(rs6478108 and rs426389), *NKX2-3* (rs11190140 and rs10883365), and *NOD2/CARD15* (rs17221417, rs2066843, and rs2076756) were combined because these SNPs were in high linkage disequilibrium with each other ($r^2 \geq 0.95$) in the HapMap CEU samples. Finally, we performed a meta-analysis for 45 SNPs located at 33 loci by using a total of 4852 to 31,125 subjects. For an easy understanding of the risk direction, we calculated the OR and 95% CI of each SNP according to the risk allele in the GWAS for CD. A *P*-value less than 0.0015 (0.05/33) was considered statistically significant after applying Bonferroni correction.

RESULTS

We found evidence of heterogeneity among the studies for 19 SNPs: rs2476601 (*PTPN22*), rs2274910 (*ITLN1*), rs2241880-rs10210302-rs3828309 (*ATG16L1*), rs4613763-rs17234657 (5p13), rs2188962 (*LOC441108*), rs10077785 (*LOC441108*), rs4958847 (*IRGM*), rs6908425 (*CDKAL1*), rs1456893 (7p12), rs1551398 (8q24), rs6478108-rs4263839 (*TNFSF15*), rs17582416 (*CCNY-CREM*), rs10995271 (*ZNF365*), rs10761659 (*ZNF365*), rs7927894 (*C11orf30*), rs2872507 (*ORMDL3*), rs2542151 (*PTPN2*), rs1736135 (21q21), and rs762421 (*ICOSLG*). Therefore, the pooled ORs and 95% CIs were calculated using a random-effect model in these variants. We found a significant publication bias at rs9292777 on 5p13 locus (Egger's $P = 0.02$) and excluded this SNP from the analysis.

Among the 45 SNPs included in the meta-analysis, 35 SNPs located at 30 loci were investigated by more than five studies. Among the 33 loci examined in this study, we found significant associations with UC in 14 loci and nominal associations ($P < 0.05$) in 11 loci. We confirmed the associations of 17 susceptibility loci which are commonly associated with both CD and UC in the previous study[20]: *IL23R, KIF21B, BSN-MST1*, 5p13, *LOC441108, IRGM, IL12B, CCR6*, 7p12, *JAK2, TNFSF15, CCNY-CREM, NKX2-3, ORMDL3, STAT3*, 21q21, and *ICOSLG* (Supporting Information Table 2). Moreover, we found associations with UC in eight additional loci (Table 2): *GCKR* (rs780094, $P = 2.47 \times 10^{-2}$, OR 1.05), *ATG16L1* (rs2241880-rs10210302-rs3828309, $P = 4.70 \times 10^{-2}$, OR 1.05), *CDKAL1* (rs6908425, $P = 7.68 \times 10^{-3}$, OR 1.10), *ZNF365* (rs10761659, $P = 4.67 \times 10^{-4}$, OR 1.14), *LRRK2-MUC19* (rs11175593, $P = 1.54 \times 10^{-2}$, OR 1.21), *C13orf31* (rs3764147, $P = 1.80 \times 10^{-2}$, OR 1.07), *PTPN2* (rs2542151, $P = 2.49 \times 10^{-2}$, OR 1.08), and *SBNO2* (rs4807569, $P = 1.72 \times 10^{-2}$, OR 1.06). For all loci showing association, the directions of risk alleles for UC were all the same as those for CD. The OR of *IL23R* locus was relatively high (rs11209026, OR 1.62, 95% CI: 1.48–1.77), whereas ORs of other loci were modest ranged from 1.05–1.22.

DISCUSSION

We comprehensively reviewed the published studies that examined the CD susceptibility loci in UC patients and performed a meta-analysis to clarify the common genetic factors for both diseases. We found associations at 25 out of 33 candidate loci. Among them, we confirmed the associations in 17 loci reported in the previous GWAS,[20] and this study found an additional eight common susceptibility loci for CD and UC, namely, *GCKR, ATG16L1, CDKAL1, ZNF365, LRRK2-MUC19, C13orf31, PTPN2*, and *SBNO2*. Among these additionally identified loci, *GCKR* and *LRRK2-MUC19* have never shown nominal association with UC in any single studies performed to date. Although the genetic risk of each locus was modest, many genes or loci will contribute to the pathogenesis of both CD and UC.

Previous GWAS identified that the autophagy-related genes are associated with the susceptibility of CD.[6,7,9,10,13] In contrast to the strong association with CD, previous association studies for UC showed inconsistent results in these autophagy-related genes.[16,21–24,29–31,56] Our meta-analysis demonstrated nominal association with *ATG16L1* by using 11,466 cases and 19,659 controls ($P = 4.7 \times 10^{-2}$, OR 1.05, 95% CI: 1.00–1.10). Other autophagy-related genes also showed associations with UC in this study ($P = 1.54 \times 10^{-2}$, OR 1.21, 95% CI: 1.03–1.41 for *LRRK2-MUC19*; $P = 1.18 \times 10^{-3}$, OR 1.14, 95% CI: 1.05–1.24 for *IRGM*). These findings suggest a possibility that autophagy might contribute to the development of both UC and CD, but its effect may be weaker for UC.

There is another possibility that the association of autophagy-related genes are caused by the contamination of colonic CD cases because rs2241880-rs10210302-rs3828309 (*ATG16L1*) and rs4958847 (*IRGM*) showed heterogeneity among the studies. However, we could not find any consistent set of studies that contributed to this heterogeneity. Moreover, when we assume the possibility of this misclassification for *ATG16L1*, colonic CD cases should be included in more than 20% of UC cases based on the assumption of a case–control study of 11,466 cases and 19,659 controls, an allele test model, a risk allele frequency of 0.571 based on the HapMap-CEU population, an allelic OR of colonic CD for 1.25,[13] a statistical power of 0.80, and a *P*-value of 0.05. Since the diagnosis of UC was made by the established guidelines in each study, we think the association of autophagy-related genes in this study might not be caused by the misclassification of colonic CD cases in the previous studies.

Recent genetic studies have revealed shared genetic components of different immune-related diseases.[66] For the shared susceptibility genes between CD and UC, previous studies have shown the importance of the common pathogenesis of the IL-23/Th17 signaling pathway, which promotes inflammation in the adaptive immune response.[14]

TABLE 2. Results of Meta-analysis for Eight Additionally Identified Common Susceptibility Loci for CD and UC

			Number		RAF			Combined		Heterogeneity		Publication Bias
	Allele* [1/2]	Study	Case	Control	Case	Control	OR (95%CI)	*P*	OR (95% CI)	*P*	I^2 Statistics	*P*
GCKR												
rs780094	T/C	Anderson (2009)	2464	4002	0.40	0.38	1.07(0.99-1.16)	2.47E-02	1.05(1.00-1.09)	0.48	0	0.40
		Franke (2010)	1043	1703	0.42	0.40	1.10(0.99-1.23)					
		McGovern (2010) GWAS#1	723	2880	—	—	1.03(0.88-1.20)					
		McGovern (2010) GWAS#2	948	1408	—	—	1.00(0.94-1.07)					
		McGovern (2010) GWAS#3	1022	2503	—	—	1.08(0.96-1.21)					
		Total	6200	12496								
ATG16L1												
rs2241880	G/A	Büning_1 (2007)	296	707	0.52	0.51	1.10(0.89-1.35)	4.70E-02	1.05(1.00-1.10)	0.11	0.31	0.43
rs10210302	T/C	Roberts (2007)	466	591	0.51	0.50	1.05(0.87-1.25)					
rs3828309	G/A	Glas (2008)	507	1615	0.55	0.52	1.15(0.98-1.36)					
		Lappalainen (2008)	459	190	0.46	0.47	0.96(0.75-1.23)					
		Franke (2008)	1077	1793	0.55	0.53	1.19(1.01-1.41)					
		Fisher (2008)	1739	1491	0.54	0.52	1.08(0.97-1.20)					
		Lakatos (2008)	149	149	0.54	0.50	1.26(0.91-1.74)					
		Okazaki (2008)	117	310	0.50	0.48	1.02(0.61-1.68)					
		Fowler (2008)	543	1244	0.48	0.51	0.87(0.75-1.01)					
		Newman (2009)	402	1005	—	—	1.19(1.00-1.41)					
		Weersma_1 (2009)	1120	1350	0.55	0.56	0.95(0.84-1.08)					
		Palomino—Morales (2009)	414	666	0.54	0.51	1.10(0.92-1.32)					
		Márquez_1 (2009)	368	745	0.51	0.53	0.93(0.78-1.12)					
		Sventoraityte (2010)	123	186	0.53	0.48	1.26(0.91-1.75)					
		McGovern (2010) GWAS#1	723	2880	—	—	1.08(0.95-1.22)					
		McGovern (2010) GWAS#2	948	1408	—	—	0.91(0.79-1.05)					
		McGovern (2010) GWAS#3	1022	2503	—	—	1.08(0.95-1.22)					
		McGovern (2010) Rep#1	993	826	—	—	1.08(0.94-1.23)					
		Total	11466	19659								
CDKAL1												
rs6908425	C/T	Franke (2008)	1102	1794	0.81	0.79	1.18(1.01-1.39)	7.68E-03	1.10(1.02-1.18)	0.13	0.39	0.33
		Anderson (2009)	2453	4034	0.80	0.77	1.18(1.08-1.29)					
		Weersma_2 (2009)	1442	1045	0.81	0.78	1.18(0.99-1.41)					
		McGovern (2010) GWAS#1	723	2880	—	—	1.05(0.90-1.22)					
		McGovern (2010) GWAS#2	948	1408	—	—	1.03(0.91-1.16)					
		McGovern (2010) GWAS#3	1022	2503	—	—	1.11(0.95-1.30)					
		McGovern (2010) Rep#1	993	826	—	—	0.91(0.76-1.09)					
		Total	8683	14490								
ZNF365												
rs10995271	C/G	Törkvist (2010)	935	1460	—	—	1.03(0.89-1.18)	1.37E-01	1.07(0.97-1.17)	0.02	0.68	0.26
		Franke (2010)	1043	1703	0.44	0.40	1.19(1.07-1.33)					

(*Continued*)

TABLE 2. (*Continued*)

	Allele* [1/2]	Study	Number		RAF		OR (95%CI)	Combined		Heterogeneity		Publication Bias
			Case	Control	Case	Control		*P*	OR (95% CI)	*P*	I^2 Statistics	*P*
		McGovern (2010) Rep#1	993	826	—	—	1.09(0.95-1.24)					
		McGovern (2010) Rep#2	1016	754	—	—	1.00(0.96-1.05)					
		Total	3987	4743								
rs10761659	G/A	Franke (2008)	1088	1775	0.58	0.54	1.10(1.02-1.19)	4.67E-04	1.14(1.05-1.23)	0.24	0.28	NA
		Fisher (2008)	1807	1549	0.57	0.54	1.19(1.07-1.31)					
		Total	2895	3324								
LRRK2—MUC19												
rs11175593	T/C	Anderson (2009)	3026	1132	0.02	0.01	1.31(0.99-1.74)	1.54E-02	1.21(1.03-1.41)	0.70	0	0.08
		Törkvist (2010)	935	1460	—	—	1.11(0.68-1.80)					
		Franke (2010)	1043	1703	0.02	0.02	1.18(0.83-1.70)					
		McGovern (2010) Rep#1	993	826	—	—	1.31(0.97-1.76)					
		McGovern (2010) Rep#2	1016	754	—	—	0.94(0.62-1.43)					
		Total	7013	5875								
C13orf31												
rs3764147	G/A	Anderson (2009)	2424	4017	0.22	0.21	1.07(0.98-1.18)	1.80E-02	1.07(1.01-1.13)	0.39	0.03	0.78
		Törkvist (2010)	935	1460	—	—	1.22(1.04-1.42)					
		Franke (2010)	1043	1703	0.25	0.25	1.02(0.89-1.15)					
		McGovern (2010) Rep#1	993	826	—	—	1.04(0.90-1.20)					
		McGovern (2010) Rep#2	1016	754	—	—	1.02(0.89-1.16)					
		Total	6411	8760								
PTPN2												
rs2542151	G/T	Franke (2008)	1005	1779	0.19	0.15	1.33(1.11-1.59)	2.49E-02	1.08(1.00-1.16)	0.14	0.37	0.06
		Fisher (2008)	1735	1488	0.17	0.17	1.07(0.93-1.22)					
		McGovern (2010) GWAS#1[a]	723	2880	—	—	1.14(0.95-1.36)					
		McGovern (2010) GWAS#2[a]	948	1408	—	—	1.04(0.90-1.20)					
		McGovern (2010) GWAS#3[a]	1022	2503	—	—	1.03(0.85-1.24)					
		McGovern (2010) Rep#1	993	826	—	—	1.00(0.92-1.09)					
		McGovern (2010) Rep#2	1016	754	—	—	1.13(0.94-1.35)					
		Total	7442	11638								
SBNO2												
rs4807569	C/A	Anderson (2009)	2425	4047	0.22	0.20	1.10(1.00-1.20)	1.72E-02	1.06(1.01-1.12)	0.57	0	0.85
		Franke (2010)	1043	1703	0.25	0.24	1.03(0.90-1.17)					
		McGovern (2010) GWAS#1[b]	723	2880	—	—	1.00(0.85-1.18)					
		McGovern (2010) GWAS#2[b]	948	1408	—	—	1.03(0.93-1.13)					
		McGovern (2010) GWAS#3[b]	1022	2503	—	—	1.15(0.99-1.33)					
		Total	6161	12541								

*Allele "1" denotes the reported risk allele.
†OR and 95% CI were calculated using the random-effect model because of the heterogeneity among the studies.
[a]rs1893217 is absolutely linked with rs2542151 ($r^2 = 1.0$).
[b]rs2024092 is absolutely linked with rs4807569 ($r^2 = 1.0$).
RAF, risk allele frequency; OR, odds ratio; CI, confidence interval; NA not applicable.

Many genetic variants including in this pathway such as *IL23R, IL12B, JAK2*, and *STAT3* are associated with susceptibility for both diseases. Among the eight additionally identified common susceptibility loci for CD and UC, several genes are reported to be associated with various diseases or traits: *C13orf31* is associated with leprosy.[67] *PTPN2* is associated with type 1 diabetes[68,69] and celiac disease.[70] *CDKAL1* is a susceptibility gene for type 2 diabetes.[71–73] *GCKR* is implicated in metabolic traits such as triglyceride,[74–76] fasting glucose,[77] and serum uric acid.[78] However, there is little information how these genes affect the development of CD and UC. Functional analysis of these genes will provide further understanding of the common pathogenesis of CD and UC.

When we compared our results with those of a recent meta-analysis for UC,[20] we could not find a significant association in the 6q21 locus. In the present study we performed a meta-analysis using the data of rs7746082 that showed the strongest association with CD at the 6q21 locus.[13] However, the GWAS meta-analysis estimated the association using the data of rs6938089, best proxy SNP for rs7746082.[20] Although the r^2 value between rs7746082 and rs6938089 is 0.60 for the HapMap CEU population (release #27, build 36), there is a possibility that the hidden causative variant at the 6q21 locus might be different between CD and UC. Further detailed analysis is necessary to clarify the effect of the 6q21 locus on susceptibility to CD and UC.

Significant publication bias was observed at rs9292777 on 5p13 locus. The funnel plot showed that the largest study[21] had the largest OR, whereas the OR of the smaller studies were all shifted to the smaller ones. Based on this asymmetrical distribution of OR, we excluded this SNP in this study.

In conclusion, in addition to the reported common susceptibility loci, we identified eight common susceptibility loci for CD and UC by a meta-analysis of published studies using more than 30,000 subjects. Our data indicate that UC and CD share many genetic factors with small effect. These findings will help to clarify the common pathway involved in the development of both diseases.

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Meta-analysis of genome-wide association studies identifies eight new loci for type 2 diabetes in east Asians

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We conducted a three-stage genetic study to identify susceptibility loci for type 2 diabetes (T2D) in east Asian populations. We followed our stage 1 meta-analysis of eight T2D genome-wide association studies (6,952 cases with T2D and 11,865 controls) with a stage 2 *in silico* replication analysis (5,843 cases and 4,574 controls) and a stage 3 *de novo* replication analysis (12,284 cases and 13,172 controls). The combined analysis identified eight new T2D loci reaching genome-wide significance, which mapped in or near *GLIS3*, *PEPD*, *FITM2-R3HDML-HNF4A*, *KCNK16*, *MAEA*, *GCC1-PAX4*, *PSMD6* and *ZFAND3*. *GLIS3*, which is involved in pancreatic beta cell development and insulin gene expression[1,2], is known for its association with fasting glucose levels[3,4]. The evidence of an association with T2D for *PEPD*[5] and *HNF4A*[6,7] has been shown in previous studies. *KCNK16* may regulate glucose-dependent insulin secretion in the pancreas. These findings, derived from an east Asian population, provide new perspectives on the etiology of T2D.

T2D is a major public health problem with a rapidly rising global prevalence[8]. The development of T2D is influenced by diverse factors, and decades of epidemiological studies have linked obesity, hypertension and dyslipidemia with the risk of T2D[9]. It is also known that T2D has considerable heritability. Within only the last 3 years, genetic studies have produced a rapidly lengthening list of loci harboring disease-predisposing variations[10]. To date, genetic variants at 45 loci have been identified for T2D[10,11]. Despite these advances toward a better understanding of the genetic basis of T2D, its heritability has not been fully explained[12]. In addition, most of the T2D loci were detected initially in population samples of European origin, with the exceptions of *KCNQ1*, *UBE2E2* and *C2CD4A-C2CD4B*, which were first identified in studies of east Asian populations[13–15]. Additional efforts involving east Asian populations identified variants associated with T2D at the *SPRY2*, *PTPRD* and *SRR* loci[5,16,17]. However, these associations need more validation from additional studies of east Asians as well as in studies of other populations. A large meta-analysis in east Asians would be expected to identify new genetic associations and provide insights into T2D pathogenesis. In addition to differences in the allele frequencies between east Asians and Europeans, which may affect the power to detect associations in these populations, T2D epidemiology also differs considerably between European populations and east Asian populations. In east Asians, the rates of diabetes are often higher at lower average body mass indices (BMIs)[18], suggesting that some different pathways may be involved in pathogenesis of T2D in east Asians and Europeans.

To discover new T2D loci, we conducted a three-stage association study in individuals of east Asian descent (**Supplementary Fig. 1**). We performed the stage 1 meta-analysis by combining eight T2D genome-wide association studies (GWAS) participating in the Asian Genetic Epidemiology Network (AGEN) consortium (6,952 cases and 11,865 controls) with association data for 2,626,356 imputed and genotyped autosomal SNPs, and we used the inverse-variance method

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for fixed effects for the statistical analyses (**Supplementary Table 1**). All imputed and genotyped SNPs (minor allele frequency (MAF) > 0.01) passed quality control filters in each of the eight stage 1 datasets prior to conducting the meta-analysis (**Supplementary Table 2**). The genomic control inflation factor (λ) for the meta-analysis was 1.046 (and was less than 1.062 for each of the individual studies), indicating that the results seen in stage 1 were probably not the result of population stratification (**Supplementary Fig. 2**). Individuals from each component study that participated in stage 1 mainly clustered together with the samples from the CHB/JPT HapMap population in the principal component analysis plot (**Supplementary Fig. 3**), further showing the similarity in ethnicity between the stage 1 samples. Most signals showing strong evidence for T2D associations were in known T2D genes (**Fig. 1**). Stage 1 *P* values, odds ratios (ORs) and average risk allele frequencies for 45 previously reported T2D-associated SNPs are listed in **Supplementary Table 3**.

After removing known T2D variants, we selected 297 SNPs from independent loci from the stage 1 meta-analysis based on our arbitrary inclusion criteria for *in silico* follow-up replication: meta-analysis $P < 5 \times 10^{-4}$ (based on the divergence between the observed and expected *P* values on the quantile-quantile plot; **Supplementary Fig. 2**), heterogeneity $P > 0.01$ and at least seven studies having been included in the meta-analysis (**Supplementary Table 4**). We took a total of 3,756 SNPs, including the 297 selected SNPs and their proxies ($r^2 > 0.8$ based on phase 2 CHB/JPT HapMap data), forward to stage 2 (*in silico* replication) in three independent GWAS (5,843 cases and 4,574 controls). After a meta-analysis that combined stage 1 and 2 data for 3,756 SNPs, we selected the 19 SNPs that showed the most compelling evidence for association (stage 1 and 2 combined $P < 10^{-5}$) (**Supplementary Table 5**) for stage 3 *de novo* genotyping in up to 12,284 cases and 13,172 controls recruited from five independent studies (**Supplementary Tables 1** and **2**). This resulted in eight new T2D loci that reached genome-wide significance in the combined meta-analysis across all three stages (**Table 1** and **Fig. 2**).

Three of these T2D-associated loci were previously associated with metabolic traits or related diseases or were suggestively associated with T2D. We detected one such locus within an intron of *GLIS3*, a gene that is highly expressed in islet beta cells. The coding product of this gene, a Krüppel-like zinc finger transcription factor, has been proposed as a key player in the regulation of pancreatic beta cell development and insulin gene expression[1,2]. SNPs in high linkage disequilibrium (LD) with this locus have been implicated in association with type 1 diabetes (T1D)[19] and fasting plasma glucose[3]. The second such locus, on 19q13, is located in an intron of *PEPD*. Several SNPs (lead SNP: rs10425678) in this gene were previously associated with T2D in a Japanese population[5]. However, the SNP in *PEPD* identified in our study (rs3786897) is not in LD with those identified in the Japanese population ($r^2 = 0.008$, $D' = 0.143$ between rs3786897 and rs10425678 based on phase 2 CHB/JPT HapMap data), and our GWAS data do not support an association for T2D with rs10425678 ($P = 0.528$). The third such signal is near *FITM2-R3HDML-HNF4A*. *FITM2* may be involved in lipid droplet accumulation[20], and the function of *R3HDML* is not known. Mutations in *HNF4A* cause maturity onset diabetes of the young type 1 (ref. 21). Common variants in the P2 promoter region of this gene (rs1884613 and rs2144908) have been associated with T2D in a population-specific manner[6,22]. The SNP near *FITM2-R3HDML-HNF4A* identified in our study (rs6017317) is not in strong LD with the *HNF4A* P2 promoter SNPs ($r^2 = 0.23$–0.25, $D' = 0.50$–0.54 between rs6017317 and rs1884613 or rs2144908 based on phase 2 CHB/JPT HapMap data), indicating that rs6017317 is a new T2D signal in the 20q13.12 region where *HNF4A* resides.

The other five loci reaching genome-wide significance in our study have not previously been reported in the context of any metabolic traits, including the loci mapped in or near *KCNK16*, *MAEA*, *GCC1-PAX4*, *PSMD6* and *ZFAND3*. *KCNK16*, which is expressed predominantly in the pancreas, encodes a potassium channel protein containing two pore-forming P domains[23]. In pancreatic β cells, potassium channels that are inhibited by ATP regulate glucose-dependent insulin secretion. Among the variants in strong LD with the signal reaching genome-wide significance in *KCNK16* (rs1535500) is rs11756091 ($r^2 = 0.977$, $D' = 1.0$ based on phase 2 CHB/JPT HapMap data), which encodes a substitution of proline to histidine in two isoforms of KCNK16. This variant or others influencing *KCNK16* may result in the defective regulation of potassium channel activity that contributes to the etiology of T2D[24]. *MAEA* encodes a protein that has a role in erythroblast enucleation and in the development of mature macrophages[25]. A gene-set analysis of the stage 1 *P* values using GSA-SNP[26] indicated that *MAEA* belongs to a group of genes that previously showed significant association with T2D and includes *IDE*, which is located at a known T2D susceptibility locus[27] (stage 1 $P = 1.41 \times 10^{-7}$ for rs6583826 at the *IDE* locus in this study). The GRIP-domain–containing protein that is encoded by *GCC1* might have a role in the organization of the trans-Golgi network, which is involved in membrane transport[28]. *PAX4*, which is only 30 kb away from *GCC1*, is a good candidate for T2D given its involvement in pancreatic islet development. *PAX4* was recently implicated in a Japanese individual with maturity onset diabetes of the young[29]. The expression product of *PSMD6*, which acts as a regulatory subunit of the 26S proteasome, is probably involved in the ATP-dependent degradation of ubiquitinated proteins[30]. Although the function of *ZFAND3* has not been fully elucidated, it is noteworthy that a member of the same gene family, *ZFAND6*, is present along with *FAH* at a previously detected T2D locus[31]. We examined whether eight new loci are potentially associated with T2D through an effect on obesity, as is the case with *FTO*[32]. All of the T2D association signals we initially detected remained after adjustment for BMI (**Supplementary Table 6**), indicating that the associations with T2D of these eight loci are not mediated through an effect on obesity.

In addition to the eight loci reaching genome-wide significance, we identified two loci showing moderate evidence (combined $P < 10^{-6}$) of association with T2D, including *WWOX* and *CMIP* loci (**Table 1**). We obtained the association results for these ten loci in GWAS data from up to 47,117 European samples generated by the DIAGRAM consortium (DIAGRAM+ is the current version of dataset)[31].

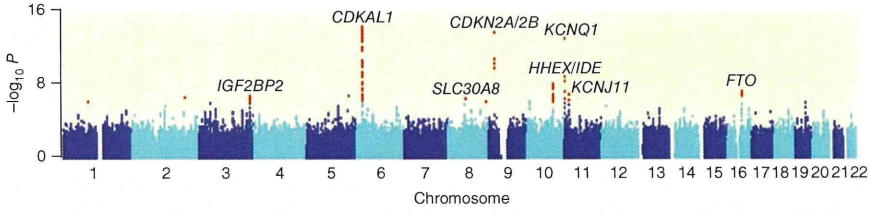

Figure 1 Genome-wide Manhattan plot for the east Asian T2D stage 1 meta-analysis. Shown are the $-\log_{10} P$ values using the trend test for SNPs distributed across the entire autosomal genome. The red dots at each locus indicate the signals with $P < 10^{-6}$ detected in the genome-wide meta-analysis. A total of 1,934,619 SNPs that were present in at least five stage 1 studies were used to generate the plot.

Table 1 Eight new T2D loci reaching genome-wide significance from a combined meta-analysis of stages 1, 2 and 3

SNP	Chr.	Position (bp)	Nearby gene	Risk allele	Other allele	Stage 1 (discovery)[a]		Stage 2 (*in silico* replication)[b]		Stage 3 (*de novo* replication)[c]		Combined (stages 1, 2 and 3)[d]	
						OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Loci showing strong evidence of association with T2D													
rs6815464	4	1,299,901	*MAEA*	C	G	1.09 (1.04–1.14)	8.21×10^{-4}	1.13 (1.07–1.20)	3.67×10^{-5}	1.16 (1.11–1.20)	4.15×10^{-15}	1.13 (1.10–1.16)	1.57×10^{-20}
rs7041847	9	4,277,466	*GLIS3*	A	G	1.09 (1.04–1.14)	1.29×10^{-4}	1.09 (1.03–1.15)	2.20×10^{-3}	1.11 (1.07–1.15)	2.89×10^{-9}	1.10 (1.07–1.13)	1.99×10^{-14}
rs6017317	20	42,380,380	*FITM2-R3HDML-HNF4A*	G	T	1.10 (1.05–1.15)	2.43×10^{-5}	1.07 (0.99–1.15)	8.42×10^{-2}	1.10 (1.06–1.14)	3.96×10^{-7}	1.09 (1.07–1.12)	1.12×10^{-11}
rs6467136	7	126,952,194	*GCC1-PAX4*	G	A	1.12 (1.06–1.18)	6.47×10^{-5}	1.11 (1.04–1.18)	2.09×10^{-3}	1.10 (1.05–1.15)	2.31×10^{-5}	1.11 (1.07–1.14)	4.96×10^{-11}
rs831571	3	64,023,337	*PSMD6*	C	T	1.11 (1.06–1.17)	4.85×10^{-6}	1.06 (1.00–1.13)	4.46×10^{-2}	1.08 (1.05–1.12)	1.41×10^{-5}	1.09 (1.06–1.12)	8.41×10^{-11}
rs9470794	6	38,214,822	*ZFAND3*	C	T	1.11 (1.05–1.17)	1.45×10^{-4}	1.09 (1.02–1.17)	1.48×10^{-2}	1.16 (1.09–1.23)	3.20×10^{-6}	1.12 (1.08–1.16)	2.06×10^{-10}
rs3786897	19	38,584,848	*PEPD*	A	G	1.14 (1.08–1.20)	3.74×10^{-6}	1.05 (0.99–1.12)	1.28×10^{-1}	1.11 (1.04–1.17)	5.46×10^{-4}	1.10 (1.07–1.14)	1.30×10^{-8}
rs1535500	6	39,392,028	*KCNK16*	T	G	1.11 (1.06–1.16)	5.34×10^{-6}	1.07 (1.01–1.15)	3.33×10^{-2}	1.06 (1.02–1.10)	3.50×10^{-3}	1.08 (1.05–1.11)	2.30×10^{-8}
Loci showing moderate evidence of association with T2D													
rs16955379[e]	16	80,046,874	*CMIP*	C	T	1.13 (1.07–1.20)	2.20×10^{-5}	1.10 (1.03–1.17)	6.59×10^{-3}	1.05 (1.01–1.10)	2.19×10^{-2}	1.08 (1.05–1.12)	2.84×10^{-7}
rs17797882	16	77,964,419	*WWOX*	T	C	1.12 (1.05–1.18)	1.76×10^{-4}	1.09 (1.02–1.16)	1.21×10^{-2}	1.06 (1.01–1.11)	1.61×10^{-2}	1.08 (1.05–1.12)	9.49×10^{-7}

[a]Up to 6,952 cases and 11,865 controls. [b]Up to 5,843 cases and 4,574 controls. [c]Up to 12,284 cases and 13,172 controls. [d]Up to 25,079 cases and 29,611 controls. [e]The proxy SNP rs9930117 ($r^2 = 1$) was genotyped in the stage 3 CAGE study.

The DIAGRAM-generated results for these loci indicated that three loci, including the *FITM2-R3HDML-HNF4A* (rs6017317: $P = 1.47 \times 10^{-2}$, OR = 1.07), *CMIP* (rs16955379: $P = 3.33 \times 10^{-2}$, OR = 1.20) and *MAEA* (using the proxy SNP for rs6815464, rs11247991 ($r^2 = 0.96$): $P = 6.56 \times 10^{-3}$, OR = 1.19) loci, were modestly associated with T2D, whereas a locus in *GLIS3* (rs7041847: $P = 6.43 \times 10^{-2}$, OR = 1.04) was nominally associated with T2D. The direction of effect was consistent in four (*PSMD6*, *PEPD*, *WWOX* and *KCNK16*) of the six loci that were not replicated in DIAGRAM+ (**Supplementary Table 5**).

We analyzed the functional connections among the 10 new T2D genes and the 28 known T2D genes that we replicated in this study (**Supplementary Table 3**) using GRAIL[33]. The connection results highlighted notable biological functions for sets of genes within T2D-associated regions (**Supplementary Fig. 4** and **Supplementary Tables 7** and **8**). For example, *KCNK16* has strong connections with previously known T2D genes encoding potassium channels (*KCNJ11* and *KCNQ1*), implying that is has a physiological role in the regulation of potassium transport in pancreatic cells.

We examined the association between each new T2D SNP and the expression of genes within 1 Mb of these SNPs by an expression quantitative trait locus (eQTL) analysis using the data from the MuTHER consortium. One SNP (rs3786897) in an intron of *PEPD* was highly associated with the mRNA expression of *PEPD* in the adipose tissue of 776 individuals of European ancestry ($P_{eQTL} = 2.14 \times 10^{-8}$) (**Supplementary Table 9**). However, this SNP did not show an association with T2D in populations of European ancestry, thus the importance of this finding is unclear.

We considered the possibility that autoimmune diabetes (rather than T2D) may be driving some of the signals that we observed. First, the cases from all the studies we examined predominantly had adult-onset diabetes (age of disease onset ≥30 years), and none of the clinically diagnosed subjects had T1D, which is defined by the presence of acute ketosis and the continuous requirement of insulin beginning within 1 year after diagnosis. Second, we researched the associations for all known T1D-associated variants in our dataset. Only a small number of loci showed association after this analysis (**Supplementary Table 10**). These results are in distinct contrast to those for known T2D-associated variants, many of which replicated in our study (**Supplementary Table 3**), further suggesting that our findings are most relevant to T2D. Third, as variants close to the *GLIS3* locus have been shown to be associated with T1D[19], we examined the association between rs7041847 and diabetes in four studies ($n = 8,383$) in which individulas with positive glutamic acid decarboxylase (GAD) antibodies had been excluded (as individuals with T1D are positive for GAD antibodies, whereas individuals with T2D are not) (data not shown). In each study, the associations between this SNP and diabetes were the same as the association found when we included all the samples (meta-analysis $P = 3.4 \times 10^{-4}$, OR = 1.12). This finding, along with the fact that SNPs at the *GLIS3* locus also show associations with fasting plasma glucose in nondiabetic adults[3] and in healthy children and adolescents[4], is consistent with the hypothesis that SNPs at this locus may affect fasting glucose homeostasis rather than the immune system. Taken together, it is unlikely that a substantial proportion of the positive associations observed in our study were driven by autoimmune diabetes.

This study is the largest GWAS meta-analysis, to our knowledge, conducted for T2D in east Asians. Findings from this study highlight not only previously unknown biological pathways but also population-specific loci for T2D. The association of rs9470794 in *ZFAND3* with T2D seems to be highly specific to east Asian populations (**Supplementary Table 5**), whereas the association of

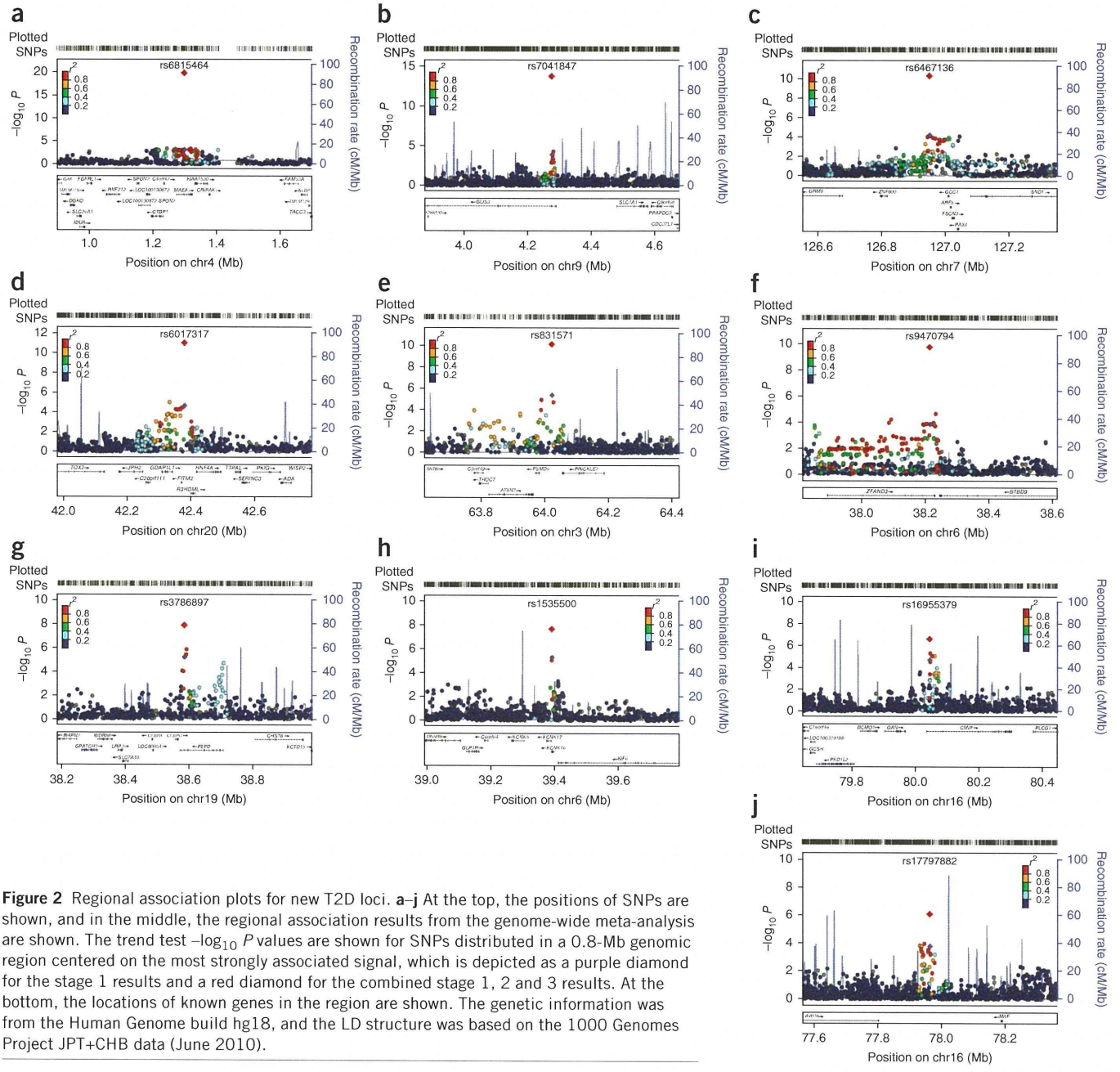

Figure 2 Regional association plots for new T2D loci. **a–j** At the top, the positions of SNPs are shown, and in the middle, the regional association results from the genome-wide meta-analysis are shown. The trend test $-\log_{10} P$ values are shown for SNPs distributed in a 0.8-Mb genomic region centered on the most strongly associated signal, which is depicted as a purple diamond for the stage 1 results and a red diamond for the combined stage 1, 2 and 3 results. At the bottom, the locations of known genes in the region are shown. The genetic information was from the Human Genome build hg18, and the LD structure was based on the 1000 Genomes Project JPT+CHB data (June 2010).

rs11634397 near *ZFAND6* seems to be specific to European populations (**Supplementary Table 3**). We observed a substantial difference in the risk allele frequencies of both loci between the two continental (Asian and European) populations (rs9470794: risk allele frequency (RAF) = 0.32 for the Asian CHB/JPT HapMap population compared to RAF = 0.12 for the European CEU HapMap population; rs11634397: RAF = 0.07 for CHB/JPT compared to RAF = 0.64 for CEU). Although these loci are related to T2D differently in the two populations (the *ZFAND3* locus is specific to Asians, whereas the *ZFAND6* locus to Europeans), these results lead to speculation that the broader A20 domain-containing zinc finger protein family has a role in the etiology of T2D. Additional population-specific T2D loci were also suggested by our analysis, for example, *WWOX* (rs17797882) (**Supplementary Table 5**) in east Asians and *ZBED3* (rs4457053) (**Supplementary Table 3**) in Europeans. Despite the lack of clear physiological evidence on T2D pathogenesis, these findings may provide clues to help understand T2D phenotypes characteristic of each population, for example, the high rates of diabetes seen at lower average BMIs in east Asians.

URLs. IMPUTE, http://mathgen.stats.ox.ac.uk/impute/impute.html; MACH, http://www.sph.umich.edu/csg/abecasis/MACH/; BEAGLE, http://faculty.washington.edu/browning/beagle/beagle.html; METAL, http://www.sph.umich.edu/csg/abecasis/Metal; WGAViewer, http://compute1.lsrc.duke.edu/softwares/WGAViewer/; SNAP, http://www.broadinstitute.org/mpg/snap/; LocusZoom, http://csg.sph.umich.edu/locuszoom/; GenABEL, http://www.genabel.org/; ProbABEL, http://www.genabel.org/packages/ProbABEL.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Study subjects. Stage 1 subjects were drawn from eight T2D GWAS participating in the AGEN consortium, which was organized to enable genetic studies on diverse complex traits in 2010. These eight studies included 6,952 cases with T2D and 11,865 controls from the Korea Association Resource Study (KARE), the Singapore Diabetes Cohort Study (SDCS), the Singapore Prospective Study Program (SP2), the Singapore Malay Eye Study (SiMES), the Japan Cardiometabolic Genome Epidemiology Network (CAGE), the Shanghai Diabetes Genetic Study (SDGS), the Taiwan T2D Study (TDS) and the Cebu Longitudinal Health and Nutritional Survey (CLHNS). Subjects in stage 2 included 5,843 cases with T2D and 4,574 controls from three independent GWAS, the BioBank Japan Study (BBJ), the Health2 T2D Study (H2T2DS) and the Shanghai Jiao Tong University Diabetes Study (SJTUDS), for *in silico* replication analysis. Stage 3 included up to 12,284 cases with T2D and 13,172 controls from five different studies, the Japan Cardiometabolic Genome Epidemiology Network (CAGE), the Shanghai Diabetes Study I/II (SDS I/II), the Chinese University of Hong Kong Diabetes Study (CUHKDS), the National Taiwan University Hospital Diabetes Study (NTUHDS) and the Seoul National University Hospital Diabetes Study (SNUHDS), for *de novo* replication analysis. The study design and T2D diagnosis criteria used in each study included in stages 1, 2, and 3 are described in **Supplementary Table 1** and the **Supplementary Note**. Each study obtained approval from the appropriate institutional review boards of each participating institution, and written informed consent was obtained from all participants. The three-stage design of the overall study is depicted in **Supplementary Figure 1**.

Genotyping and imputation. Subjects for the stage 1 and 2 analyses were genotyped with high-density SNP typing platforms that covered the entire human genome. In most of the studies, only unrelated samples with missing genotype call rates below 5% were included for subsequent GWAS analyses. For the genome-wide association meta-analysis, each study participating in stages 1 and 2 performed SNP imputation. IMPUTE, MACH or BEAGLE (see URLs) were used, together with haplotype reference panels from the JPT and CHB samples that are available in the HapMap database (JPT+CHB+CEU and/or YRI, in some studies) on the basis of HapMap build 36 (release 21, 22, 23a or 24). Only imputed SNPs with high genotype information content (proper info > 0.5 for IMPUTE and Rsq > 0.3 for MACH and BEAGLE) were used for the association analysis. Genotyping for the stage 3 analysis was carried out using TaqMan, Sequenom MassARRAY or the Beckman SNP Stream method. All SNPs included in stage 3 had a genotype success rate of >98% (**Supplementary Table 2**).

Statistical analyses, analysis tools and SNP prioritization for stages 2 and 3. Associations between SNPs and T2D were tested by logistic regression with an additive model (1 degree of freedom) after adjustment for sex. Other adjustments were permitted according to the situations in the individual studies. The meta-analysis was performed using an inverse-variance method assuming fixed effects, with a Cochran's *Q* test to assess between-study heterogeneity. METAL software (see URLs) was used for all meta-analyses. A plot of the negative log of the association results from the stage 1 meta-analysis, by chromosome, was generated using WGAViewer software (see URLs).The quantile-quantile plot was constructed by plotting the distribution of observed *P* values for the given SNPs against the theoretical distribution of the expected *P* values for T2D[34]. The genomic control inflation factor, λ, was calculated by dividing the median χ^2 statistics by 0.456 (ref. 35) for individual GWAS, as well as for the stage 1 GWAS meta-analysis. We did not correct for genomic control in the stage 1 analyses because the inflation was modest, suggesting that population structure is unlikely to cause substantial inflation of the stage 1 results (**Supplementary Table 2**). The selection criteria for the lead SNPs to take forward to stage 2 *in silico* replication analysis were as follows: (i) stage 1 meta-analysis $P < 5 \times 10^{-4}$ (based on the divergence between the observed and expected *P* values on the quantile-quantile plot; **Supplementary Fig. 2**); (ii) heterogeneity $P > 0.01$; and (iii) at least seven studies having been included in the stage 1 meta-analysis (**Supplementary Table 4**). After removing known variants associated with T2D, proxies for each lead SNP ($r^2 > 0.8$) were selected using the SNAP software (see URLs). The replication genotyping for stage 3 was performed for the new SNPs having a stage 2 combined $P < 10^{-5}$. Regional association results from genome-wide meta-analysis were plotted using LocusZoom software (see URLs) for SNPs reaching genome-wide significance from the combined meta-analysis of stages 1, 2 and 3.

Principal components analysis. A list of 76,534 common SNPs across the Illumina 550, 610 and 1M and Affymetrix 5.0 and 6.0 arrays were first selected. This set of SNPs in the Asian (CHB+JPT) HapMap II samples was then trained to generate a list of 44,524 SNPs having pairwise LD < 0.3 in a sliding window of 50 SNPs. Individuals from each component study and from HapMap II were plotted based on the first two eigenvectors produced by the principal components analysis.

eQTL analysis. Gene expression information from 776 adipose tissues, 667 skin tissues and 777 lymphoblastoid cell lines was obtained from the MuTHER consortium[36]. The eQTL data for eight of the ten T2D loci identified in this study were available in the MuTHER dataset. Most of those loci passed the filtering criteria, such as MAF > 5% and INFO > 0.8, except for rs16955379, which has MAF = 1.5% in the MuTHER data set. Two of the ten loci that were used in the eQTL analysis, rs6815464 (on chromosome 4) and rs17797882 (on chromosome 16), are not included in the MuTHER data set. Association between each SNP with a significant association to T2D and the normalized mRNA expression values of genes within 1 Mb of the lead SNP were performed with the GenABEL and ProbABEL package (see URLs) using the polygenic linear model incorporating a kinship matrix in GenABEL followed by the ProbABEL mmscore test with imputed genotypes. A multiple-testing correction was applied to the *cis* association results. *P* value thresholds of $P = 5.06 \times 10^{-5}$ in adipose tissue, $P = 3.81 \times 10^{-5}$ in skin and $P = 7.80 \times 10^{-5}$ in lymphoblastoid cell lines correspond to an estimated genome-wide false discovery rate of 1%.

Gene relationships among implicated loci (GRAIL) analysis. A GRAIL analysis was performed as described previously[31,33]. A total of 38 genes within T2D-associated regions were selected for the analysis. Among these genes, 28 were from the previously implicated set (**Supplementary Table 3**), and the other 10 genes were newly implicated in this study (**Table 1**). PubMed abstracts published after December 2006 were omitted from the analysis to reduce confounding by results from T2D GWAS.

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Interleukin-13 Damages Intestinal Mucosa via TWEAK and Fn14 in Mice—A Pathway Associated With Ulcerative Colitis

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BACKGROUND & AIMS: TWEAK, a member of the tumor necrosis factor (TNF) superfamily, promotes intestinal epithelial cell injury and signals through the receptor Fn14 following irradiation-induced tissue damage and during development of colitis in mice. Interleukin (IL)-13, an effector of tissue damage in similar models, has been associated with the pathogenesis of ulcerative colitis (UC). We investigated interactions between TWEAK and IL-13 following mucosal damage in mice. **METHODS:** We compared patterns of gene expression in intestinal tissues from wild-type and TWEAK knockout mice following γ-irradiation. Intestinal explants from these mice were used to detect cell damage induced by IL-13 and TNF-α. Levels of messenger RNA for IL-13, TWEAK, and Fn14 were measured in mucosal samples from patients with UC. **RESULTS:** Based on gene expression analysis, TWEAK mediates γ-irradiation–induced epithelial cell cycle arrest and apoptosis. However, TWEAK alone did not induce damage or apoptosis of primary intestinal epithelial cells. On the other hand, exogenous IL-13 activated caspase-3 in naïve intestinal explants; this process required TWEAK, Fn14, and secretion of endogenous TNF-α which was mediated by ADAM17. Conversely, activation of caspase by exogenous TNF-α required IL-13, TWEAK, and Fn14. In mucosa from patients with UC, messenger RNA levels of IL-13, TWEAK, and Fn14 increased with level of disease severity. **CONCLUSIONS: IL-13–induced damage of intestinal epithelial cells requires TWEAK, its receptor (Fn14), and TNF-α. IL-13, TNF-α, TWEAK, and Fn14 could perpetuate and aggravate intestinal inflammation in patients with UC.**

Keywords: Ulcerative Colitis; IBD; Diagnostic Marker; Cytokine Signaling; Cell Death.

Interleukin (IL)-13 is a cytokine produced by T-helper 2 (Th2) cells that has pleiotropic effects on a variety of target cell types. In the gastrointestinal tract, IL-13 is a well-established mediator of intestinal epithelial cell (IEC) damage in contexts of injury and inflammatory disease.[1,2] We previously showed that IL-13 was up-regulated in the intestine after γ-irradiation in mice and its blockade reduced IEC damage.[3] Growing evidence supports that IL-13 mediates IEC damage in human ulcerative colitis (UC),[4] and IL-13 also has been shown to mediate tissue damage in murine models of colitis,[5] including oxazolone-induced colitis[6] and trinitrobenzene sulfonic acid–induced chronic-type colitis.[7] IL-13 induced the detachment of the intercellular junctions and disturbed arrangement of β-catenin after addition to intestinal explants[3] and a colon carcinoma cell line HT29/B6, as well as apoptosis of this cell line.[1] Both mechanisms are believed to be responsible for the epithelial cell damage and defect in barrier function mediated by IL-13. However, the precise molecular bases of IL-13–induced IEC damage have not been delineated.

TWEAK is a cytokine of the tumor necrosis factor (TNF) ligand superfamily that is expressed by inflammatory cells, including activated T cells and innate immune cell types.[8,9] Like TNF, TWEAK is a type II transmembrane homotrimer that can function as a soluble cytokine with diverse biological roles, including proinflammatory activity, angiogenesis, and regulation of cell survival, proliferation, and death.[10] TWEAK mediates these effects through its receptor, fibroblast growth factor–inducible molecule (Fn14), which is expressed by epithelial and mesenchymal cells, as well as by progenitor cells,[10] and signals via the nuclear factor κB and mitogen-activated protein kinase pathways.[11,12] Interestingly, Fn14 is expressed at low levels in normal tissue and highly up-regulated in contexts of tissue injury and regeneration, supporting a physiologic role for this pathway in coordinating acute inflammation and tissue repair. Fn14 is also highly up-regulated in chronic inflammatory disease, supporting a role for this pathway in promoting end organ pathology in chronic disease settings. The molecular mechanism of TWEAK/Fn14 involvement in the tissue injury is yet to be clarified. A recent study reported that the TWEAK/Fn14 pathway enhanced the apoptotic effect of TNF-α on tumor cells by inducing degradation of a cIAP1-TRAF2 complex in tumor cells.[13] In agreement with

Abbreviations used in this paper: IEC, intestinal epithelial cell; IL, interleukin; KO, knockout; SPF, specific pathogen-free; TACE, tumor necrosis factor converting enzyme; Th, T helper; TNF, tumor necrosis factor; WT, wild-type.

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this, TWEAK primes tumor cells for TNFR1-mediated cell death.[14] These studies, however, do not fully elucidate the mechanism underlying the TWEAK/Fn14 pathway in the pathophysiology of inflammatory diseases.

We previously reported that TWEAK promotes IEC death after γ-irradiation in mice, as evidenced by reduced IEC death in TWEAK or Fn14 gene knockout (KO) mice.[15] In addition, TWEAK enhances intestinal inflammation, ulcers, and pathological tissue remodeling in mice by acting through Fn14 up-regulated on IECs to promote the production of inflammatory chemokines and matrix metalloproteinases and potentially directly mediating IEC damage.[15] TWEAK/Fn14 signaling apparently aggravated and perpetuated inflammation; however, TWEAK alone is not a potent inducer of IEC death. Thus, other effector molecules appear to be tightly involved in this pathway. Because IL-13 was also up-regulated in the tissue damage model, we hypothesized that the TWEAK/Fn14 pathway may be a key mechanism underlying IL-13–mediated IEC death. In this study, we show that IL-13–induced apoptosis of IECs requires endogenous TWEAK/Fn14 as well as endogenous TNF-α as a novel molecular mechanism of IL-13–induced tissue damage and remodeling.

Materials and Methods

Mice

TWEAK[16] or Fn14[17] KO mice with BALB/c background were used for this study, except crossing IL-10 KO mice. IL-10 KO × TWEAK KO or IL-10 KO × Fn14 KO double KO mice were generated in our facility by crossing TWEAK KO mice or Fn14 KO mice with C57BL/6 background with IL-10 KO mice with C57BL/6 background obtained from The Jackson Laboratory (Bar Harbor, ME). All experimental protocols were approved by the local institutional animal care and use committees. For details, see Supplementary Materials and Methods.

Total RNA Purification, Gene Profiling, and Reverse-Transcription Polymerase Chain Reaction

RNA was isolated from snap-frozen mouse tissues and subjected to gene expression profiling performed with automated probe labeling, hybridization, and scanning as previously described.[15] Analysis of Affymetrix data was described in Supplementary Materials and Methods. The human specimens were provided with informed consent under the approval of this research project by the local ethics committee. Normal mucosa was obtained from patients who had colectomy for colorectal cancer. Primers and methods for quantitative reverse-transcription polymerase chain reaction are described in Supplementary Materials and Methods.

Primary Tissue Culture

The mouse jejunum tissues were washed with phosphate-buffered saline and cut into 1-cm length, opened, placed in 24-well plates, and kept in RPMI 1640 with 5% fetal calf serum, 7.5% gelatin, and antibiotics. Tissues were then cultured with 40 ng/mL of recombinant IL-13, IL-4, or TNF-α (PeproTech Inc, Rocky Hill, NJ) or 100 ng/mL of recombinant TWEAK (prepared by Biogen Idec, Cambridge, MA) at 37°C, 5% CO_2. For human mucosa, 100 ng/mL cytokines was added to the culture. The tissues were gently washed with phosphate-buffered saline and were frozen with liquid nitrogen and stored at −80°C until use. In some experiments, colons were taken and cultured as previously described with media alone, IL-13, TNF-α or IL-6 (R&D Systems, Minneapolis, MN). Methods for histologic analysis for cultured tissues and detection of apoptosis are described in Supplementary Materials and Methods.

Results

The TWEAK/Fn14 Pathway Is Necessary but Not Sufficient to Induce IEC Death

Recently we have shown that TWEAK/Fn14 promotes IEC death after 3-Gy irradiation in mice.[15] Corresponding expression analysis showed that TWEAK messenger RNA (mRNA) was constitutively present in both the small and large intestine as expected. TWEAK mRNA tended to increase at 24 hours after γ-irradiation (Supplementary Figure 1*A*). Fn14 mRNA was also detected in naïve small and large intestine and significantly up-regulated 6 to 24 hours in jejunum and colon (Supplementary Figure 1*A*). We further assessed the role of the TWEAK/Fn14 pathway under conditions of a larger dose of γ-irradiation and found that Fn14 KO mice exhibited increased numbers of surviving cells (Supplementary Figure 1*B*).

Gene transcript expression profiling was conducted in intestinal tissue from wild-type (WT) and TWEAK KO mice after 3-Gy irradiation (Figure 1 and Supplementary Results). WT tissue exhibited a pattern of decreased expression of cell cycle genes related to DNA replication and mitosis, indicating decreased cell division as compared with naïve mice. In contrast, cell cycle genes and apoptotic genes were not altered in KO mice, reflecting epithelial cell survival and almost normal cell turnover. These results show the significant role of the TWEAK/Fn14 pathway in stress-induced cell cycle arrest and subsequent cell death.

Although the previous result indicates that endogenous TWEAK strongly promotes IEC death after γ-irradiation in vivo, TWEAK alone has been known to be a weak inducer of apoptosis.[18] TWEAK did not activate caspase-3 (Supplementary Figure 2*A*). Consistent with this, injection of an Fc-TWEAK fusion protein in naïve mice did not induce significant IEC death (Supplementary Figure 2*B*). We have previously shown that neutralization of IL-13 in γ-irradiation injury protects epithelial cells from apoptosis and promotes regeneration as seen in TWEAK or Fn14 KO mice. Thus, the pathologic role of the TWEAK/Fn14 pathway after DNA damage mirrors that of IL-13.[3,15] Based on this, we investigated whether there is interplay between the IL-13 and the TWEAK/Fn14 pathway in inducing IEC damage.

IL-13–Induced Apoptosis and Dissociation of IEC Intercellular Junctions Is Dependent on the TWEAK/Fn14 Pathway

We and others have previously shown that IL-13 induced the loss of IEC intercellular junctions in vitro.[1,3] In mouse primary intestinal explants, IL-13 induced the loss of β-catenin from the lateral wall and disturbed tight

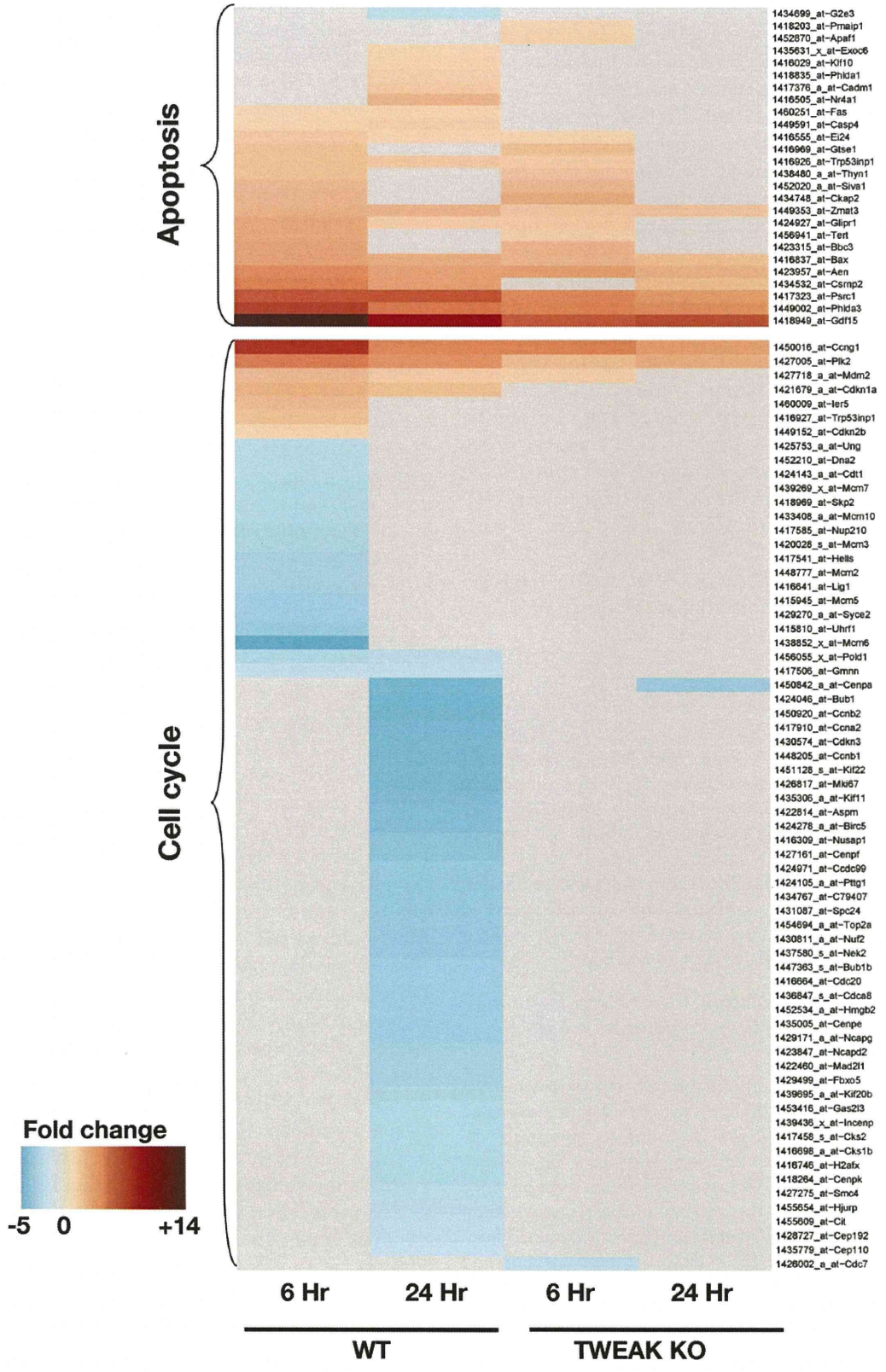

Figure 1. Gene expression profiling after γ-irradiation. Heat map shows the fold changes in gene expression in the colon of irradiated WT mice as compared with untreated WT mice and irradiated TWEAK KO mice as compared with untreated TWEAK KO mice at 6 and 24 hours after irradiation. Each *row* corresponds to an individual gene named on the *right*, with clusters of apoptotic and cell cycle genes as bracketed. Positive (*red*) and negative (*blue*) fold changes are as indicated by intensity on the scale bar, with *gray* indicating no significant change. Data were generated with either 4 or 5 mice per group.

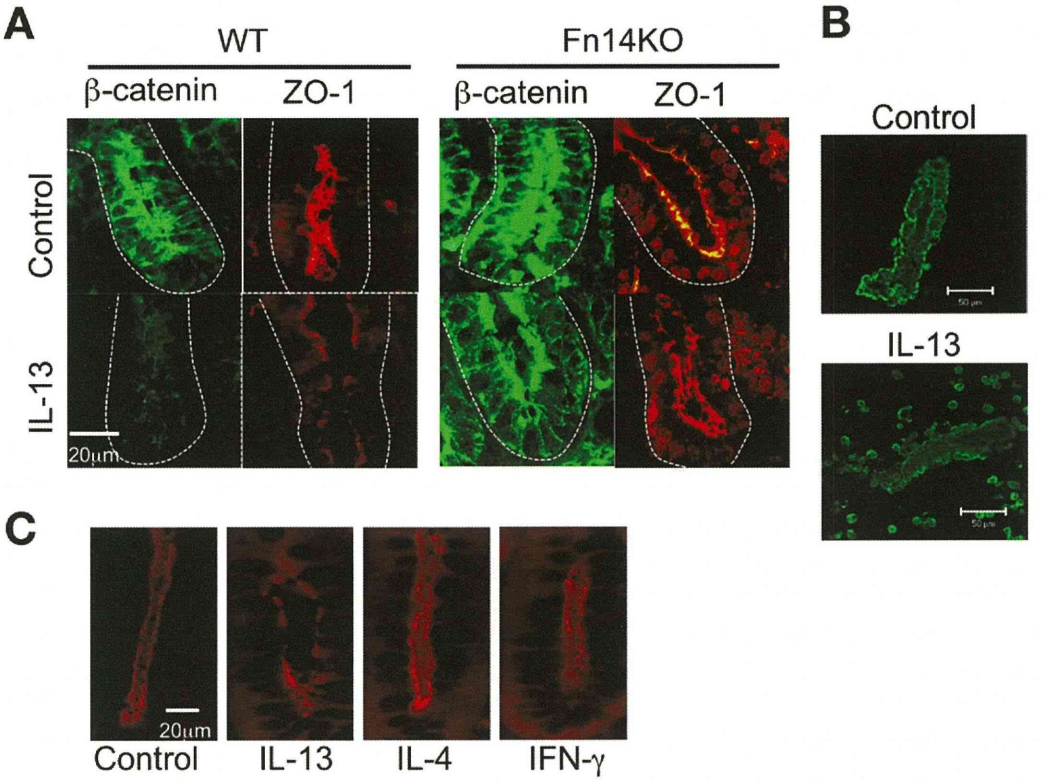

Figure 2. IL-13–induced loss of IEC intercellular junctions is dependent on Fn14. (*A*) Primary culture of jejunum from WT or Fn14 KO mice was performed in the presence or absence (no cytokine) of IL-13 for 4 hours, fixed with formalin, and stained for β-catenin and ZO-1. The shape of a crypt is outlined by a *dotted line*. (*B*) WT crypts were dissociated and incubated as previously described. Sections were prepared from collected crypt pellets and stained for β-catenin. (*C*) Human colonic mucosa was cultured with cytokines for 4 hours and stained with anti–ZO-1 antibody. Images are representative of 3 mice or human cases.

junctions as shown by ZO-1 staining (Figure 2*A*). Of note, these changes were not induced by IL-13 in Fn14 KO tissue. IL-13 appeared to act directly on the IECs, without a requirement for mesenchymal cells (Figure 2*B*). A similar effect of IL-13, but not IL-4 or interferon gamma, on ZO-1 was shown in human colon tissue (Figure 2*C* and data not shown). Thus, IL-13 induced a loss of epithelial cell layer integrity mediated by Fn14.

Using the same culture system, we found that IL-13 but not IL-4 induced apoptosis, with the magnitude comparable to that induced by TNF-α (Figure 3*A* and *B*). Interestingly, IL-13–induced apoptosis was markedly reduced in Fn14 KO explants. In addition, TNF-α–induced IEC apoptosis was partially Fn14 dependent, apparently because IECs located around the crypt bottom showed less apoptosis in TNF-α–treated Fn14 KO tissue (Figure 3*A* and *B*). Thus, at 4 hours of culture, both intercellular adhesion defects and apoptosis were observed in response to IL-13, both of which were dependent on Fn14. To find out if IL-13–induced loss of intercellular junction is independent of the apoptosis pathway or not, we tested the effect of caspase inhibitors. As shown in Figure 3*C*, caspase-3 inhibitors inhibited loss of β-catenin in IL-13–treated cells. This result indicated that apoptosis signal transduction occurred before the loss of intercellular junction.

In this intestinal explant system, a low but detectable level of spontaneous caspase-3 activation was seen by 40 minutes in untreated (no cytokine) WT and Fn14 KO cultures. IL-13 did not induce significant caspase-3 activation at the 15-minute time point but clearly did so in WT cultures at 40 minutes. In contrast, IL-13–induced caspase-3 activation in Fn14 or TWEAK KO tissue remained comparable to the spontaneous activation level (Figure 4*A* and *B*). This result was confirmed by the measurement of caspase-3 activity (Figure 4*C*) and blocking of TWEAK by anti-TWEAK antibody or IL-13 by IL-13Rα2-Ig chimera (Supplementary Figure 3*A*). There was no detectable effect of IL-4 (Figure 4*A*). TNF-α induced caspase-3 activation by 15 minutes, which was maintained at 40 minutes. In contrast to IL-13, the early phase of TNF-α–induced caspase-3 activation but not the later phase was dependent on Fn14 (Figure 4*A* and *B* and Supplementary Figure 3*B* and *C*). Activation patterns of caspase-8 and caspase-9 were also similar to those seen in caspase-3 (Supplementary Figure 4). On the other hand, caspase-2, which was reported to mediate anoikis of epithelial cells isolated from the intestine,[19] was activated in cultures without cytokine at 40 minutes in both WT and Fn14 KO tissue, but not activated by either IL-13 or TNF-α in this system (Supplementary Figure 4*A*). From these results, we concluded that IL-13 primarily induced apoptosis by activation of caspase-8, caspase-9, and caspase-3 cascade, before the death signal by anoikis. Cell death induced by IL-13 or TNF-α did not require new gene expression, including Fn14, TWEAK, or TNF-α, because caspase-3 activation was not

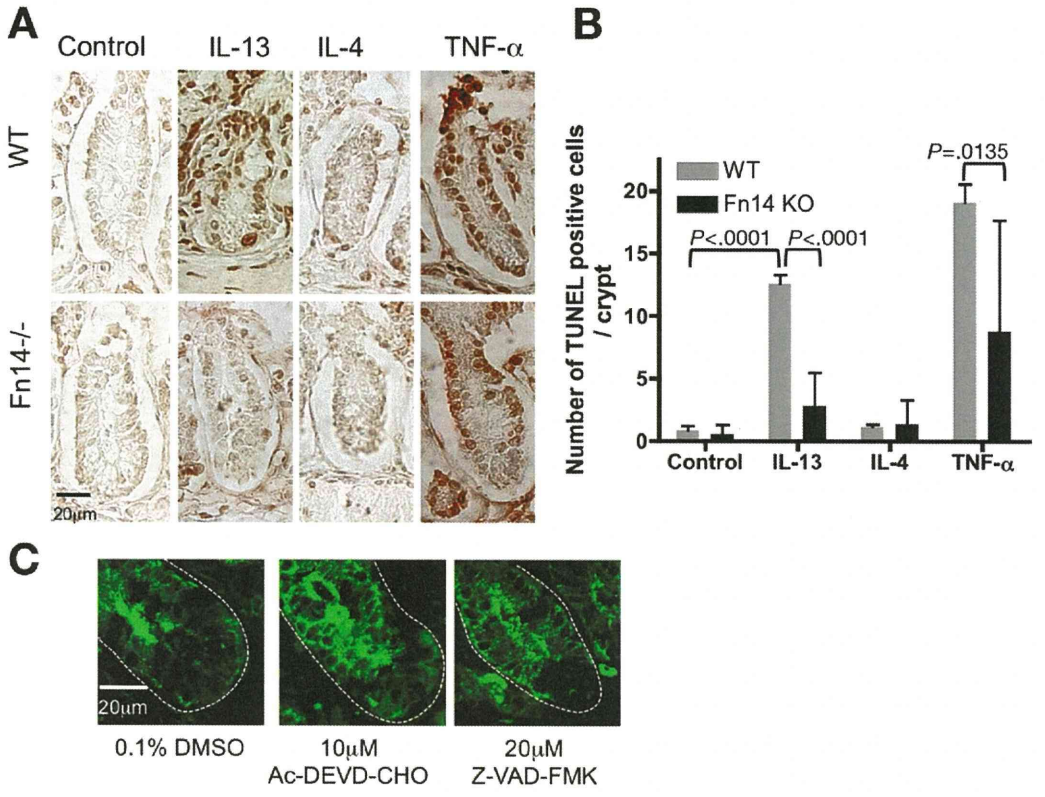

Figure 3. IL-13–induced apoptosis of IECs is dependent on Fn14. Primary culture of jejunum from WT or Fn14 KO mice was performed in the presence of cytokines for 4 hours and (*A*) terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining shown. (*B*) TUNEL-positive cells were enumerated in ~10 to 50 crypts from each section prepared from 3 mice, and data are shown as mean ± SD. (*C*) In addition to IL-13, indicated caspase inhibitors with 0.1% dimethyl sulfoxide were added to the culture as described in Figure 2.

affected by the presence of the protein synthesis inhibitor cycloheximide (Figure 4*D*).

IL-13/TNF-α Axis in IEC Apoptosis

Having shown that both exogenous IL-13– and TNF-α–induced apoptosis have an Fn14-dependent component, we investigated the interplay between IL-13 and TNF-α. IL-13–induced caspase-3 activation was also suppressed by neutralization of TNF-α, and no additional effect was seen when both TNF-α and Fn14 were blocked (Figure 4*E*). This indicated that IL-13 essentially requires both endogenous TNF-α and the TWEAK/Fn14 pathway to induce apoptosis. For caspase-3 activation by exogenous TNF-α, early-phase but not late-phase activation was dependent on IL-13 as well as Fn14, with no additional effect seen when both IL-13 and Fn14 were blocked by neutralization of IL-13 and using Fn14 KO tissue (Figure 4*F* and Supplementary Figure 3*C*). This indicated that early- but not late-phase exogenous TNF-α–induced apoptosis requires both endogenous IL-13 and Fn14.

The requirement for TNF-α for IL-13–induced cell death was unexpected, because this tissue was from normal mice without detectable levels of spontaneous secretion of TNF-α (Figure 5*A* and *B*). Because IL-13 induced apoptosis without protein synthesis or up-regulation of TNF-α transcripts (data not shown) in 40 minutes, we hypothesized that IL-13 may activate a latent form of TNF-α by promoting shedding mediated by TNF-α converting enzyme (TACE). Indeed, IL-13 induced secretion of TNF-α into the culture supernatant, which was efficiently suppressed with a TACE inhibitor, TAPI-2 (Figure 5*A*). IL-13–induced TNF-α shedding was also dependent on TWEAK/Fn14 (Figure 5*B*). TAPI-2 suppressed caspase-3 activation (Figure 5*C*). Thus, IL-13 induced activation of endogenous TNF-α in a TWEAK/Fn14-dependent manner, which resulted in the TNF-α–mediated cell death (Supplementary Figure 5).

Interplay of IL-13, TNF-α and Fn14 at the Transcriptional Level

When mouse naïve colon was cultured with IL-13 and TNF-α, the expression of Fn14 increased, while IL-6 did not (Supplementary Figure 6). These results suggest that IL-13, TNF-α and Fn14 in concert induce IEC cell death by acting at multiple levels, at the transcriptional level as well as at the posttranscriptional level.

Spontaneous Colitis in IL-10 KO Mice Was Dependent on the TWEAK/Fn14 Pathway

In addition to our previous report of a tissue-damaging role for the TWEAK/Fn14 pathway in acute TNBS-induced colitis, we further investigated the role of TWEAK/Fn14 using IL-10 KO mice. When IL-10 KO mice were moved from a specific pathogen-free (SPF) to an ex-SPF area (HEPA filter is not equipped, see Supplementary Materials and Methods) at 6 weeks of age, they

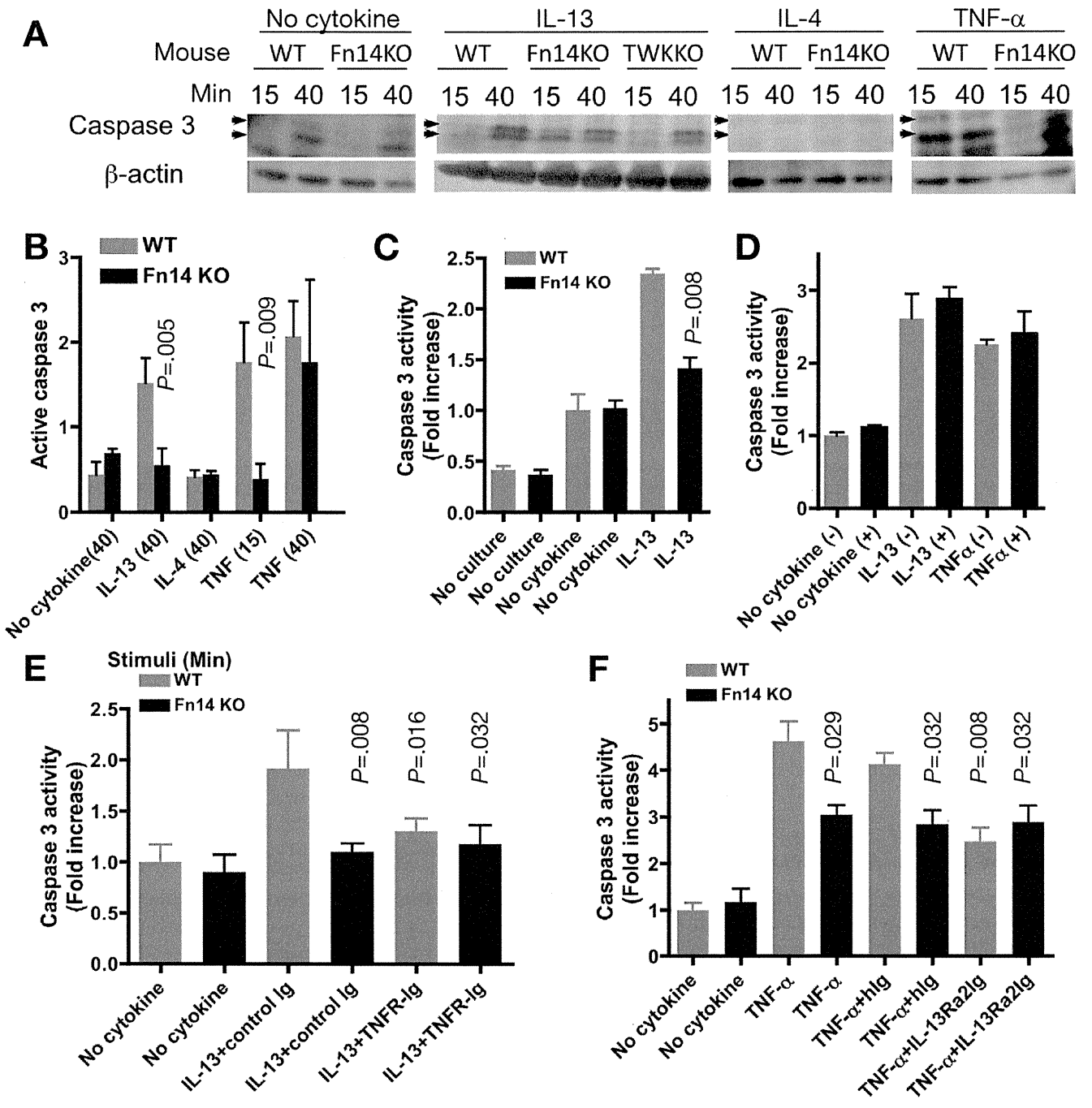

Figure 4. IL-13–induced caspase activation is dependent on Fn14. Jejunum tissue was incubated in the presence of indicated cytokines or without (No cytokine) for indicated times, and protein extract was subjected to immunoblotting for caspases or assay for caspase-3 activity. (*A*) Tissues from WT, Fn14 KO, or TWEAK (TWK) KO mice were incubated with cytokines and probed with anti-activated caspase-3 antibody. Representative photos from experiments repeated 3 times are shown. (*B*) Activated caspase-3 was detected as in *panel A* and quantified by densitometry with normalization with β-actin signal, with stimuli and time point (15 or 40 minutes) as indicated. Data from 3 independent experiments are shown as mean ± SD. (*C*) Caspase-3 activity was measured using WT or Fn14 KO fresh tissues (no culture) or cultured for 40 minutes with IL-13 or without (No cytokine). (*D*) Caspase-3 activity in WT tissues incubated with (+) or without (−) 0.1 μg/mL of cycloheximide. (*E* and *F*) Caspase-3 activity after (*E*) 40 minutes of culture with IL-13 or (*F*) 15 minutes of culture with TNF-α. In *panels E* and *F*, inhibitors were added to the cultures as indicated: murine TNF receptor-Ig (TNFR-Ig) or the murine control antibody clone P1.17 (Control Ig), and IL-13 receptor α2-Ig (IL-13Ra2-Ig) or its control human IgG (hIg). In *panels C* to *F*, data are shown as mean ± SD of 5 cultures as relative activity, fold relative to no cytokine. *P* values are shown for significant differences between inhibitor and its control reagent or between WT and Fn14 KO tissues.

showed severe colitis as determined by thick colon with elongated crypts and cell infiltration within 2 to 4 weeks. Within about 8 weeks in an ex-SPF facility (14 weeks old), colitis with splenomegaly and sacral lymph node enlargement became obvious (Figure 6*A* and *B* and Supplementary Results). In 8-week-old IL-10 KO mice, just at the onset of the colitis, TNF-α and IL-17 but not IL-13 were up-regulated. IL-13 mRNA was strikingly up-regulated in

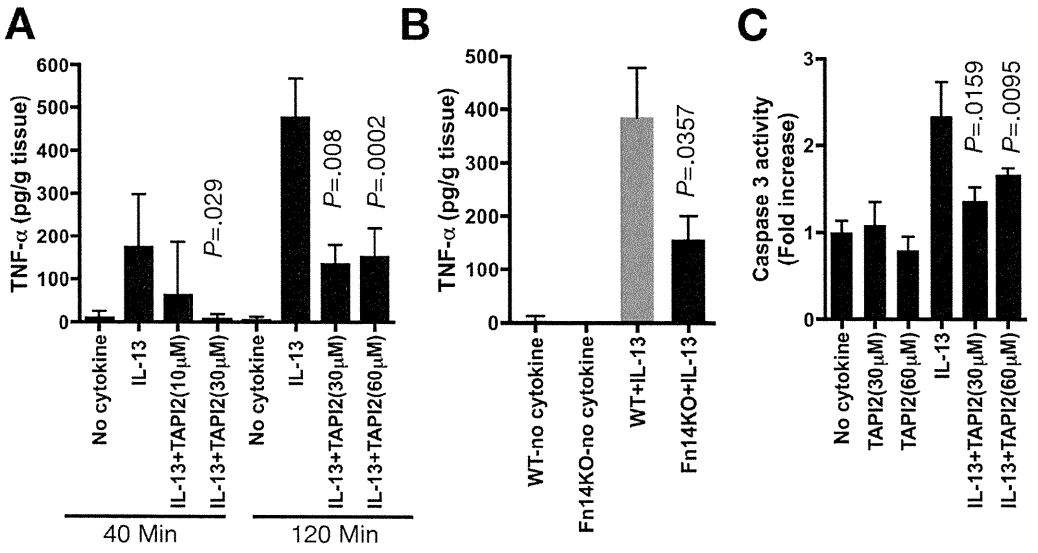

Figure 5. IL-13 induced TNF-α shedding. (*A*) WT tissues were cultured with IL-13 as in Figure 4 for indicated time with TAPI-2, and TNF-α in the culture supernatant was measured. (*B*) WT or Fn14 KO tissues were cultured for 80 minutes with or without IL-13 and shed TNF-α was measured. (*C*) WT tissues were incubated for 120 minutes and caspase-3 activity was measured. Data are shown as mean ± SD of 5 cultures. *P* values are shown for significant differences between inhibitor and its control or between WT and Fn14 KO tissues.

older IL-10 KO mice together with significant up-regulation of Fn14 but not TWEAK (Figure 6*C* and *D*). In contrast, IL-10 KO × TWEAK KO or IL-10 KO × Fn14 double KO mice were all asymptomatic, and up-regulation of TNF-α or IL-17 was not seen, and IL-13 mRNA was not detected in their colon (Figure 6*B* and *C*). These results clearly show the major role of TWEAK/Fn14 in colitis.

Up-regulated Expression of TWEAK, Fn14, and IL-13 in the Mucosa of Human UC

In UC mucosa, Th2-like responses are dominant but not Th1-type cytokines, in contrast to high levels of Th1-type cytokines, including TNF-α in Crohn's disease.[20] High levels of IL-13 are produced by the lamina propria cells from subjects with UC but not Crohn's disease, and the possible pathologic role of IL-13 in UC is supported by the ability of IL-13 to induce epithelial apoptosis and disrupt barrier function of the human epithelial cell line.[4] Our new finding, the requirement of Fn14 in IL-13–induced apoptosis, strongly suggests that TWEAK and Fn14 also play roles in the mucosal damage in UC. In all normal mucosa from 10 cases, the level of IL-13 transcripts was below the sensitivity of quantitative reverse-transcription polymerase chain reaction. However, in UC, IL-13 was detected in 45.5% of samples. When samples were separated into macroscopically uninvolved and involved mucosa, which was with ulcer, inflammatory polyps, or atrophic changes, the frequency of samples with IL-13 expression was significantly higher in the involved mucosa (Figure 7*A*). Low levels of transcript for TNF-α were detected in UC mucosa but not in non–inflammatory bowel disease mucosa. In some cases, TNF-α was further up-regulated in the inflamed UC mucosa (Figure 7*A*). TWEAK and Fn14 transcripts were detected in normal mucosa, and their expression was significantly up-regulated in UC mucosa (Figure 7*B*). When paired samples consisting of the involved and uninvolved mucosa derived from the same patient were compared, the involved mucosa showed clearly higher levels of both TWEAK and Fn14 than the uninvolved mucosa (Figure 7*C*). On the other hand, in a limited number of 4 Crohn's disease mucosa tested, TWEAK transcripts were up-regulated; however, up-regulation of Fn14 expression was not seen (Figure 7*B*), and IL-13 mRNA was not detected. According to the results of Figure 7*A*, we separated UC samples into IL-13 mRNA-positive and -negative groups and compared the expression levels of TWEAK and Fn14. Both TWEAK and Fn14 showed higher expression in the IL-13–positive group, and the difference in Fn14 levels was statistically significant (Figure 7*D*). Overall, our results support the notion that the IL-13, TNF-α and TWEAK/Fn14 pathways act interdependently to aggravate IEC damage and perpetuate the inflammation in UC.

Discussion

In this study, we clearly showed a new linkage in that TWEAK/Fn14 is indispensable for IL-13–induced IEC damage. Importantly, our results indicate that IL-13 is able to trigger significant cell death using endogenous TWEAK/Fn14 and a latent form of TNF-α. In addition to establishing this novel link between IL-13 and the TWEAK/Fn14 pathway in IEC apoptosis, our data further elucidate that the proapoptotic mechanism of IL-13 in IECs is the promotion of TACE-mediated TNF-α shedding. The relevance of TWEAK/Fn14 to IEC death after γ-irradiation damage, as well as in spontaneous colitis in IL-10 KO mice, is shown based on the resistance that we observed in TWEAK or Fn14 KO mice. Our study also shows that up-regulated TWEAK/Fn14 expression is associated with elevated IL-13 levels in human UC.