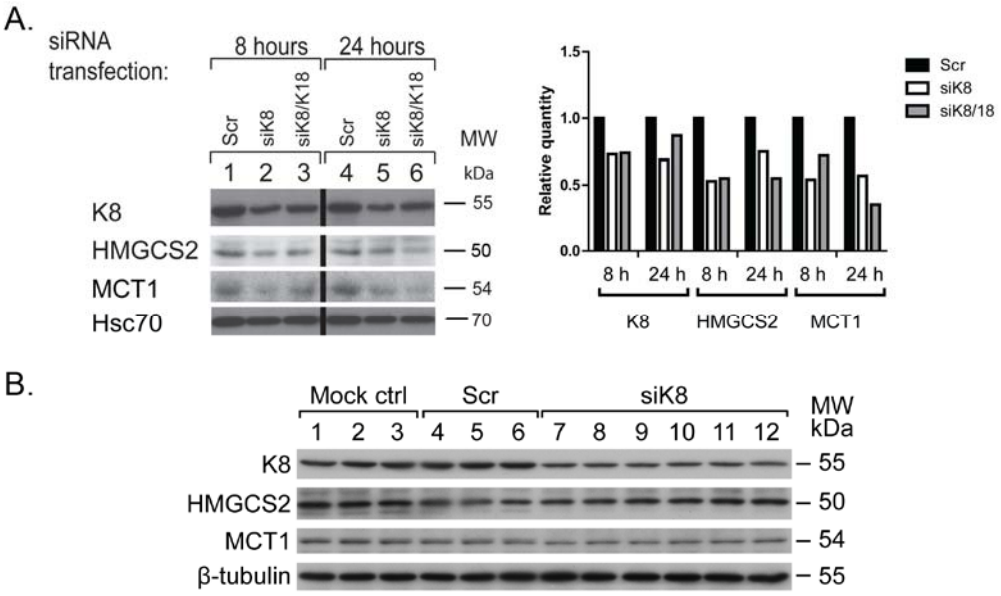
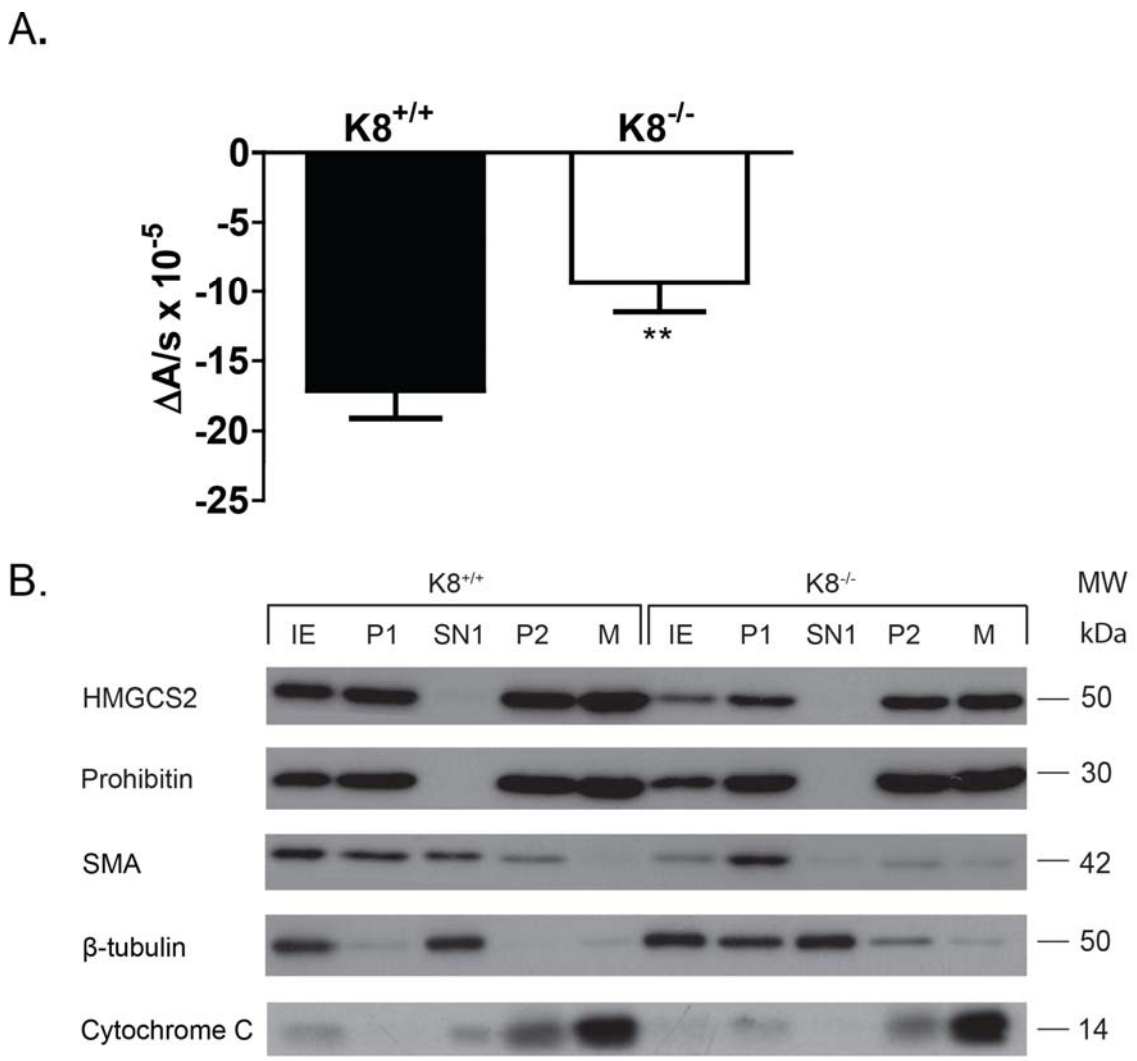
Supplemental Materials

Molecular Biology of the Cell

Helenius et al.

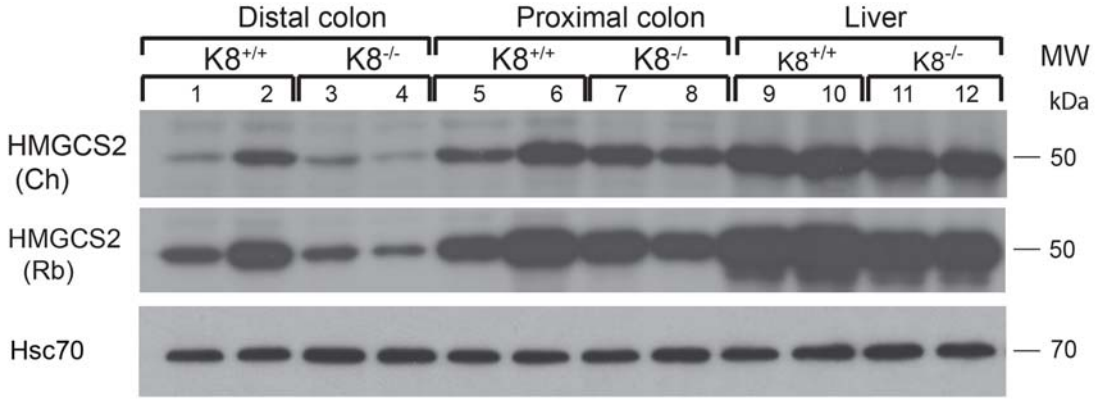


Supplemental figure 1. Silencing of K8 and K18 downregulates MCT1 and HMGCS2 expression in HT-29 and Caco-2 cells. (A) HT-29 cells were transfected for 8 and 24 hours with: scrambled siRNA control (Scr), siRNA keratin 8 (siK8, Abnova) or a combination of siRNA for K8 and siRNA K18 (siK8 + siK18). HT-29 cell lysates were analyzed by immunoblotting for K8, HMGCS2 and MCT1. Equal loading is shown by Hsc70. (B) Caco-2 cells were mock-transfected (all reagents except siRNA), transfected with scrambled siRNA control (Scr) or siRNA K8 (siK8, Eurofins Genomics). Caco-2 cell lysates were analyzed by immunoblotting for K8, HMGCS2 and MCT1. Equal loading is shown by β-tubulin.



Supplemental figure 2. Absence of K8 leads to diminished HMGCS2 enzyme activity in $K8^{-/-}$ mice. HMGCS2 activity was determined in $K8^{+/+}$ and $K8^{-/-}$ mice by measuring the loss of absorbance that occurs at 303 nm as HMGCS2 converts acetoacetyl-CoA and acetyl-CoA to HMG-CoA and CoA. The enzyme activity was calculated for $K8^{+/+}$ and $K8^{-/-}$ and expressed as change in absorbance per second (ΔA/s). The results are based on three independent experiments (each experiment involving one $K8^{+/+}$ and one $K8^{-/-}$ mouse) and represent the mean ± SD (** = $p < 0.01$) (A). Lysates of isolated colonic epithelium and different fractions collected during the isolation of mitochondria were normalized by protein assay and analyzed by immunoblotting for HMGCS2, prohibitin, SMA, β-tubulin and cytochrome c. Although $K8^{+/+}$ and $K8^{-/-}$ epithelia are characterized by different morphologies due to long crypts in $K8^{-/-}$, very comparable fractions were obtained from both genotypes (compare prohibitin and cytochrome c protein levels between $K8^{+/+}$ and $K8^{-/-}$). The down-regulation of HMGCS2 in $K8^{-/-}$ is clearly seen in isolated epithelium and in isolated mitochondria in comparison to $K8^{+/+}$. This decrease is not due to fewer mitochondria because the levels of the mitochondrial markers prohibitin and cytochrome c are unchanged in $K8^{-/-}$ in

comparison to $K8^{+/+}$. The purity of isolated mitochondria was assessed by detection of muscle (SMA) and cytoplasm (β-tubulin) and was found negligible. IE = isolated epithelium, P1 and P2 = pellet 1 and 2, SN1 = supernatant 1 and M = isolated mitochondria (B).

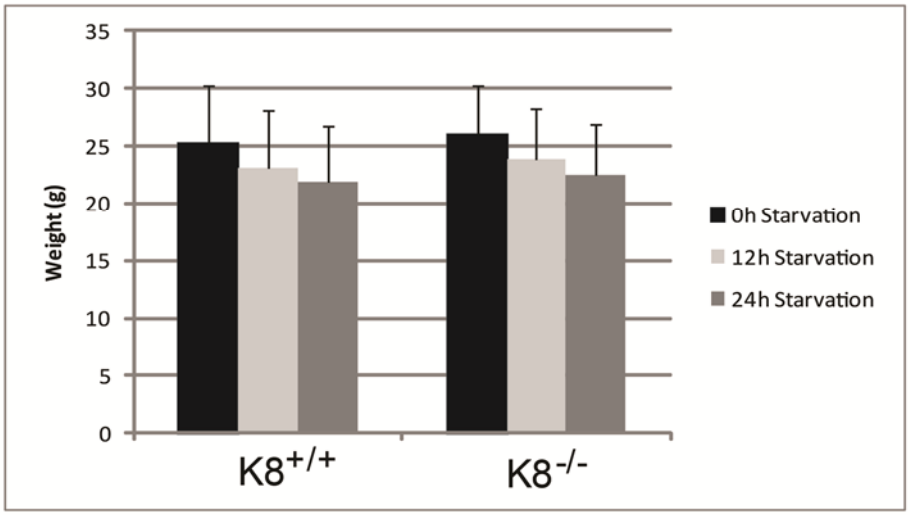


Supplemental figure 3. HMGCS2 is expressed at different levels in an organ-specific manner. Total lysates of distal and proximal colon and liver were obtained from $K8^{+/+}$ and $K8^{-/-}$ mice. Lysates were normalized by protein assay and analyzed by immunoblotting. Equal loading is shown by Hsc70. The levels of HMGCS2 were compared using two different HMGCS2 antibodies from two different hosts, chicken (Ch) and rabbit (Rb). The highest expression levels of HMGCS2 are observed in liver, the main ketogenic organ. Lower levels of HMGCS2 are expressed in the colon, with higher levels in the proximal than distal colon. However, HMGCS2 is down-regulated in $K8^{-/-}$ distal and proximal colon, whereas no down-regulation is seen in $K8^{-/-}$ liver.

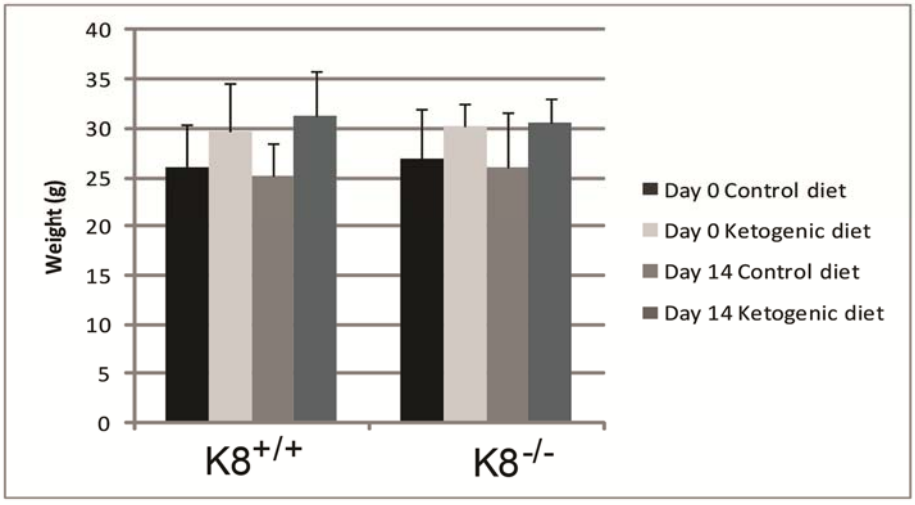
A.

Genotype/diet	Food ingested (g)		
	Female	Male	Female + Male
$K8^{+/+}$ Control diet	48.0 ± 0.9	50.9 ± 3.7	49.4 ± 2.9
$K8^{-/-}$ Control diet	48.6 ± 2.4	51.2 ± 7.4	49.6 ± 4.3
$K8^{+/+}$ Ketogenic diet	42.9 ± 9.2	41.3 ± 3.1	42.3 ± 6.7
$K8^{-/-}$ Ketogenic diet	40.3 ± 2.5	41.2 ± 2.9	40.7 ± 2.5

B.

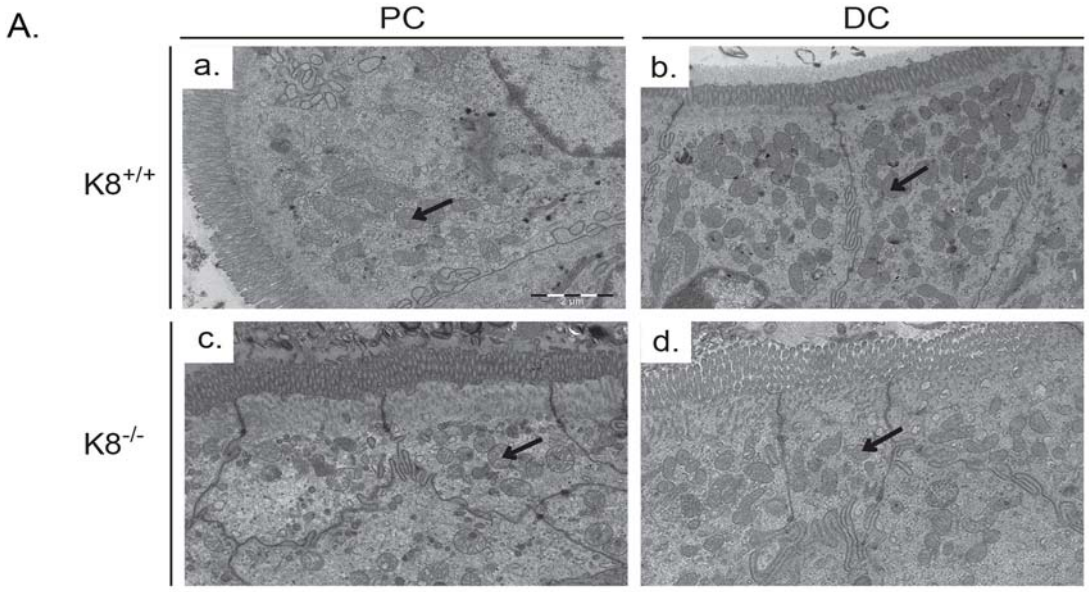


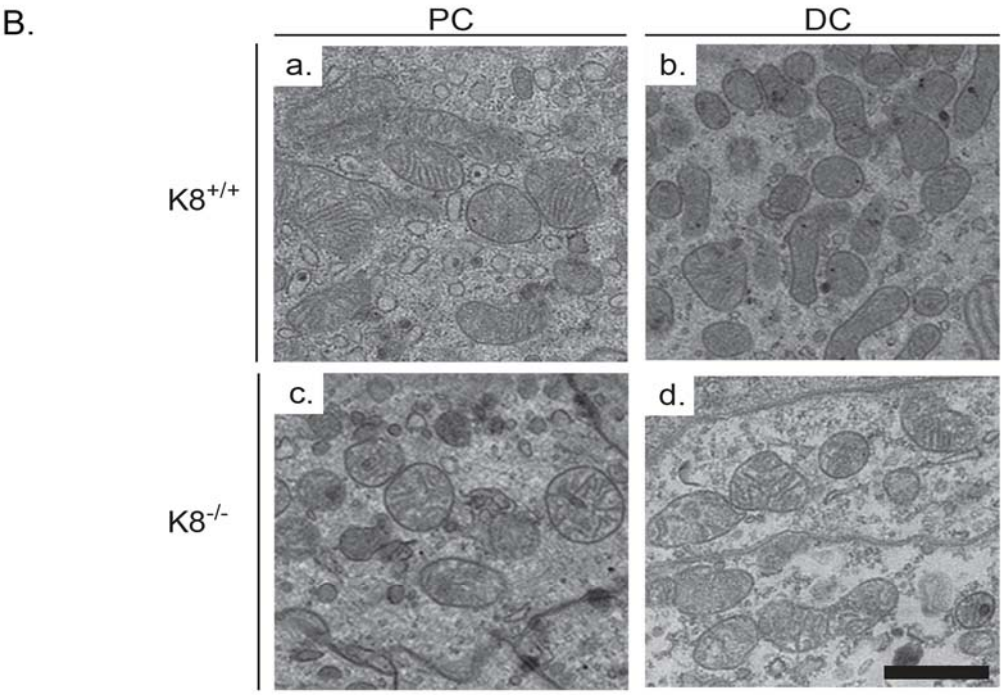
C.



Supplemental figure 4. Food ingestion and mouse body weight changes under ketogenic conditions are similar in $K8^{+/+}$ and $K8^{-/-}$. The amount of food ingested (A) by the different genotypes and gender groups during the control and ketogenic diet periods were calculated based on the daily food intake. The body weights of $K8^{+/+}$ and

$K8^{-/-}$ mice were measured after 0, 12 and 24 hours of starvation (B) and 0 and 14 days after onset of a ketogenic diet (C).



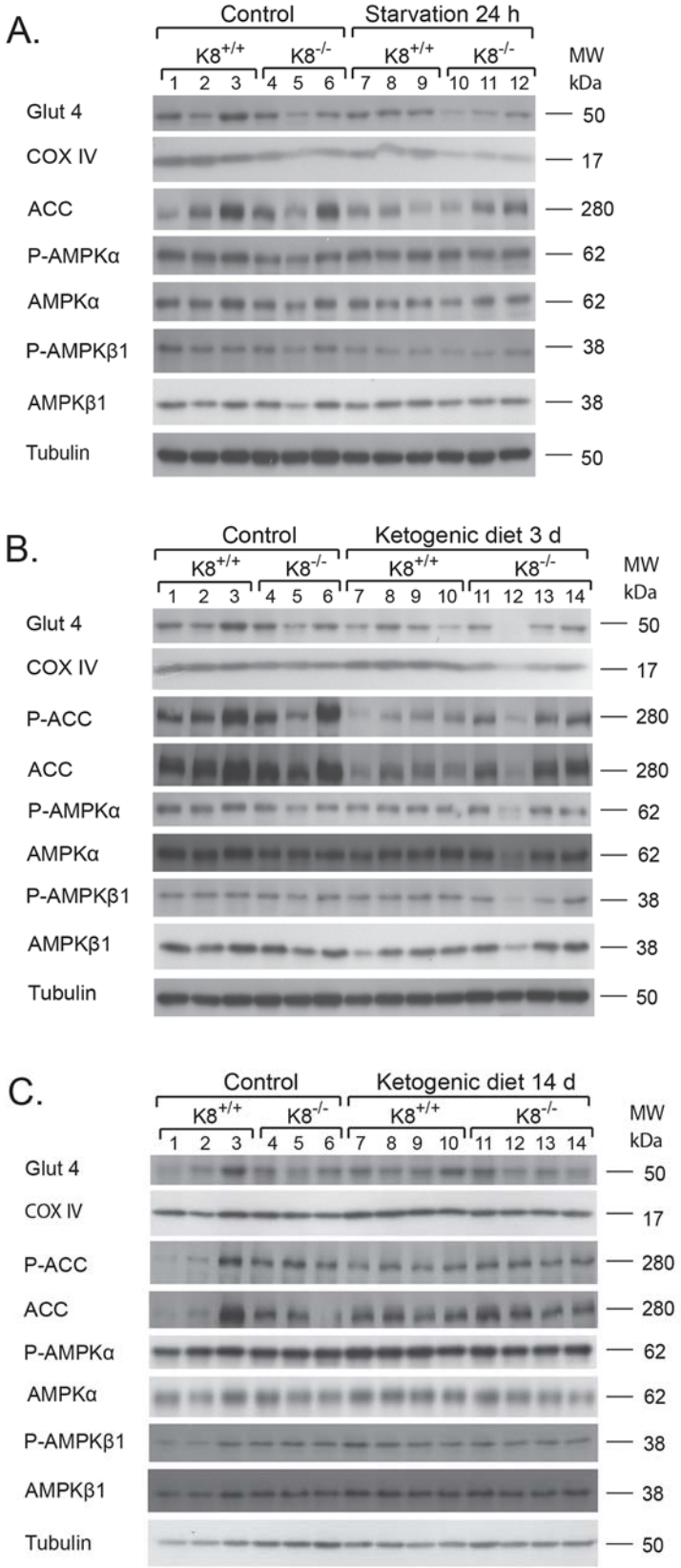


C.

Relative number of cristae per mitochondria	PC	DC
$K8^{+/+}$	1.00 ± 0.2	1.00 ± 0.25
$K8^{-/-}$	0.67 ± 0.2*	0.98 ± 0.18

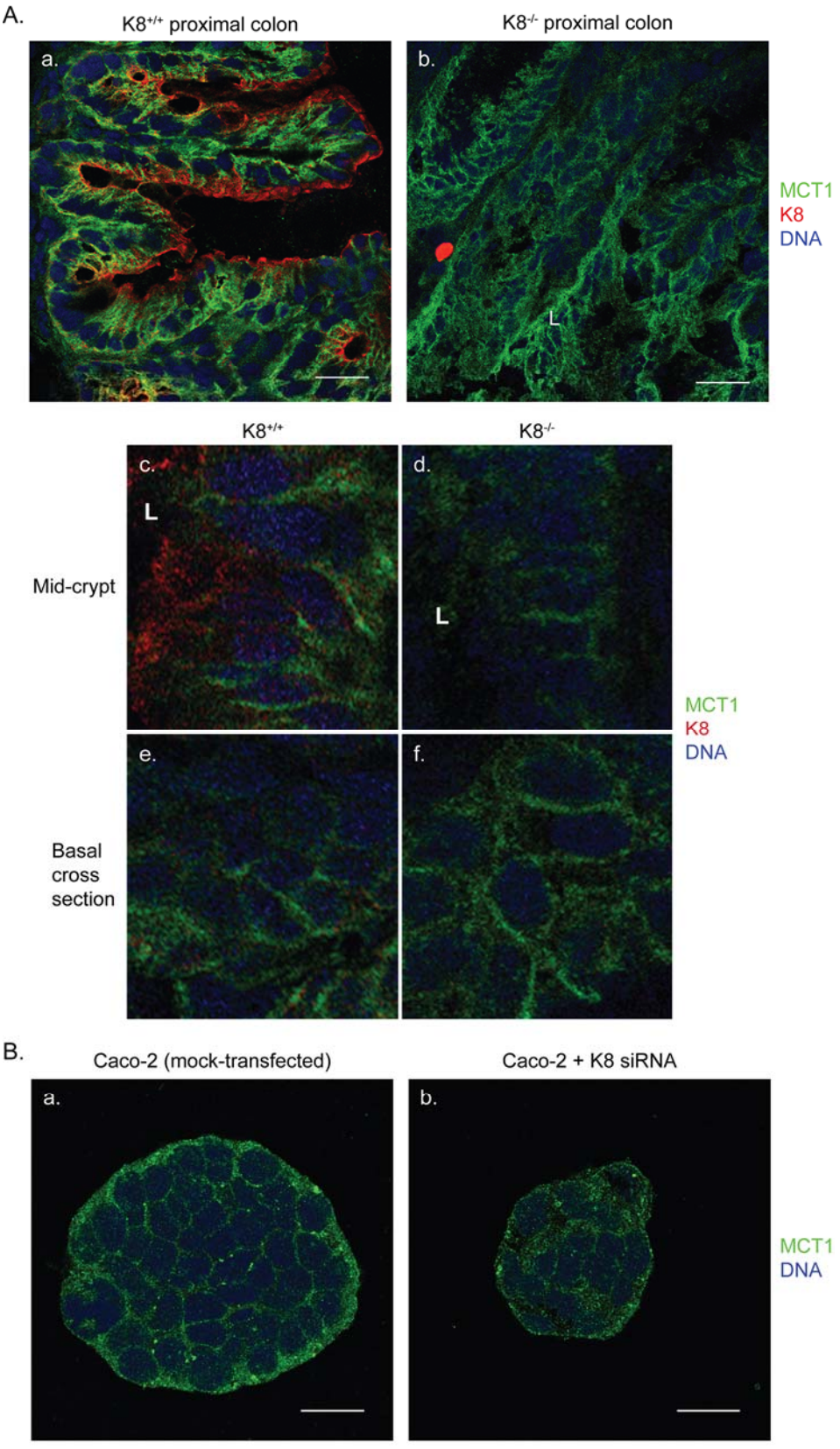
Supplemental figure 5. $K8^{-/-}$ colonocyte mitochondria are normal size and location but have fewer cristae. Colonocyte mitochondria were analyzed in $K8^{+/+}$ and $K8^{-/-}$ PC and DC by transmission electron microscopy (A, B, C). No differences were seen in mitochondrial size or localization, whereas there was a significantly lower number of

cristae per mitochondria section area in $K8^{-/-}$ PC (C) compared to $K8^{+/+}$. n = 3 mice, 6 mitochondria/genotype, average ± SD, *= $p<0.05$ compared to $K8^{+/+}$ PC.



Supplemental figure 6. Minor differences in energy homeostasis between $K8^{-/-}$ mice subjected to normal and ketogenic conditions. Total lysates of equal parts of distal and

proximal colons from $K8^{+/+}$ and $K8^{-/-}$ mice subjected to normal and different ketogenic conditions (A: 24 h of starvation, B: 3 days of ketogenic diet, C: 14 days of ketogenic diet) were analyzed for a panel of proteins related to cellular energy metabolism.



Supplemental figure 7. MCT1 localization remains unchanged while the levels of MCT1 are decreased in K8$^{-/-}$ proximal colon and in K8 siRNA-treated Caco-2 cells. A. K8$^{+/+}$ (a, c, e) and K8$^{-/-}$ (b, d, f) proximal colon were cryosectioned and analyzed by immunostaining for MCT1 (green), K8 (red) and DNA (blue). The blown-up images in c-f show representative areas of mid-crypt lateral plasma membrane MCT1 staining, and MCT staining in the plasma membrane near the basal part of crypt cells from K8$^{+/+}$ and K8$^{-/-}$ distal colon, showing similar MCT1 distribution independent of the presence of K8. B. Caco-2 cells were mock-transfected (a) or K8 siRNA-treated (b) for 72 hours and analyzed by immunostaining for MCT1 (green), and nuclei (blue). K8 siRNA treatment decreases K8-levels with 50-70% (see Supplemental Figure 1B. Scale bars: 25 μm, L = lumen.

Supplemental Table 1.List of differentially expressed proteins in K8$^{-/-}$ colonocytes

Spot number	Accession number	Down-regulated protein ID in K8$^{-/-}$	Ratio of change	Role/location
365	Q811J3	Iron-responsive element-binding protein 2	-1.8	regulation of iron metabolism /cytoplasm
368		Iron-responsive element-binding protein 2	-1.7	
623	P53690	Matrix metalloproteinase 14	-1.2	breakdown of extracellular matrix/cytoplasm, membrane
906	P17563	Selenium binding protein	-4.5	selenium binding/cytoplasm, membrane, nucleus
916	P26443	Glutamate dehydrogenase	-1.7	ATP-GTP-binding, NAD+ and NAD(P)+ activity/mitochondrial inner membrane, mitochondrial matrix
1032	P54869	HMGCS2	-3.3	ketone bodies production/mitochondrion
1033		HMGCS2	-3.4	
1035		HMGCS2	-4.7	
1044		HMGCS2	-4.2	
1637	P34896	Serine hydroxymethyl-transferase	-2.1	conversion of serine and glycine/cytoplasm
1840		Phosphoglyceratedehydratase 1	-3.9	lyase/cell membrane, cytoplasm, membrane
1872	P13634	Carbonic	-1.8	one-carbon metabolic

		anhydrase I		processes/cytoplasm
1889		Carbonic anhydrase I	-8.1	
1933	Q8BU95	Enoylcoenzyme A hydratase 1	-2.6	fatty acids metabolism/mitochondrion
1970	P11352	Glutathione peroxidase	-2.7	protection from oxidative damage/cytoplasm
2058	P08228	Superoxide dismutase	-1.7	protection from oxidative damage/cytoplasm

Spot number	**Accession number**	**Up-regulated protein ID in K8$^{-/-}$**	**Ratio of change**	**Role/location**
60	Q61316	Heat shock 70-related protein APG-2	1.2	chaperone-mediated protein complex assembly/cytoplasm
132	BC010445.1	GRP94/ERP99	1.3	processing and transport of secreted proteins /endoplasmatic reticulum
204	Q01853	Valosin containing protein	1.2	transport and fusion of vesicles/cytoplasm endoplasmic reticulum, nucleus
276	Q9DBT9	Dimethylglycine dehydrogenase	2.1	amino acids metabolism/ mitochondrion
277	Q5EBH4	Dimethylglycine dehydrogenase	1.5	amino acids metabolism/ mitochondrion
438	Q9JKX3	Transferrin receptor protein 2	1.9	regulation of iron levels in blood/ cell membrane, cytoplasm, membrane
439		Transferrin receptor protein 2	2.7	
444		Transferrin receptor protein 2	4.0	
460	Q8BZA7	GRP78	1.4	Endoplasmatic reticulum integrity and stress induced autophagy/cell membrane, membrane
482	P38647	GRP75	1.2	transport of mitochondrial proteins from the cytoplasm to mitochondria/mitochondrion, nucleolous
486		GRP75	1.2	
522	NP_112442	Hsc71	1.5	chaperone
526		Hsc71	1.1	
543		Hsc71	1.3	
749	P63038	Hsp60	1.5	chaperone/mitochondrion matrix

752		Hsp60	1.5	
755		Hsp60	0.1	
952	P47738	Mitochondrial ALDH	1.2	oxidationof aldehydes/ mitochondrion
1093	P16460	Argininosuccinate synthase 1	2.0	arginine biosynthetic pathway/mitochondrion
1096		Argininosuccinate synthase 1	2.0	
1424	Q64467	Glyceraldehyde 3-phosphate dehydrogenase	1.6	glycolysis/cytoplasm
1425	P16858	Glyceraldehyde 3-phosphate dehydrogenase	1.3	glycolysis/cytoplasm, cytoskeleton, nucleus

Supplemental table 1.Complete list of differentially expressed proteins in $K8^{-/-}$ colonocytes. In the $K8^{-/-}$colonocytes, major down-regulated proteins were as expected K8 and K19 and their fragments. When compared to $K8^{+/+}$, the novel most down-regulated protein in $K8^{-/-}$ was the rate-limiting enzyme of ketogenesis, HMGCS2, and its 4 isoforms (spots 1032, 1033, 1035 and 1044).