ratios that were calculated using the original values.

Additional file 2: Figure S2. Comparison between the Ct- and Lt-OATP1B3 mRNA levels in each colon and lung cancer tissues. Fold differences between Ct- and Lt-OATP1B3 mRNA levels were calculated in individual colon cancer (A) and lung cancer (B) patients who showed positive Ct-OATP1B3 mRNA expression in cancer tissue, where the Lt-OATP1B3 mRNA level was set to the baseline. The Lt-OATP1B3 mRNA levels in normal tissues were tentatively corrected as 10^3 copies/ng total RNA (identical to the QL value) if the mRNA level was the QL. The values obtained from an individual cancer tissue were connected by a line. The gray lines indicate the fold differences that were calculated using the corrected values, while the blue lines indicate the fold differences that were calculated using the original values.

Additional file 3: Figure S3. Examination of Ct-OATP1B3 protein expression in the soluble membrane fractions of colon cancer cells. Western blotting was performed using the soluble membrane fraction of LS180 or HCT116 cells with rabbit anti-OATP1B3 serum that was developed by immunizing a rabbit with the synthesized epitope peptide (LEFLNNGEHFVPSAGTD). A control rabbit serum was also used for comparison. The soluble membrane fraction of human liver tissue (HL) was used as a positive control. Na^+/K^+ ATPase was used as a membrane marker.

Additional file 4: Figure S4. Examination of Ct-OATP1B3 protein expression in the whole cell lysates of colon cancer cells. Western blotting was performed using the whole cell lysates of LS180 or HCT116 cells with (A) rabbit polyclonal anti-OATP1B3 antibodies (Sigma) or (B) rabbit anti-OATP1B3 serum (developed in this study). The soluble membrane fraction of human liver tissue (HL) was used as a positive control. β-actin was used as a loading control.

Additional file 5: Figure S5. Transient expression and functional analysis of Ct-OATP1B3 in HCT116 cells. (A) Immunohistochemistry was performed to examine the protein expression of each OATP1B3 isoform using HCT116 cells transiently transfected with the Lt1B3/p3.1 (Lt-1B3/HCT116), Ct1B3-C/pBapo (Ct-1B3-C/HCT116), Ct1B3-v1/pBapo (Ct-1B3-v1/HCT116), or an empty vector (mock/HCT116). The representative results that were obtained from there independent experiments are shown. (B) The CCK-8 (1 μM) uptake by Lt-1B3/HCT116, Ct-1B3-C/HCT116, Ct-1B3-v1/HCT116, or mock/HCT116 was examined by transport analysis in the absence (black bar) or presence (white bar) of BSP (100 μM). The uptake level of CCK-8 in Lt-1B3/HCT116, Ct-1B3-C/HCT116 or Ct-1B3-v1/HCT116 cells was represented as the relative ratio to that observed in mock/HCT116 cells. The experiment was performed three times in duplicate.

Additional file 6: Figure S6. Stable expression and functional analysis of Ct-OATP1B3 in HEK293 cells. (A) Either Ct-OATP1B3 or Lt-OATP1B3 mRNA expression level in human pooled hepatocytes (Hep50), Lt-1B3/

HEK, Ct-1B3-C/HEK, Ct-1B3-v1/HEK and mock/HEK, was determined by qPCR using the primer set that could detect all mRNA isoforms, and the results were normalized using those of GAPDH. Each mRNA expression level is shown as mean ± S.D. of percentages relative to the level of pooled human hepatocytes (100%). Experiments were performed three times in duplicate. N.D. indicates that the value was too low to be calculated. (B) The protein expression of either Ct-OATP1B3 or Lt-OATP1B3 in Ct-1B3-C/HEK, Ct-1B3-v1/HEK, Lt-1B3/HEK, and mock/HEK, was examined by Western blotting using the anti-OATP1B3 antibodies. The soluble membrane fraction of human liver tissue (HL) was used as a positive control. Na^+/K^+ ATPase was used for a plasma membrane marker. (C) The CCK-8 (1 μM, left) or E_2G (0.5 μM, right) uptake by Lt-1B3/HEK, Ct-1B3-C/HEK, Ct-1B3-v1/HEK and mock/HEK was examined by transport analysis in the absence (black bar) or presence (white bar) of BSP (100 μM). The uptake level of each substrate in Lt-1B3/HEK, Ct-1B3-C/HEK or Ct-1B3-v1/HEK was represented as the relative ratio to that observed in mock/HEK cells. The experiment was performed three times in duplicate.

Additional file 7: Figure S7. Receiver operating characteristic (ROC) analysis for Ct-OATP1B3 mRNA in colon cancer patients. To assess the diagnostic potency of Ct-OATP1B3 mRNA in terms of its ability to discriminate cancer tissues from normal tissues, the ROC curve was generated on the basis of Ct-OATP1B3 mRNA levels in the matched-pairs of colon cancer and normal tissues (n = 39). During this analysis, the mRNA levels under the QL value were set to 10^3 copies/ng total RNA (identical to the QL value) and used in the calculation. The area under the ROC curve (AUC) along with its 95% confidence intervals (CI) was analyzed using Prism 6 (GraphPad Software, La Jolla, CA). The AUC is 0.930 (95% CI = 0.865-0.994; P <0.0001).

Additional file 8: Supplemental materials and methods.

Abbreviations

BSP: Bromosulfophthalein; CCK-8: Cholecystokinin-octapeptide sulfated; Ct- and Lt-OATP1B3: Cancer-type and liver-type organic anion transporting polypeptide 1B3, respectively; E_2G: Estradiol-17β-D-glucuronide; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; Hep50: Fifty donor-pooled human hepatocytes; QL: The quantification limit; qPCR: Quantitative real-time polymerase chain reaction; SLCO: Solute carrier organic anion transporter; T/N ratio: Tumor/normal expression ratio.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YS, TF, and KC participated in the design of the study. YS, SI, MN, MH, and AK performed the experiments. OS, TK, SM and IY provided the research materials and analyzed the results. YS, TF, KK and KC analyzed the results and wrote the manuscript. All authors read and approved the final manuscript.

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Trithorax complex component Menin controls differentiation and maintenance of T helper 17 cells

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Epigenetic modifications, such as posttranslational modifications of histones, play an important role in gene expression and regulation. These modifications are in part mediated by the Trithorax group (TrxG) complex and the Polycomb group (PcG) complex, which activate and repress transcription, respectively. We herein investigate the role of Menin, a component of the TrxG complex in T helper (Th) cell differentiation and show a critical role for Menin in differentiation and maintenance of Th17 cells. Menin$^{-/-}$ T cells do not efficiently differentiate into Th17 cells, leaving Th1 and Th2 cell differentiation intact in in vitro cultures. Menin deficiency resulted in the attenuation of Th17-induced airway inflammation. In differentiating Th17 cells, Menin directly bound to the *Il17a* gene locus and was required for the deposition of permissive histone modifications and recruitment of the RNA polymerase II transcriptional complex. Interestingly, although Menin bound to the *Rorc* locus, Menin was dispensable for the induction of *Rorc* expression and permissive histone modifications in differentiating Th17 cells. In contrast, Menin was required to maintain expression of *Rorc* in differentiated Th17 cells, indicating that Menin is essential to stabilize expression of the *Rorc* gene. Thus, Menin orchestrates Th17 cell differentiation and function by regulating both the induction and maintenance of target gene expression.

RNAPII | asthma | chromatin

Naive CD4 T cells adopt distinct cell fates including differentiation into T helper 1 (Th1), Th2, Th17, and regulatory T cells, and direct immune responses to facilitate the elimination of microorganisms (1, 2). Effector functions of these Th cells are defined by production of their signature cytokines and expression of lineage-specific transcription factors. Th1 cells express T-bet (encoded by the *Tbx21* gene) and produce IFN-γ (3), and Th2 cells express GATA-3 and secrete interleukin 4 (IL-4), IL-5, and IL-13 (4–6). Th17 cells were identified by their ability to produce IL-17A and express high amounts of the RAR-related orphan receptor-γ, named RORγt, that is essential for Th17 differentiation (7–10). Although Th17 cells contribute to host defense against fungi and extracellular bacteria, the pathogenicity of IL-17–producing T cells has been recognized not only in autoimmune diseases but also in allergic diseases (11–13).

Although lineage-specific transcription factors are key regulators of helper T-cell differentiation, epigenetic modifications, such as the methylation of DNA and posttranslational modifications of histones, also play crucial roles (14, 15). Trithorax group (TrxG) and Polycomb group (PcG) genes were originally discovered in *Drosophila melanogaster* as activators and repressors of Homeobox genes, respectively (16). It has been recognized that epigenetic modification and chromatin accessibility mediated by the PcG or TrxG complexes is a critical factor for the commitment of helper T-cell lineages (17, 18). Mixed-lineage leukemia (MLL), which is a mammalian homolog of the *Drosophila* trithorax, controls the maintenance of Th2 cytokine gene expression by memory Th2 cells (19). MLL forms a multicomponent complex that includes Menin, and mediates its epigenetic transcriptional effector functions via SET domain-dependent histone methyltransferase activity (20). MLL specifically methylates lysine 4 in the N-terminal tail on histone H3, a modification typically associated with transcriptionally active regions of chromatin (16). Menin protein is encoded by multiple endocrine neoplasia 1 (*Men1*), and mutation of this gene is the cause of multiple endocrine neoplasia type 1 in humans (21). Menin is a highly specific partner for MLL proteins and is an essential component required for DNA binding of the TrxG/MLL complex (22). The binding of the Menin/TrxG complex is required for the maintenance of *Gata3* expression and Th2 cytokine production in established Th2 cells (23), and the same mechanism was also recently found to function in human Th2 cells (24). However, it remains unclear whether the Menin/TrxG complex is involved in the differentiation and maintenance of other Th cell subsets. We herein show that Menin-deficient (Menin$^{-/-}$) T cells displayed reduced ability to differentiate into Th17 cells in vitro, and that development of Th17 cell-mediated airway inflammation was attenuated in mice transferred with Menin$^{-/-}$ Th17 cells. We found that Menin recruitment to the *Il17a* locus was crucial for histone modification, RNA polymerase II (RNAPII) accumulation, and the subsequent expression of *Il17a* mRNA. The binding of Menin to the *Rorc* gene locus was required for the long-term maintenance of *Rorc* expression. Thus, these data point to a mechanism by which Menin

Significance

Epigenetic modifications, including various histone modifications, play important roles in regulating gene expression. The Trithorax group (TrxG) complex induces permissive histone modifications to activate transcription. We herein investigate the role for Menin, a component of the TrxG complex, in T helper (Th) cell differentiation, and find a critical role for Menin in differentiation and maintenance of Th17 cells. Menin is required for Th17 cell differentiation in vitro through the direct regulation of *Il17a* expression. Menin controls IL-17–mediated pathology in vivo. Menin is also required to maintain expression of *Rorc*, the gene encoding RORγt, a key transcription factor for Th17 cell function. Thus, Menin orchestrates Th17 cell differentiation and function by regulating both induction and maintenance of target gene expression.

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regulates both the induction of Th17 differentiation and maintenance of Th17 cell function after differentiation.

Results

Menin Is Required for Th17 Cell Differentiation. Menin is an essential component of the MLL/TrxG complex that is required for DNA binding (25). In the context of Th2 cells, we have reported that Menin is crucial for the maintenance of *Gata3* expression and the function of Th2 cells after differentiation (23). However, it remains unclear whether the Menin/TrxG complex is involved in the differentiation or maintenance of function of Th17 cells. To address this question, we assessed the ability of Menin$^{-/-}$ naive CD4 T cells to differentiate into Th1, Th2, and Th17 cells. In in vitro Th1/Th2 cultures, Th1 and Th2 cell differentiation of Menin$^{-/-}$ T cells were not impaired as evidenced by IFN-γ and IL-4 production, respectively (Fig. S1 *A* and *B*) (23). In contrast, a dramatic reduction in the number of IL-17A–producing cells was observed in Menin$^{-/-}$ Th17 cell cultures (Fig. 1*A*, *Left* and *Center*). Likewise, a substantial decrease in *Il17a* mRNA expression was found in Menin$^{-/-}$ Th17 cells (Fig. 1*A*, *Right*). Moreover, at all concentrations of IL-6 tested, Menin$^{-/-}$ Th17 cells showed less IL-17A–positive cells compared with WT controls (Fig. S1*C*). As IL-1β, especially in synergy with IL-23, plays an essential role in the induction or expansion of IL-17A producers both in murine and human systems (26, 27), we examined whether IL-17A production by Menin$^{-/-}$ T cells was normalized by IL-1β and IL-23 under Th17 culture conditions. As shown in Fig. 1*B*, Menin$^{-/-}$ CD4 T cells showed decreased numbers of IL-17A–producing cells and reduced expression of *Il17a* even in the presence of IL-1β and IL-23. Menin$^{-/-}$ CD4 T cells showed a tendency for increased IFN-γ–producing cells in the culture, although anti–IFN-γ neutralizing antibody was added in this condition (Fig. 1*B*). The strength of T-cell receptor (TCR) signaling is also known to regulate IL-17 production (28, 29). We therefore investigated whether alteration of TCR stimulation could affect the reduced generation of IL-17–producing cells in Menin$^{-/-}$ T-cell cultures. OVA-specific DO11.10 TCR transgenic (Tg) CD4 T cells from WT or Menin-deficient mice were stimulated with various concentrations of antigenic OVA peptide together with antigen-presenting cells (APCs). Fig. 1*C* shows that, in Menin$^{-/-}$ CD4 T-cell cultures, the generation of IL-17A–producing cells was markedly reduced together with a slight increase in IFN-γ–producing cells at all concentrations of OVA peptide tested. Menin$^{-/-}$ CD4 T cells showed decreased generation of IL-17A–producing cells even at the early time points of the culture (day 2 and day 3; Fig. S1 *D* and *E*). Slightly accelerated cell division was detected in Menin$^{-/-}$ CD4 T cells compared with WT CD4 T cells (Fig. S1*F*). Knockdown experiments using *Men1* siRNA in peripheral CD4 T cells confirmed that Menin is required for the differentiation of Th17 cells (Fig. 1*D*). Together, these results indicate that Menin is required for efficient differentiation of Th17 cells.

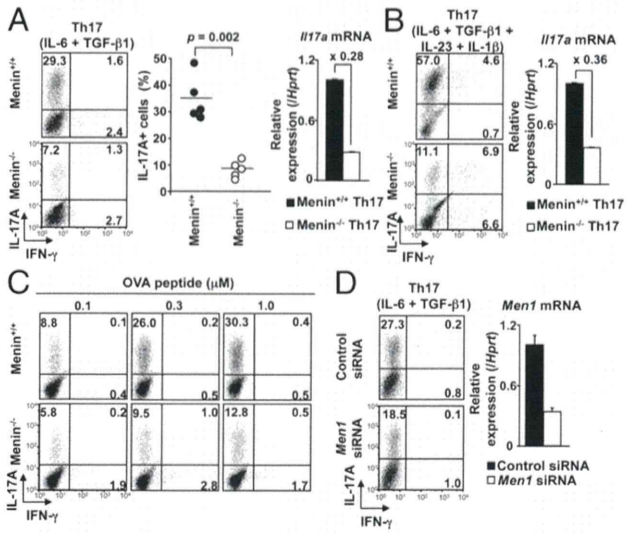

Fig. 1. Menin is required for Th17 cell differentiation. (*A*) Naive CD4 T cells from WT or Menin-deficient (Menin$^{-/-}$) mice were cultured under Th17 conditions for 5 d. The cultured cells were restimulated with phorbol 12-myristate 13-acetate plus ionomycin for 4 h, and IL-17A protein expression was analyzed by intracellular staining (*Left*). IL-17A protein expression data from five independent experiments are shown with mean values (*Center*). Expression *Il17a* mRNA was determined by quantitative RT-PCR (*Right*). The levels of transcripts normalized to *Hprt* signal in Menin$^{-/-}$ cells were depicted as the fold changes compared with those in WT cells. Mean values with SDs ($n = 3$) are shown. (*B*) Naive CD4 T cells were cultured under Th17 conditions in the presence of IL-23 (10 ng/mL) and IL-1β (10 ng/mL) for 5 d. The cultured cells were harvested and tested for intracellular staining (*Left*) and quantitative RT-PCR analysis (*Right*) as described in *A*. (*C*) Naive CD4 T cells from WT or Menin-deficient DO11.10 OVA-specific TCR Tg mice were cultured with splenic APCs under Th17 conditions in the presence of the indicated concentrations of OVA peptides (0.1–1.0 μM) for 6 d. IL-17A– and IFN-γ–secreting cells were assessed by intracellular staining. Three independent experiments were performed with similar results (*B* and *C*). (*D*) Control and *Men1* siRNA were transfected into naive CD4 T cells from WT mice. These naive CD4 T cells were cultured under Th17 condition for 1 or 2 d before analysis. The IL-17A–producing cells (*Left*) and mRNA expression level of *Men1* (*Right*) were assessed by intracellular staining on day 2 or quantitative RT-PCR on day 1, respectively. Two independent experiments were performed with similar results.

OVA-Induced Neutrophilic Airway Inflammation Is Attenuated in Mice Transferred with Menin$^{-/-}$ Th17 Cells and in Menin-Deficient Mice. Based on our in vitro results, we reasoned that Menin could be an important factor regulating IL-17–dependent pathology in vivo. Some patients with severe asthma appear to have IL-17A–mediated airway inflammation with increased airway neutrophils, mucous cell metaplasia in airway epithelial cells, and increased airway hyperreactivity (30). Therefore, we next used a model of airway inflammation in which Th17 cells are key mediators of neutrophilic inflammation and pathology (31). We adoptively transferred Th17 cells from DO11.10 TCR Tg WT or Menin-deficient mice into syngeneic BALB/c recipient mice. First, we accessed the accumulation of transferred CD4 T cells before and after OVA challenge (Fig. S2*A*). Comparable numbers of Menin$^{-/-}$ T cells were engrafted in the lung, and 1 d after the last OVA challenge, a substantial increase was detected in the numbers of WT CD4 T cells but not Menin$^{-/-}$ CD4 T cells (Fig. S2*B*). There was little difference in the expression level of homing receptors between WT and Menin$^{-/-}$ Th17 cells (Fig. S2*C*). We assessed airway inflammation in BALB/c recipient mice, which were adoptively transferred with Th17 cells from DO11.10 TCR Tg WT or Menin-deficient mice followed by OVA inhalation (Fig. S2 *D* and *E*). The levels of IL-17A in bronchoalveolar lavage (BAL) fluid samples from mice that received Menin$^{-/-}$ Th17 cells were dramatically reduced ($P < 0.01$) in comparison with BAL fluid samples from mice that received WT Th17 cells (Fig. 2*A*). The total number of infiltrating leukocytes in the BAL fluid was significantly decreased ($P < 0.01$) in the group transferred with Menin$^{-/-}$ Th17 cells (Fig. 2*B*). Moreover, we detected a large increase in the number of neutrophils in the BAL fluid from mice transferred with WT Th17 cells that was absent from the mice transferred with Menin$^{-/-}$ Th17 cells (Fig. 2*B*). The mRNA expression levels of *Muc5ac*, *Muc5b*, and *Gob5*, molecular markers for goblet cell hyperplasia and mucus production, were decreased in the lungs of mice receiving Menin$^{-/-}$ Th17 cells (Fig. 2*C*). Consistent with these findings, deletion of Menin in Th17 cells resulted in diminished infiltration of mononuclear cells around the peribronchiolar and perivascular regions of the

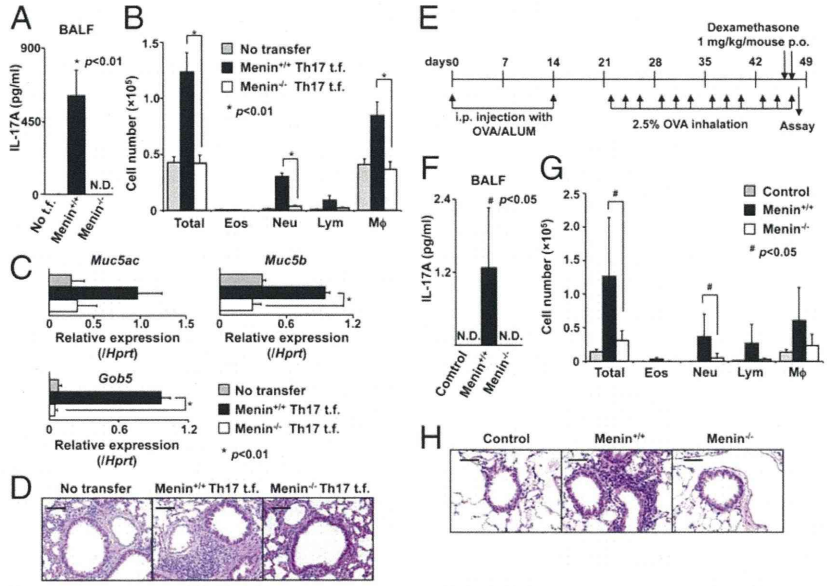

Fig. 2. Neutrophilic airway inflammation is attenuated by deficiency of Menin. (*A*) Airway inflammation was induced as described in *Materials and Methods* and Fig. S2*D*. The concentration of IL-17A in the BAL fluid was measured by ELISA. Mean values with SDs ($n = 3$) are shown (*$P < 0.01$). N.D., under the detection levels; t.f., transfer. (*B*) The cell number of eosinophils (Eos), neutrophils (Neu), lymphocytes (Lym), and macrophages (Mϕ) in the BAL fluid are shown. Mean values with SDs ($n = 3$) are shown (*$P < 0.01$). (*C*) The data represent the mean values of the indicated gene expression in the lungs of mice that received WT or Menin$^{-/-}$ Th17 cells (*$P < 0.01$). (*D*) The level of OVA-induced airway inflammation in recipient mice was examined by histological analysis (H&E staining). (Scale bars: 50 μm.) Data are representative of at least three independent experiments (*A–D*). (*E*) A schematic overview of the study protocol for the induction of steroid-resistant neutrophilic inflammation. p.o., per oral. (*F*) The concentration of IL-17A in the BAL fluid was measured by cytometric bead array (CBA). Mean values with SDs ($n = 6$) are shown (#$P < 0.05$). (*G*) The cell number of eosinophils (Eos), neutrophils (Neu), lymphocytes (Lym), and macrophages (Mϕ) in the BAL fluid are shown. Mean values with SDs ($n = 3$ for control group, $n = 6$ for WT, and $n = 4$ for Menin$^{-/-}$ group) are shown (#$P < 0.05$). (*H*) Antigen-induced leukocyte infiltration into the lungs was evaluated by H&E staining. (Scale bars: 50 μm.)

lungs (Fig. 2*D*). Next, to determine whether the impaired ability of transferred Menin$^{-/-}$ Th17 cells to induce airway inflammation is due to impaired expansion of Th17 cells or decreased IL-17A production in Th17 cells, we examined how many WT Th17 cells needed to be transferred to induce inflammation at the same level as transfer of 1×10^6 Menin$^{-/-}$ Th17 cells. We found that the number of neutrophils in the BAL fluid from mice transferred with 0.2×10^6 WT Th17 cells was comparable to that from the mice transferred with 1×10^6 Menin$^{-/-}$ Th17 cells (Fig. S2*F*). In the lungs of these recipient mice, the number of WT CD4 T cells was significantly lower ($P < 0.05$) than that of Menin$^{-/-}$ CD4 T cells (Fig. S2*G*). These results indicate that WT Th17 cells in the lung could induce inflammation with smaller number of cells than Menin$^{-/-}$ Th17 cells. Thus, we concluded that the impaired ability of transferred Menin$^{-/-}$ Th17 cells to induce airway inflammation was most likely due to decreased IL-17A production in Th17 cells rather than impaired expansion of Th17 cells in lung. Next, we examined neutrophilic airway inflammation directly in CD4-Cre^{+}Menin$^{fl/fl}$ mice by using a previously reported steroid-resistant neutrophilic airway inflammation model (Fig. 2*E*) (32). The levels of IL-17A in BAL fluid samples from CD4-Cre^{+}Menin$^{fl/fl}$ mice were dramatically reduced ($P < 0.05$) in comparison with BAL fluid samples from WT mice (Fig. 2*F*). In CD4-Cre^{+}Menin$^{fl/fl}$ mice, the total number of infiltrating leukocytes in the BAL fluid was significantly decreased ($P < 0.05$) compared with that in WT mice (Fig. 2*G*). Moreover, CD4-Cre^{+}Menin$^{fl/fl}$ mice showed a significant decrease in neutrophils in the BAL fluid compared with WT mice. Histological analysis also revealed that infiltration of mononuclear cells around the peribronchiolar and perivascular regions of the lungs was dependent on the ability of CD4 T cells to express Menin (Fig. 2*H*). Thus, Menin is required for the induction of Th17 cell-mediated neutrophilic airway inflammation in vivo.

Menin Does Not Control the Expression of Other Key Transcription Factors That Can Regulate Th17 Cell Differentiation. To further investigate the nature of the defect in IL-17A production in Menin$^{-/-}$ T cells, we next focused on the transcription factors involved in Th17 cell differentiation. Th17 cell differentiation is associated with the expression of several transcription factors (33). Despite the decrease of *Il17a* in Menin$^{-/-}$ Th17 cells, mRNA expression levels of all of these transcription factors including *Rorc* appeared to be normal in Menin$^{-/-}$ Th17 cells (Fig. 3*A* and Fig. S3*A*). The protein level of RORγt was also comparable (Fig. S3*B*, *Top*). IL-6–mediated phosphorylation of STAT3 in Menin$^{-/-}$ Th17 cells was equivalent to those in WT Th17 cells (Fig. S3*B*, *Middle*). Moreover IL-6–mediated phosphorylation of STAT3 in freshly isolated Menin$^{-/-}$ CD4 T cells was not altered (Fig. S3*C*). These results indicate that the expression of key transcription factors for Th17 cell differentiation, including the protein encoded by *Rorc*, was not affected by Menin deficiency.

Menin Is Required for the Formation of Permissive Histone Modifications at the *Il17a* Gene Locus. To further understand the possible mechanism whereby Menin functions to regulate *Il17a* expression, we next assessed the binding of Menin and the histone modification states around the *Il17a* and *Rorc* gene loci together with the *Actb* and *Ccl2* loci as heritably active and silent genes in Menin$^{-/-}$ Th17 cells by chromatin immunoprecipitation (ChIP) assays (Fig. S4 *A* and *B*). Five-day culture of WT cells under Th17-inducing conditions resulted in enhanced Menin binding at both the *Il17a* and *Rorc* gene loci compared with control Th2-inducing conditions (Fig. 3*B*). The accumulation of Menin at the *Il17a* gene locus was detected even after 48 h of stimulation (Fig. S4*C*). Levels of histone H3 trimethylated at Lys4 (H3-K4Me3) and histone H3 acetylated at Lys9 (H3-K9Ac), which frequently correlate with transcriptional activation, were decreased at the *Il17a* gene locus in Menin$^{-/-}$ Th17 cells compared with WT cells 5 d after TCR stimulation (Fig. 3*C*, *Left*). In addition,