Supplemental Information

CD98 expression modulates intestinal homeostasis, inflammation, and colitis-associated cancer in mice

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Supplemental data

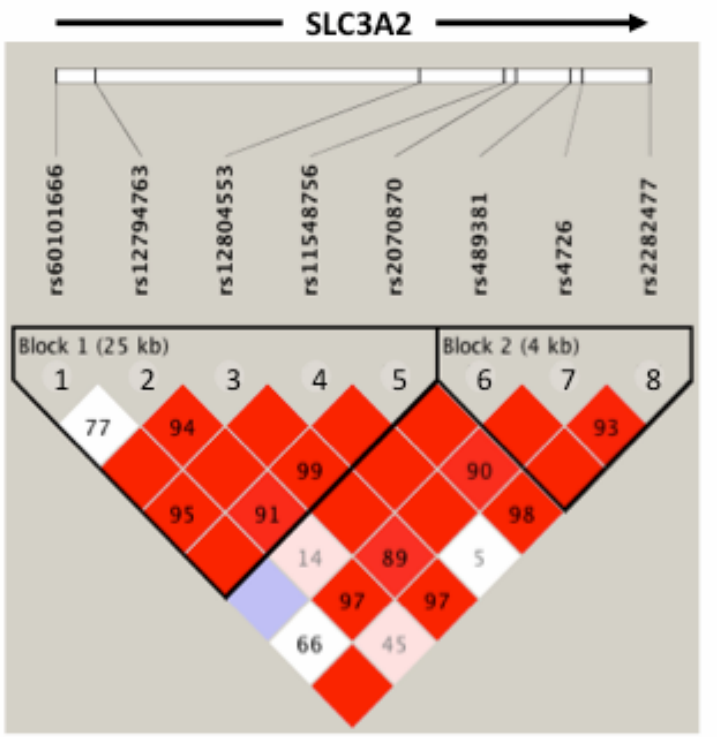
Table S1. IEC-specific CD98 over-expression results in dysregulated gene expression in mouse jejunum

Total RNA was extracted from the jejunums of WT, Tg and Tg-HE littermates, pooled from 4 mice/group and subjected to a cDNA microarray analysis as described in Methods. Dysregulated genes in Tg (A) or Tg-HE (B) groups with fold changes of at least 1.5-fold relative to WT mice were selected. Dysregulated genes were annotated using the DAVID Bioinformatics Resources 2008 (C). (-) fold change indicates down-regulated genes; __ indicates not found. Supplied as an Excel file.

Table S2: IEC-specific CD98 over-expression results in dysregulated gene expression in mouse colon

Total RNA was extracted from the colons of WT, Tg and Tg-HE littermates, pooled from 4 mice/group and subjected to a cDNA microarray analysis as described in Methods. Dysregulated genes in Tg (A) or Tg-HE (B) groups with fold changes of at least 1.5-fold relative to WT mice were selected. Dysregulated genes were annotated using the DAVID Bioinformatics Resources 2008 (C). (-) fold change indicates down-regulated genes; __ indicates not found. Supplied as an Excel file.

Figure S1



Single marker associations									
			Ctrls (N=630)	CD (N=618)		UC (N=630)		IBD (N=1248)	
SNP nr	SNP	allele	freq	freq	P	freq	P	freq	P
1	rs60101666	A	0.007	0.015	0.073	0.014	0.082	0.014	0.053
2	rs12794763	G	0.134	0.154	0.160	0.150	0.247	0.152	0.142
3	rs12804553	G	0.710	0.721	0.529	0.721	0.549	0.721	0.477
4	rs11548756	A	0.033	0.036	0.674	0.036	0.661	0.036	0.622
5	rs2070870	T	0.708	0.725	0.347	0.721	0.475	0.723	0.339
6	rs489381	A	0.091	0.112	0.087	0.106	0.229	0.109	0.096
7	rs4726	C	0.767	0.769	0.911	0.774	0.672	0.772	0.756
8	rs2282477	T	0.778	0.805	0.098	0.799	0.197	0.802	0.087

Haplotype associations									
			Ctrls (N=630)	CD (N=618)		UC (N=630)		IBD (N=1248)	
Block	SNPs	haplo	freq	freq	P	freq	P	freq	P
1	1.2.3.4.5	GTGGT	0.545	0.537	0.644	0.533	0.672	0.535	0.564
1	1.2.3.4.5	GTTGC	0.288	0.274	0.455	0.276	0.471	0.274	0.373
1	1.2.3.4.5	GGGGT	0.132	0.150	0.216	0.149	0.237	0.149	0.178
1	1.2.3.4.5	GTGAT	0.024	0.020	0.534	0.023	0.711	0.022	0.725
2	6.7.8	GCT	0.455	0.464	0.643	0.474	0.363	0.469	0.427
2	6.7.8	GTT	0.232	0.229	0.838	0.220	0.496	0.224	0.608
2	6.7.8	GCC	0.220	0.193	0.081	0.195	0.121	0.194	0.055
2	6.7.8	ACT	0.091	0.112	0.084	0.106	0.229	0.109	0.095

Figure S1. Analysis of *SLC3A2* polymorphism in IBD

Eight SNPs were genotyped on 1248 cases and 630 controls, and tested for association with IBD, Crohn's disease (CD) and ulcerative colitis (UC). Linkage disequilibrium (LD) and haplotype block structure as from Haploview analysis were shown in the upper panel. The markers were ordered according to their position in the *SLC3A2* gene, and direction of *SLC3A2* transcription was reported on top. The numbers in each box corresponded to LD coefficient D' between markers (only values for LD < 100% were shown). The results of the statistic analysis were included in tables on the bottom, where *P* values for tests of association for both single markers and haplotypes were reported, together with allele and haplotype frequencies in cases and controls.

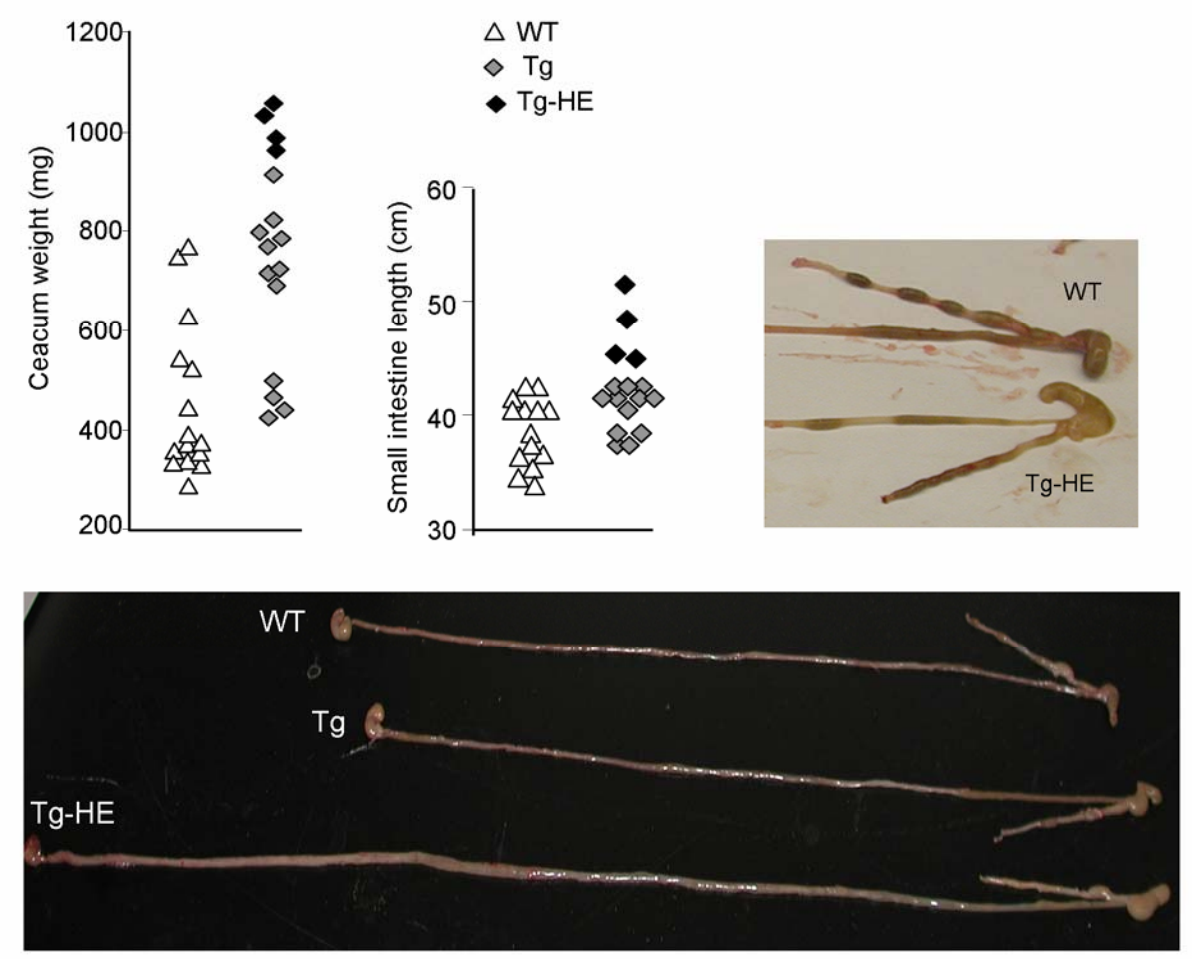


Figure S2. Characterization of transgenic mice with IEC-specific CD98 over-expression

Ceacum weight and small intestine length of WT, Tg and Tg-HE littermates.

Figure S3

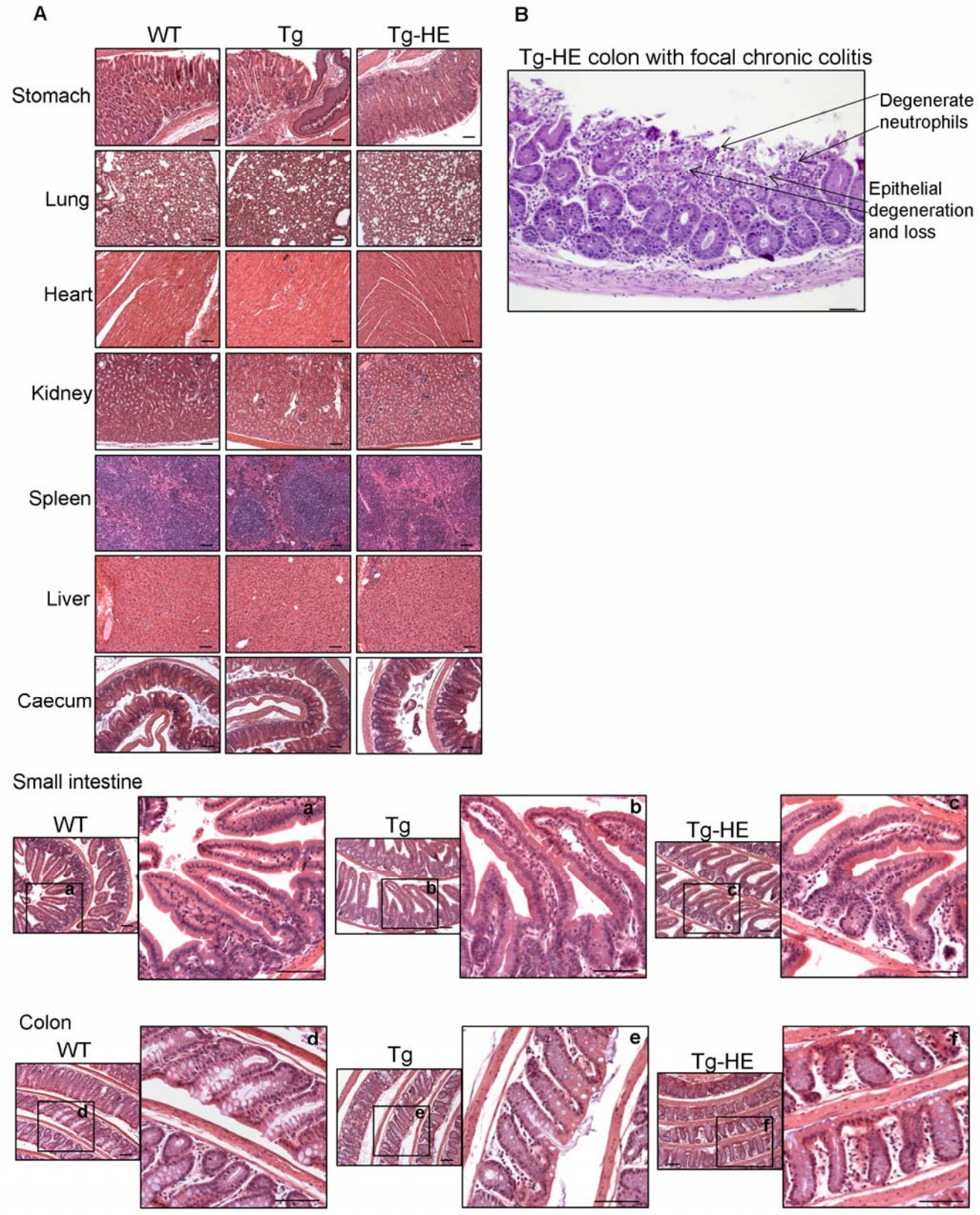

A
WT
Tg
Tg-HE
Stomach
Lung
Heart
Kidney
Spleen
Liver
Caecum
B
Tg-HE colon with focal chronic colitis
Degenerate neutrophils
Epithelial degeneration and loss
Small intestine
WT
Tg
Tg-HE
Colon
WT
Tg
Tg-HE

Figure S3. Histological examination of different tissues from WT and Tg mice

Tissue samples from WT, Tg and Tg-HE mice were collected, fixed in 10% formalin and embedded in paraffin. 5-μm sections were stained with H&E. Photomicrographs were taken using a Nikon Eclipse TS100 microscope. Enlargement of the insets in the photomicrographs is shown beside (A). Focal chronic colitis with crypt epithelial degeneration and loss associated with neutrophil infiltrates detected in Tg-HE mice (B). *Bars* = 100 μm (A) and 50 μm (B).

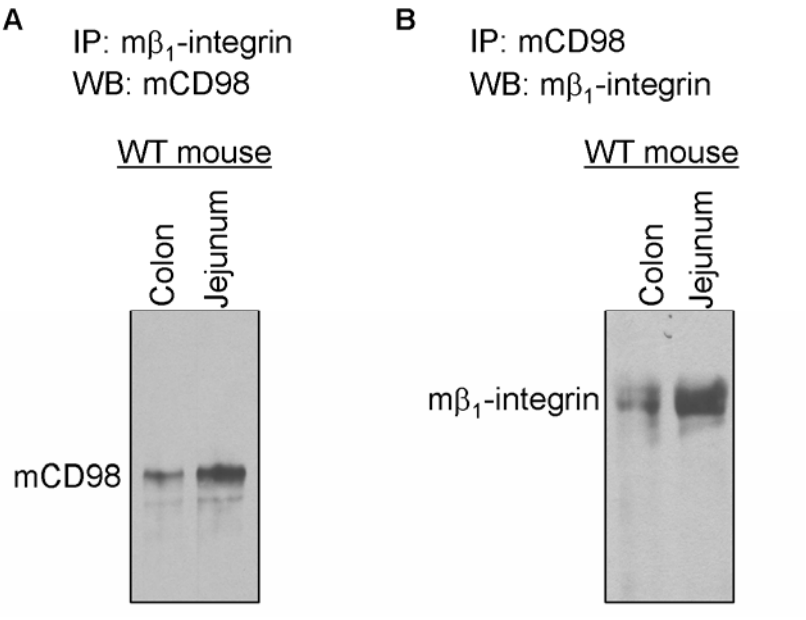


Figure S4. Co-immunoprecipitation of CD98 and β_1-integrin in mouse jejunal and colonic enterocytes

Colonic or jejunal enterocytes from WT mice were immunoprecipitated (IP) for mouse (m) β_1-integrin and Western blotted (WB) for mCD98 (A) or IP for mCD98 and then WB for mβ_1-integrin (B).

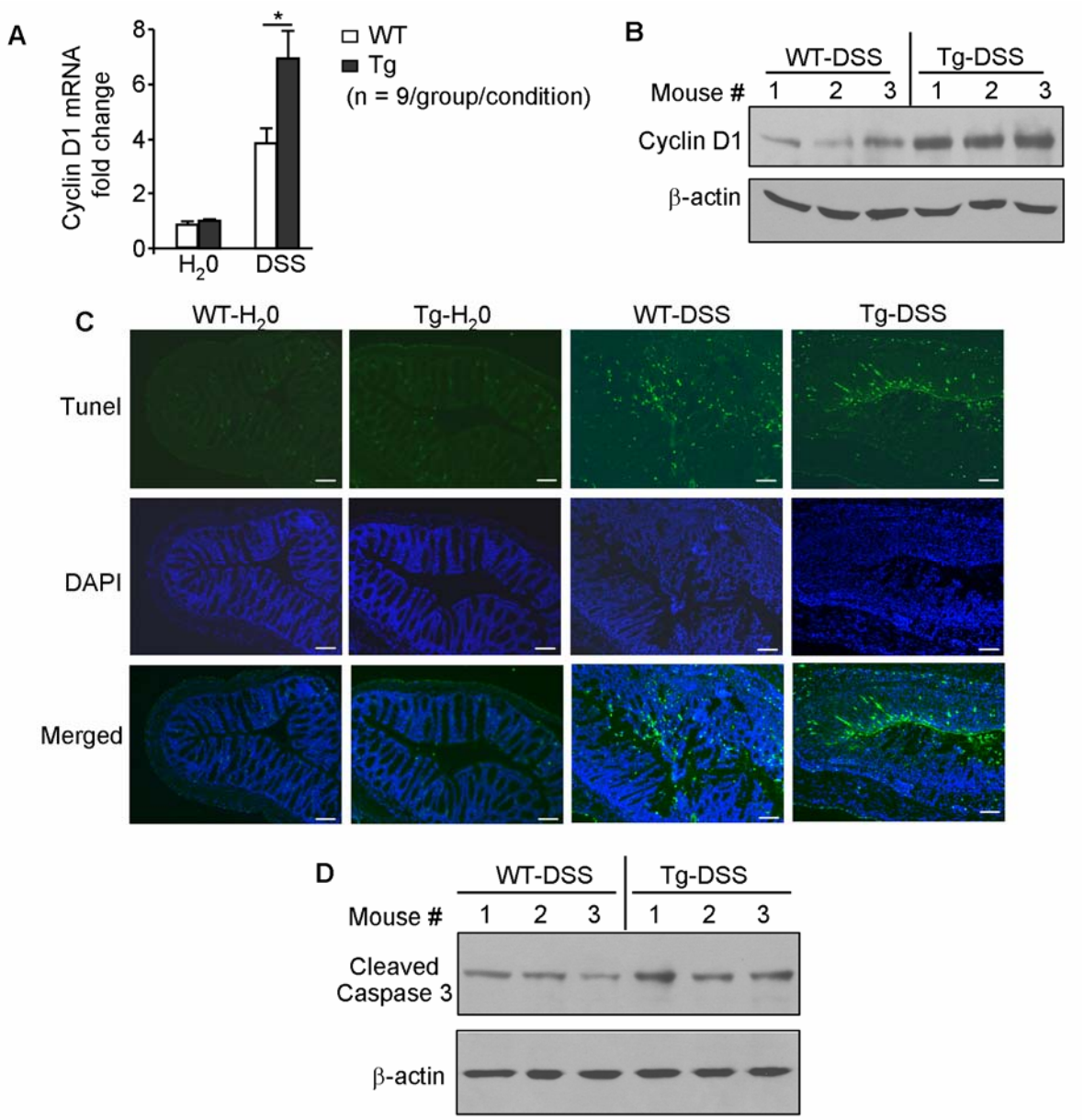


Figure S5. Effects of IEC-specific CD98 over-expression on cell proliferation and apoptosis in a mouse model of DSS-induced colitis

WT and Tg mice were administered drinking water (control) or 3.5 % DSS for 8 days. Colonic cyclin D1 mRNA and protein levels in control and DSS-treated mice were quantified by qRT-PCR (A) and Western blot (B), respectively. IEC apoptosis in DSS-treated mice was assessed by TUNEL staining of colonic sections (C) and Western blot analysis of cleaved caspase 3 levels (D). Data are means ± S.E.M from one experiment repeated twice with similar results. *$P < 0.05$. *Bars* = 100 μm.

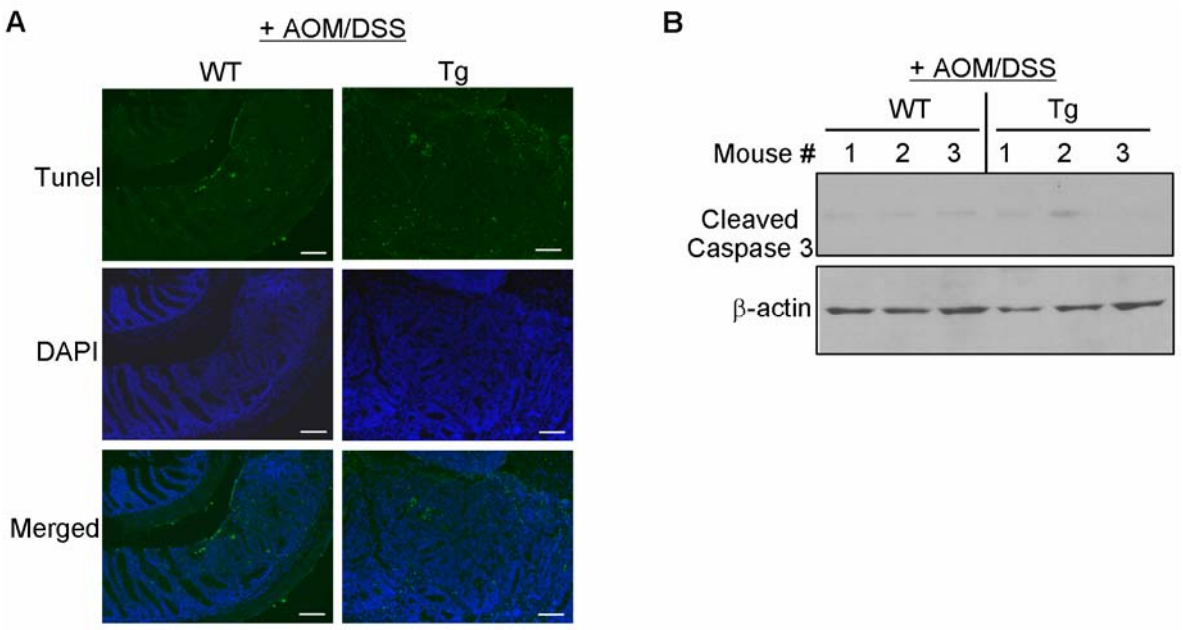


Figure S6. Effects of IEC-specific CD98 over-expression on cell apoptosis in a mouse model of colitis-associated cancer

WT and Tg mice were intraperitoneally injected with AOM (10 mg/kg body weight) and maintained for 5 days, and then subjected to 2 cycles of DSS treatment (1 cycle = 7 days of 2.5% DSS + 14 days of H_2O). IEC apoptosis in AOM/DSS-treated mice was assessed by TUNEL staining of colonic sections (A) and Western blot analysis of cleaved caspase 3 levels (B). *Bars* = 100 μm.

Figure S7

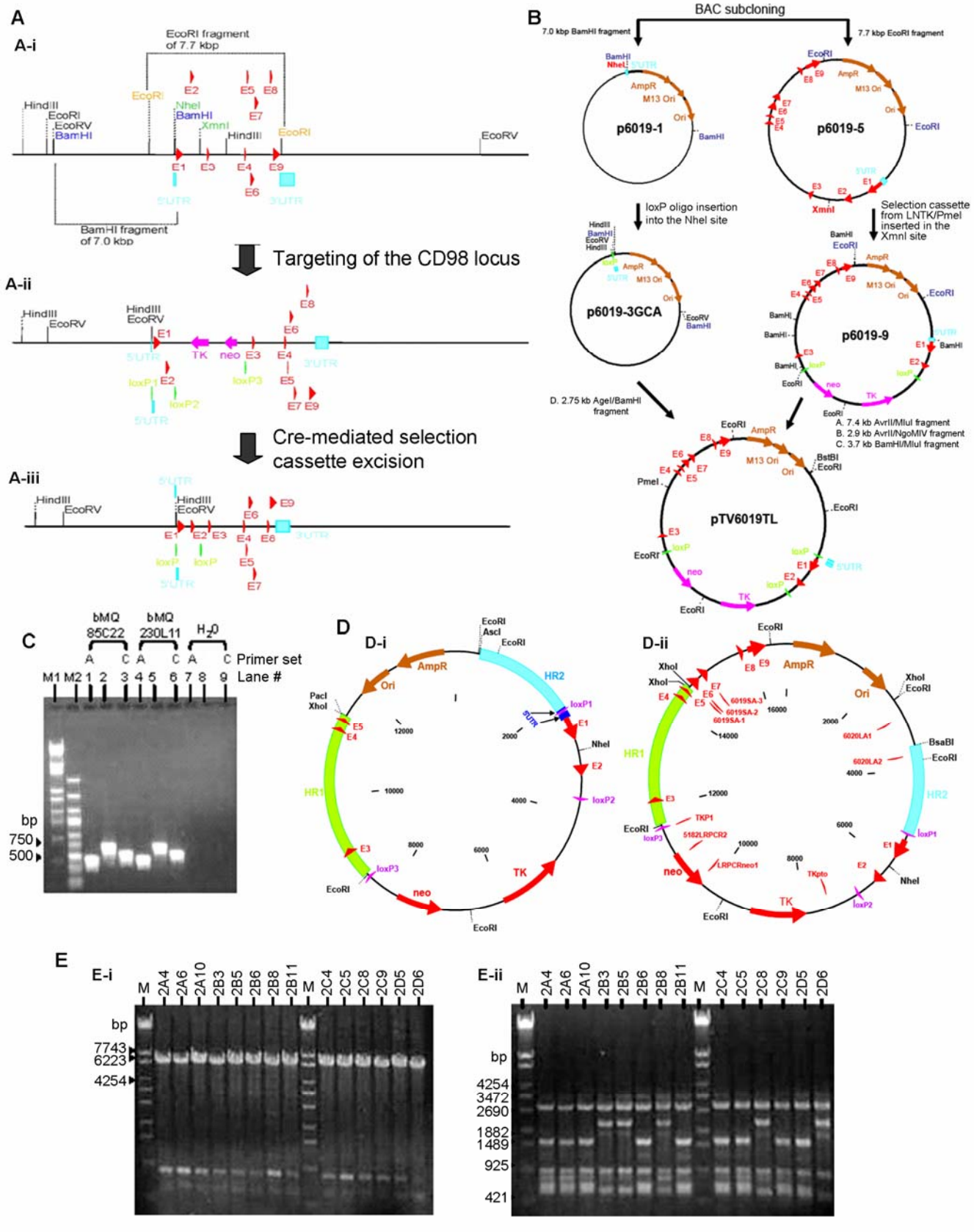

A
A-i
EcoRI fragment of 7.7 kbp
BamHI fragment of 7.0 kbp
Targeting of the CD98 locus
A-ii
Cre-mediated selection cassette excision
A-iii
B
BAC subcloning
7.0 kbp BamHI fragment
7.7 kbp EcoRI fragment
p6019-1
p6019-5
loxP oligo insertion into the NheI site
Selection cassette from LNTK/PmeI inserted in the XmnI site
p6019-3GCA
p6019-9
D. 2.75 kb AgeI/BamHI fragment
A. 7.4 kb AvrII/MluI fragment
B. 2.9 kb AvrII/NgoMIV fragment
C. 3.7 kb BamHI/MluI fragment
pTV6019TL
C
bMQ 85C22
bMQ 230L11
H2O
Primer set
Lane #
bp
750
500
D
D-i
D-ii
E
E-i
E-ii
bp
7743
6223
4254
4254
3472
2690
1882
1489
925
421

Figure S7

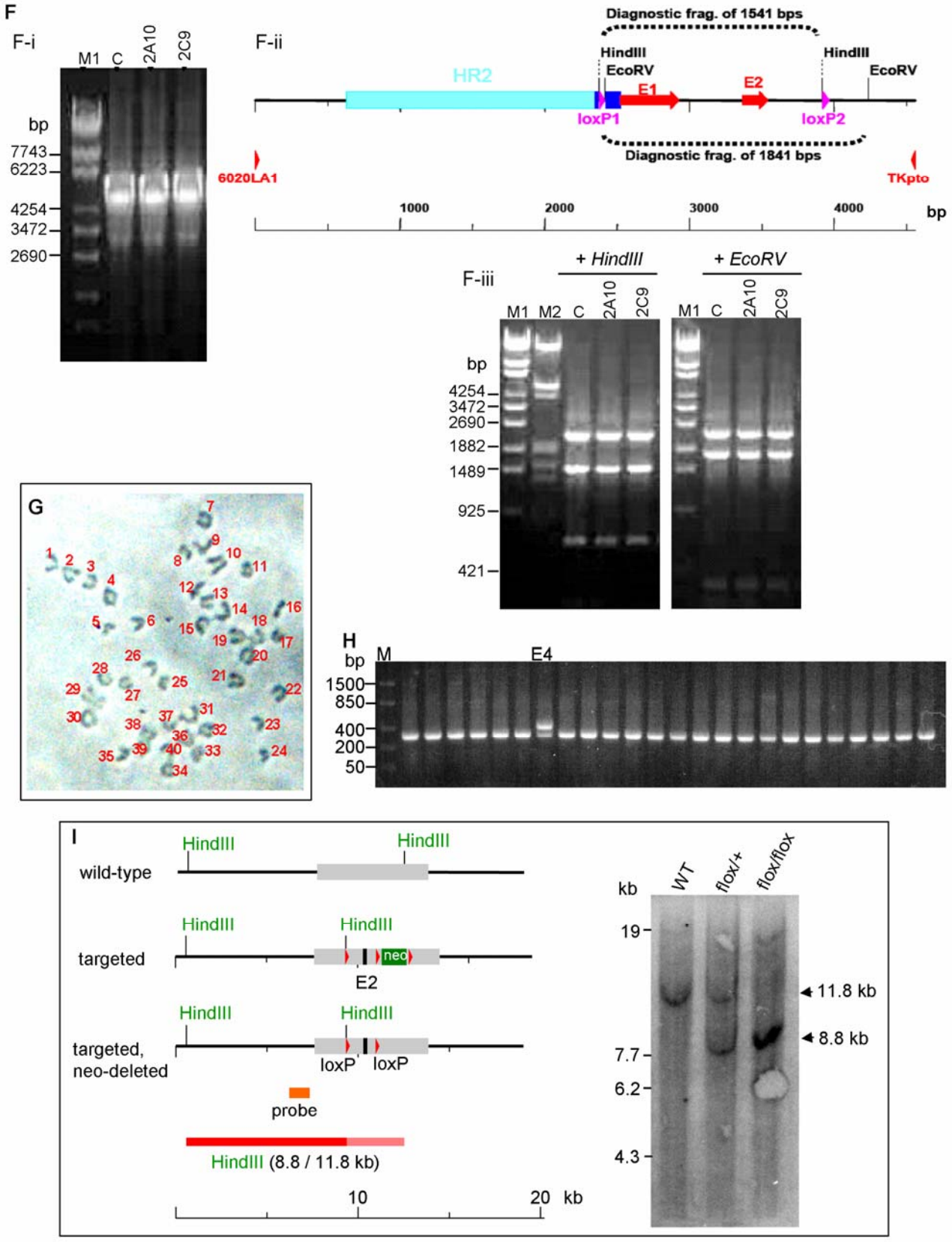

F
F-i
M1
C
2A10
2C9
bp
7743
6223
4254
3472
2690
F-ii
Diagnostic frag. of 1541 bps
HindIII
EcoRV
HR2
E1
E2
HindIII
EcoRV
loxP1
loxP2
Diagnostic frag. of 1841 bps
6020LA1
TKpto
1000
2000
3000
4000
bp
F-iii
+ HindIII
+ EcoRV
M1
M2
C
2A10
2C9
M1
C
2A10
2C9
bp
4254
3472
2690
1882
1489
925
421
G
H
bp
M
E4
1500
850
400
200
50
I
HindIII
HindIII
wild-type
HindIII
HindIII
targeted
neo
E2
HindIII
HindIII
targeted,
neo-deleted
loxP
loxP
probe
HindIII (8.8 / 11.8 kb)
10
20
kb
kb
WT
flox/+
flox/flox
19
7.7
6.2
4.3
11.8 kb
8.8 kb

Figure S7: Generation of CD98$^{flox/flox}$ mice

(A) ***Strategy for conditional targeting of the mouse SLC3A2 gene*****. A-i.** Genomic structure of a 31.4 kbp DNA fragment containing the nine exons (numbered red arrow heads), the 5' and the 3' untranslated regions (5'/3'UTR, blue boxes) of the *SLC3A2* gene, which encodes CD98. To conditionally knock-out this gene, a *loxP* (*loxP1*, green) flanked by *HindIII* and *EcoRV* sites was inserted into the *NheI* site (green, in the 5'UTR). The selection cassette (*loxP*-TK-neo-*loxP)* was cloned into the *XmnI* (green) site downstream of exon 2. To facilitate assembly of the targeting vector, a 7.7 kbp *EcoRI* and a 7.0 kbp *BamHI* fragment was sub-cloned into the plasmid pLitmus28. **A-ii**. Configuration of the targeted *CD98* locus. The *HindIII* and *EcoRV* sites were used to discriminate between the wild-type and the targeted *CD98* locus. **A-iii.** Configuration of the targeted *CD98* locus after removal of the selection cassette by *Cre* recombination, after transient expression of *Cre* recombinase in targeted ES cell clones. "Type II" cells with an excised selection cassette (following *Cre* recombination between the *loxP1* and *loxP2* sites) contain an allele which is suitable for conditional gene inactivation by *in vivo Cre* expression; other recombinatiorial events involving the other *loxP* site do also occur, a complete deletion yielding a constitutively knocked-out allele often being termed "type I".

(B) ***Assembly of the targeting vector to warrant conditional knockout of the SLC3A2 gene.*** The first *loxP* (*loxP1*) sequence was flanked by a new *HindIII* site and *EcoRV* site, thereby allowing the various alleles (targeted and wild-type) of the locus to be distinguished by Southern analysis. For example, *HindIII* digestion of the wild-type CD98 locus yielded an 11.8-kbp fragment when detected with a probe external to the targeting vector sequence, whereas that of the targeted locus yielded a shorter 8.8-kbp fragment.

(C) ***Verification of the BAC clones 85C22 and 23CL11 by PCR.*** The BAC clones were grown and purified using standard procedures. ~5 ng of BAC DNA was used as PCR templates. Expected fragment: 427 bps with primer set A (6019S1 + 6019AS4), 682 bps with primer set B (6019S2 + 6019AS5) and 529 bps with primer set C (6019S3 + 6019AS5).

(D) D-i. ***Scheme of the control vector pTV6019TL***. HR1 (green): Homology region 1; HR2 (blue): Homology region 2; neo (red arrow): Neomycin resistance gene; E1-E7 (small red arrows): Exon 1-Exon 7. The primers used for the screening were shown as thin red arrows. **D-ii.** ***Scheme of the targeting vector pTV6019NEB193***. 5'UTR: 5' untranslated region of the *SLC3A2* gene.

(E) E-i. A PCR with the 6019LA2 and LRPCRneo1 primers was used to verify if the recombination happened correctly in the HR2 (see D-i). All clones tested generated the expected fragment of 4500 bp. **E-ii.** The PCR products shown in panel A were digested with *HindIII* and *XbaI*. Size of the expected fragments (in bp): 2880, 1573, 896, 645, and 581. M: Lambda DNA/*Eco130I* Marker 16 from Fermentas.

(F) Screening PCR to detect correct recombinants in the HR2. **F-i.** Results of the screening PCR. Size of the expected fragment: 4558 bps. C: Positive control, 1 pg of the plasmid pTV6019TL. **F-ii**. The drawing shows the molecular organization of the PCR fragment generated by the primers TKpto and 6020LA1. E1: Exon1, E2: Exon2 (red arrows). **F-ii**. Results obtained after digesting the PCR products shown in F-i with *HindIII* or *EcoRV*. Clones 2A10 and 2C9, which were chosen for the next step, resulted correct. Size of the expected fragments: *HindIII* digest: 2374, 1541 and 644 bps; *EcoRV* digest: 2416, 1819 and 324 bps. M1: Lambda DNA/*Eco130I* Marker 16 from Fermentas Inc.; M2: Lambda DNA *EcoRI/HindIII* digest.

(G) Karyotype of a correct targeted ES cell clone, which contains the correct number of chromosomes (40).

(H) A PCR screening with the 5'CreDel-S1 and 3'CreDel-AS1 primers, which amplified a fragment of 301 bp for the wild-type and a 413 bp amplicon for the type-II-mutant in the positive ES clone (E4). M: Marker, FastRuler Low Range DNA Ladder, Fermentas SM1103.

(I) Confirmation of correct recombination of the floxed allele by Southern blot analysis. Southern blot performed on *HindIII*-digested genomic DNA isolated from WT, $CD98^{flox/+}$ or $CD98^{flox/flox}$ mice using the probe illustrated in the left panel showed the expected WT allele (11.8 kb) and floxed allele (8.8 kb).

See also Supplemental Methods for more detail.

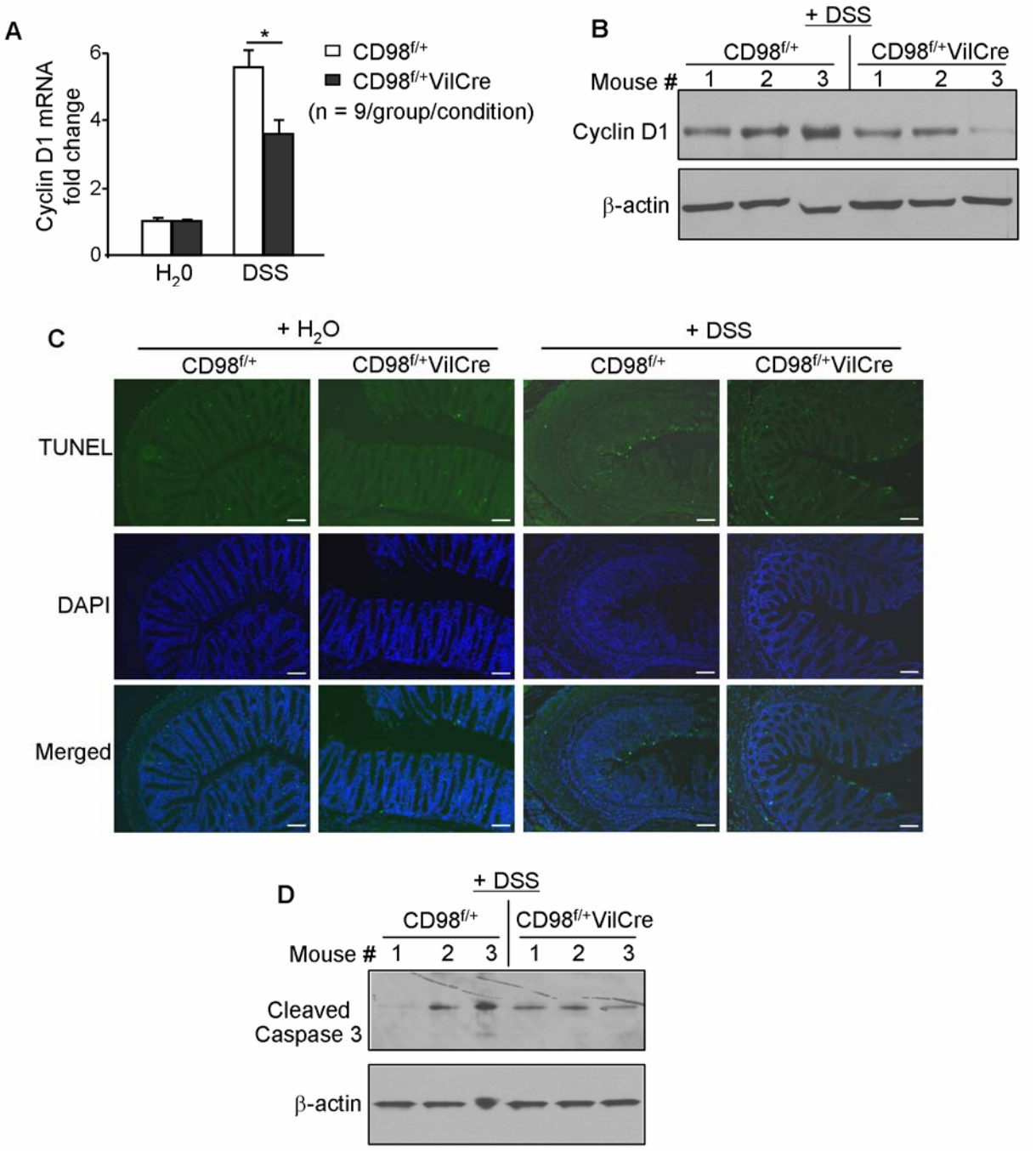


Figure S8. Effects of IEC-specific CD98 down-regulation on cell proliferation and apoptosis in a mouse model of DSS-induced colitis

$CD98^{f/+}$ and $CD98^{f/+}$VilCre mice were administered drinking water (served as control) or 3 % DSS for 9 days. Colonic cyclin D1 mRNA and protein levels in control and DSS-treated mice were assessed by qRT-PCR (A) and Western blot (B), respectively. Apoptosis of colonic epithelial cells in DSS-treated mice was assessed by TUNEL staining of colonic sections (C) and Western blot analysis of cleaved caspase 3 levels (D). Data are means ± S.E.M from one experiment repeated twice with similar results. **P* < 0.05. *Bars* = 100 μm.

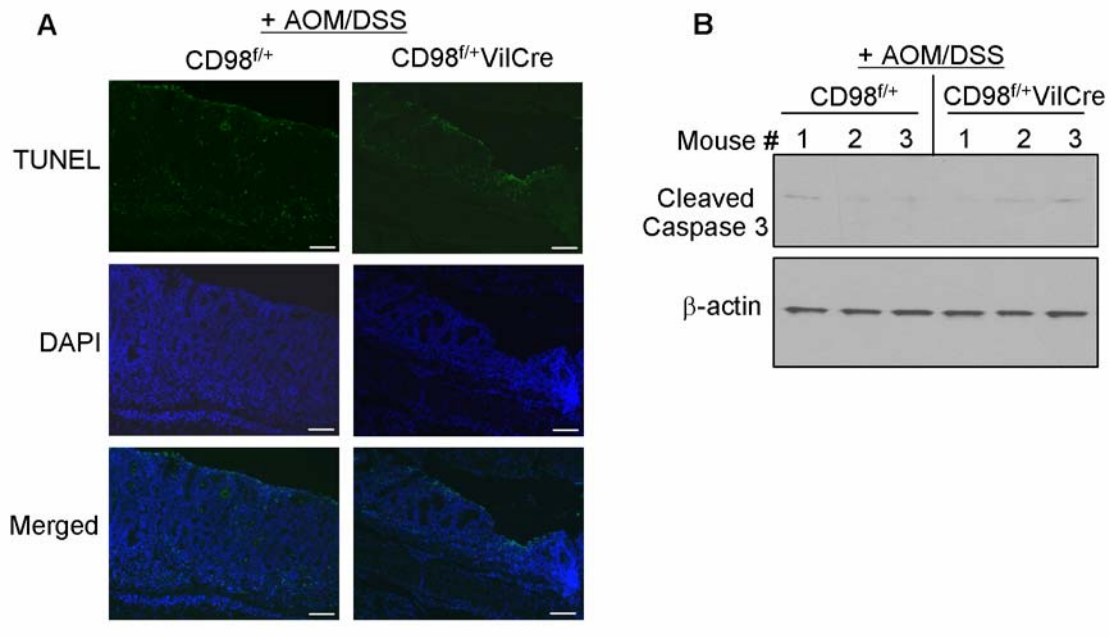


Figure S9. Effects of IEC-specific CD98 down-regulation on cell apoptosis in a mouse model of colitis-associated cancer

$CD98^{f/+}$ and $CD98^{f/+}$VillinCre mice were intraperitoneally injected with AOM (10 mg/kg body weight) and maintained for 7 days, and subjected to 2 cycles of DSS treatment (1 cycle = 8 days of 2.5% DSS + 14 days of H_2O). IEC apoptosis in AOM/DSS-treated mice was assessed by TUNEL staining of colonic sections (A) and Western blot analysis of cleaved caspase 3 levels (B). *Bars* = 100 μm.

Supplemental Methods

Generation of CD98$^{flox/flox}$ mice

CD98$^{flox/flox}$ mice were generated by flanking exons 1 and 2 with *loxP* sites following the steps below.

Strategy for conditional targeting of the SLC3A2 gene

The basic targeting vector concept for *SLC3A2* gene knockout was depicted in Figures S7A. The insertion of a *loxP* site in the 5' untranslated region (*NheI* site), and that of *loxP*-TK-neo-cassette downstream of exon 2 (*XmnI* site) warrants complete deletion of the translation start and of two exons of the *SLC3A2* gene upon Cre-recombination.

1. Molecular Cloning

A 7.7 kbp *EcoRI* and a 7.0 kbp *BamHI* fragment, to be isolated and sub-cloned from (129S7 mouse derived) bMQ-BAC85C22 or bMQ-BAC230L11 into the plasmid pLitmus28, were served as starting materials for the targeting vector construction. These steps gave rise to plasmids p6019-1 and p6019-5, respectively (Figure S7B). The targeting vector was constructed by assembling several intermediate plasmids and stepwise cloning as outlined in Figure S7B. Before electroporation, the vector was linearized by restriction digestion with *BstBI* and *EcoRI.*

Procurement of BAC clones, and verification thereof: Two independent bacterial artificial chromosome (BAC) clones were identified and purchased from Geneservice Ltd, following NCBI database information. The inserts of these BACs (129S7 mouse derived, bMQ-BAC85C22 and bMQ-BAC23CL11) were mapped to contain the entire genomic DNA sequence of the *SLC3A2* gene. The BAC's were PCR verified (Figure S7C) with primers designed to amplify different regions of the *SLC3A2* gene:

6019S1: 5'-GGC AGA AGC AGT TAG GAA GC-3'
6019S2: 5'-AAG GTG GCG GAG GAC GAG AC-3'
6019S3: 5'-GCC GTG GTT ATC ATC GTT CG-3'
6019AS4: 5'-CGC CCG AAC GAT GAT AAC CA-3'
6019AS5: 5'-AAG CCT CCA GCC TCC ACA TT-3'
6019AS6: 5'-GTG AGA CCC TAC CTG AGA AC-3'

The control vector pTV6019TL and the targeting vector pTV6019NEB193: The final targeting vector pTV6019NEB193 was constructed by ligating the large blunt-ended *BsaBI*/*XhoI*-fragment of pTV6019TL into the blunted *BamHI* site of pNEB193 (New England Biolabs Inc.). The maps of both constructs were shown in Figure S7D-i and Figure S7D-ii,

respectively. The targeting vector had homology arms of 3.4 and 3.0 kbp; the singular *loxP* site was placed in the middle of the 3.4 kbp arm.

2. Targeting

Electroporation of the pTV6019NEB193 targeting vector into ES cells: 12 μg of the linearized targeting vector pTV6019NEB193.1 (digested with *AscI* and *PacI*) was electroporated into 13 x 10^6 TC-1 embryonic stem cells (derived from 129SvEvTac blastocysts; Deng et al., 1996), and selected with 0.2 mg/ml G418. After 10 days, 400 G418-resistant clones were picked, transferred into four 96-well tissue culture plates and expanded, prior to splitting and freezing down one set of cells at -80°C, while growing up the rest in replica-plates for production of genomic DNA. PCR screening was performed using the following primers:

5182LRPCR2: 5'-TGA TAA CCC TGA GCC CCC TGA AAC*C-3'
6019SA1: 5'-AAG CAA GGA TAG GAG TGT GAT TAG A-3'
TKP1: 5'-AGC TTG GCT GGA CGT AAA CT-3'
* *Indicates the presence of a phosphorothioate (PTO) bond.*

RT-PCR screening was first performed with the primers 5182LRPCR2 and 6019SA1 (amplicon size 3699 bp), and was repeated for reconfirmation with the primers TKP1 and 6019SA1 (amplicon size 3065 bp), yielding an extremely high percentage of positive clones (116 out of 196 tested). To our knowledge, this is the highest recombination frequency reported in literature, even when compared to enhanced systems as those employing counterselection strategies.

14 PCR-positive clones were thawed, expanded and cultured in duplicate in 24-well dishes. The clones of the master plates were individually frozen, whereas those of the replica plates were used to prepare genomic DNA for further analysis. RT-PCR was used to analyze genomic DNA and determine correct recombination with the following specific primers:

6019LA2: 5'-CCA GGG CAA GGT AGG ACT C*T-3'
LRPCRneo1: 5'-AAT GGG CTG ACC GCT TCC TCG TGC TT*T-3'
* *Indicates the presence of a PTO bond*
Expected amplicon: 6020LA2/LRPCRneo1: 6574 bps

As shown in Figure S7E-i, all of the 14 clones generated amplicons of the expected size (6.57 kbp), confirming again the surprising screening results. Digestion of these fragments with *HindIII* and *XbaI* revealed that in 5 of the clones (2B3, 2B5, 2B8, 2C8, and 2D6), the distal *loxP* site (*loxP1*) was not co-integrated into the *CD98* locus together with the rest of the targeting vector (see Figure S7E-ii), presumably by homologous recombination between the selection

cassette and the distal *loxP* site (i.e., between *loxP1* and *loxP2*). Thus, 9 clones resulted correct: 2A4, 2A6, 2A10, 2B6, 2B11, 2C4, 2C5, 2C9, and 2D5.

Final test by PCR in the homology region 2 (HR2) of the clones 2A10 and 2C9. The screening PCR for correct integration in the HR2 was performed using the primer 6020LA1 and TKpto (Figure S7F).

6020LA1: 5'-TTA GGA AGG GTG CTG CTG AAA AGA CTG-3'
TKpto: 5'-CGA ACT AAA CCT GAC TAC GGC ATC TC*T-3'
* *Indicates the presence of a PTO bond.*

***Counting chromosomes in ES cells*:** Screenings were pursued with the clones that showed the best morphology. As a final test, the karyotypes of a few selected clones were verified. Figure S7G shows a representative example of ES cells from the clone 2A10, which were found to contain the correct number of chromosomes (40) in the majority of the spreads analyzed. Therefore, 2A10 was chosen for the next step, which is the *Cre*-mediated excision of the selection cassette.

3. Selection marker excision by transient *Cre* transfection in targeted ES cells

In a second round of recombination in tissue culture, ES cells were electroporated with pMC-Cre (a constitutive *Cre*-recombinase expressing plasmid) to achieve marker excision, leaving behind a *CD98* gene locus with exons 1 and 2 flanked by *loxP* sites. This step can result in the recombination of any two or all three of the residual *loxP* sites, thus, the proper recombination involving only the sites flanking the selection cassette must be properly selected and screened for.

For this purpose, 5-10 x 10^6 cells from various starting clones (2A10, 2A6, 2D5) were electroporated with 10 µg of pMC-Cre, and replated after 72 h in limiting dilutions und FIAU and/or Gancyclovir selection. This step was surprisingly difficult, yielding only 2 positive clones out of > 300 clones tested, from only one of these clones, while we usually expect about equity of clones representing elimination of the selection cassette and leaving behind two flanking *loxP* sites ("type II"), and clones representing recombination between the external *loxP* sites, equaling a constitutive knock-out ("type I"). Clones from the maternal clone 2D5 were PCR-identified (Figure S7H) using the following primers:

5'CreDel-S1: 5'-GGT TTT GGT TCT CAG GTA GG-3'
3'CreDel-AS1: 5'-GCT GCT TGT GTT GTG CTG AG-3'

These primers amplified a fragment of 301 bp in the wild-type and a 413 bp amplicon for the type-II-mutant.

4. Mouse generation by injection of targeted ES cells into blastocysts

The positive ES cell clones (type-II mutant) were microinjected into C57Bl/6 blastocysts, yielding highly chimeric agouti offspring (F0) from one of the clones (E4). The F0 male chimeras were mated with C57Bl/6 females (The Jackson Laboratory, Bar Harbor ME). About 50% of the agouti F1 offspring showed double bands of WT (301 bp) and mutant (413 bp) in a PCR involving primers 5'CreDel-S1 and 3'CreDel-AS1, evidence for Mendelian distribution of the targeted locus. The strain was congenically rebred to C57Bl/6 for 8 generations, and then interbred to select homozygous $CD98^{f/f}$ mice. $CD98^{f/f}$ mice were maintained to cross with VillinCre mice (JAX B6.SJL-Tg(Vil-Cre)997Gum/J, Stock # 004586, The Jackson Laboratory, Bar Harbor ME). The resulting offspring were genotyped by PCR of the genomic DNA extracted from tail snips for expression of $CD98^{flox}$ allele using the 5'CreDel-S1 and 3'CreDel-AS1 primers, and for expression of VillinCre transgene using a combination of the following primers:

oIMR0015: 5'-CAA ATG TTG CTT GTC TGG TG-3'
oIMR0016: 5'-GTC AGT CGA GTG CAC AGT TT-3'
oIMR1878: 5'-GTG TGG GAC AGA GAA CAA ACC-3'
oIMR1879: 5'-ACA TCT TCA GGT TCT GCG GG-3'

Cre-mediated recombination of the $CD98^{flox}$ allele was verified by PCR analysis of genomic DNA from IECs extracted from different parts of the gastrointestinal tract and from control tissues using a combination of three primers: 5'CreDel-S1, 3'CreDel-AS1 and the Cre-recombination primer 5'-GGGATAGACGGGAGTATTC-3'. PCR could give three bands of WT (301 bp), mutant (413 bp), and the 356-bp product, which indicates recombination of the floxed *slc3a2* allele.

Southern blot performed on *HindIII*-digested mouse genomic DNA for confirmation of correct homologous recombination of the floxed *slc3a2* allele.

Southern hybridization was performed using genomic DNA extracted from mouse livers as previously described (Laird et al., 1991). Twenty μg of genomic DNA were digested with *HindIII* overnight, electrophoresed on 0.7% SeaKem LE agarose in TAE, and capillary alkaline transferred to a BioRad Zeta-Probe GT membrane. The hybridization probe was PCR generated with oligonucleotide primers 6019.P1: 5'-CTC CTC TGT GCC TGG TTT ATG-3' and 6019.P2:

5'-CAC TGC CCT CCA CAA AGT AAG-3' on genomic DNA, yielding a 561 bp band. The fragment was purified with a commercial kit (GeneClean Turbo, Q-Biogene), labeled with the Stratagene Prime-It RmT Random Primer Labeling Kit according to manufacturer's instructions, and purified using Amersham Sephadex columns. Hybridization was performed in the presence of Sheared Salmon Sperm DNA with the Stratagene QuickHyb hybridization solution for 90 min at 68°C, the blots were washed in 0.1 x SSC/0.1% SDS at 60°C for 30 min, and again for 45 min at 65°C. The blots were exposed overnight on a Phosphor Storage Screen and read on a Molecular Dynamics Storm 860 PhosphorImager.

In vivo permeability assay

Barrier function was assessed using the permeability tracer FITC-labeled dextran. Age- and sex-matched mouse littermates were deprived of food and water for 4 h, and gavaged with 4 kD FITC-labeled dextran (Sigma-Aldrich Corp) at 0.6 mg/g of body weight. Blood was retro-orbitally collected after 3 h, and fluorescence intensity in serum was measured (excitation, 492 nm; emission, 525 nm; Cytofluor 2300; HITACHI F-4500 Fluorescence Spectrophotometer). FITC-dextran concentrations were determined from a standard curve generated by serial dilutions of FITC-dextran.

RT-PCR and qRT-PCR

Total RNA extracted using TRIzol (Invitrogen) or RNeasy Mini Kit (Qiagen) was reverse transcribed using the first strand cDNA synthesis kit (Fermentas). RT-PCR was performed using the GeneJET Fast PCR kit (Fermentas). qRT-PCR was performed using SYBR Green qPCR Master Mix (Fermentas) on a Mastercycler Realplex^4 (Eppendorf). 36B4 was used as housekeeping gene. Fold-induction was calculated using the *C*t method: $\Delta\Delta Ct = (Ct_{\text{Target gene}}\text{-}Ct_{\text{housekeeping gene}})_{\text{treatment}} - (Ct_{\text{Target gene}}\text{-}Ct_{\text{housekeeping gene}})_{\text{non-treatment}}$, and the final data were derived from $2^{-\Delta\Delta Ct}$.

Primers used for RT-PCR and qRT-PCR:

human CD98 for: 5′-CCAGGTTCGGGACATAGAGA-3′
human CD98 rev: 5′-GAGTTAGTCCCCGAAATCAA-3′

β-actin for: 5′-GTCACCCACACTGTGCCCATC-3′
β-actin rev: 5′-ACGGAGTACTTGCGCTCAGGA-3′

KC for: 5'-TTGTGCGAAAAGAAGTGCAG-3'

KC rev: 5'-TACAAACACAGCCTCCCACA-3'

IL-1β for: 5'-TCGCTCAGGGTCACAAGAAA-3'
IL-1β rev: 5'-CATCAGAGGCAAGGAGGAAAAC-3'

IL-6 for: 5'-ACAAGTCGGAGGCTTAATTACACAT-3'
IL-6 rev: 5'- TTGCCATTGCACAACTCTTTT C-3'

TNF-α for: 5'-AGGCTGCCCCGACTACGT-3'
TNF-α rev: 5'-GACTTTCTCCTGGTATGAGATAGCAAA-3'

IFN-γ for: 5'-CAGCAACAGCAAGGCGAAA-3'
IFN-γ rev: 5'-CTGGACCTGTGGGTTGTTGAC-3'

MCP-1 for: 5'-ACTGAAGCCAGCTCTCTCTTCCTC-3'
MCP-1 rev: 5'-TTCCTTCTTGGGGTCAGCACAGAC-3'

Mouse CD98 for: 5′-GAGGACAGGCTTTTGATTGC-3′
Mouse CD98 rev: 5′-ATTCAGTACGCTCCCCAGTG-3′

MIP-2 for: 5′-CACTCTCAAGGGCGGTCAAA-3′
MIP-2 rev: 5′-TACGATCCAGGCTTCCCGGGT-3′

Cyclin D1 for: 5′-CAGACGTTCAGAACCAGATTC-3′
Cyclin D1 rev: 5′-CCCTCCAATAGCAGCGAAAAC-3′

36B4 for: 5'-TCCAGGCTTTGGGCATCA-3'
36B4 rev: 5'-CTTTATCAGCTGCACATCACTCAGA-3'

Intestinal epithelial cell isolation

IEC isolation was performed as previously described (Flint et al., 1991).

Western blot

Cell lysates were resolved on polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). Membranes were probed with relevant primary antibodies followed by incubation with appropriate HRP-conjugated secondary antibodies (Amersham Biosciences). Blots were detected using the Enhanced Chemiluminescence Detection kit (Amersham Biosciences).

ELISA

Serum TNF-α levels were quantified using the TNF-α RSG ELISA kit (eBiosciences).

TUNEL assay

Colonic sections were deparaffinized, and apoptotic cells were detected by immunofluorescent terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) using the In Situ Cell Death Detection Kit (Roche Applied Science). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen). Sections were mounted and photographed as described above.

Myeloperoxidase (MPO) assay

Colonic tissues were homogenized in ice-cold potassium phosphate buffer (50 mM K_2HPO_4 and 50 mM KH_2PO_4 (pH 6.0)) containing 0.5% hexadecyltrimethylammonium bromide (Sigma). The homogenates were then sonicated, freeze-thawed for three times, and centrifuged at 14,000 rpm for 15 min. Supernatants (20 μl) or MPO standard were added to 1 mg/ml *o*-dianisidine hydrochloride (Sigma) and 0.0005% H_2O_2, and the change in absorbance at 460 nm was measured. One unit of MPO activity was defined as the amount that degraded 1 μmol of peroxidase per minute. The results were expressed as absorbance per milligram of protein.

Supplemental Reference

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