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Disruption of the potassium channel regulatory subunit Kcne2 causes iron-deficient anemia

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Running title: Iron-deficient anemia in *Kcne2* mutant mice

Keywords: iron, anemia; gastric pH; Kcne2

Abstract

Iron homeostasis is a dynamic process that is tightly controlled to balance iron uptake, storage and export. Reduction of dietary iron from the ferric to the ferrous form is required for uptake by solute carrier family 11 (proton-coupled divalent metal ion transporters) member 2 (Slc11a2) into the enterocytes. Both processes are proton dependent, and have led to the suggestion of the importance of acidic gastric pH for the absorption of dietary iron. Kcne2, in combination with Kcnq1, form a gastric potassium channel essential for gastric acidification. Deficiency of either *Kcne2* or *Kcnq1* results in achlorhydia, gastric hyperplasia and neoplasia, but the impact on iron absorption has not been investigated. Here we report that *Kcne2* deficient mice, in addition to the previously reported phenotypes, also present with iron-deficient anemia. Interestingly impaired function of KCNQ1 results in iron deficient anemia in Jervell and Lange-Nielsen syndrome patients. We speculate that impaired function of KCNE2 could result in the same clinical phenotype.

Introduction

Iron is imperative for human health and defects in iron homeostasis are known to result in serious pathological abnormalities such as hemochromatosis and anemia. This dynamic process requires a constant balance of iron achieved by both intake of dietary iron and successful co-ordination of iron uptake, export, and storage. Iron-deficient anemia can be caused by a lack of dietary iron, blood loss or a physiological defect affecting iron bioavailability, uptake, or transfer into the circulation. The majority of dietary iron is in the ferric form and requires reduction to the ferrous form prior to being transported by Slc11a2 located in the brush border of the enterocytes [1].

Kcne2 is a single-pass integral membrane β-subunit of a potassium ion-channel and assembles with various α-subunits. In a heterotrimeric channel with Kcnq1, Kcne2 forms a constitutive potassium ion-channel at the apical membrane of gastric parietal cells [2]. This Kcne2/Kcnq1 potassium channel provides a constant source of potassium ions into the stomach lumen, which are used by the gastric K^+/H^+-ATPase to pump hydrogen ions into the stomach lumen [3]. Point mutations in *KCNE2* have been shown to cause Long QT Syndrome 6 [4], a phenotype recapitulated in knockout mouse models of *Kcne2* [5]. In addition, *Kcne2* deficient mice have been reported to have gastric hyperplasia and neoplasia, achlorhydria [3,

6], anemia [7] and hypothyroidism [8]. Gastric pH has been suggested to be a critical determinant for dietary iron absorption, a theory supported by the observation that the *sublytic* mouse model, with a point mutation in *Atp4a* (K^+/H^+-ATPase α-subunit), has increased gastric pH and iron-deficient anemia [9].

In this study we have generated a targeted gene trap for *Kcne2* and identified that mutant male animals suffer from iron-deficient anemia.

Materials and methods

Animals

Generation of the *Kcne2*$^{tm1a(EUCOMM)Wtsi}$ allele (hereafter referred to as *Kcne2*^{tm1a}) was performed as part of the EUCOMM/KOMP projects and Sanger Mouse Genetics Project [10]. Mice were generated from ES cell clone EPD0156_2_F10 and backcrossed to C57BL/6N females with genotyping carried out as previously described [11]. Animals were housed in specific pathogen-free conditions and placed on high fat diet (Western RD 829100, Special Diet Services, U.K) from four weeks of age with *ad libitum* access to autoclaved non-acidified water and food and phenotyped according to a standard pipeline as previously reported [12]. All experiments were performed in accordance with the UK Home Office regulations, UK Animals (Scientific Procedures) Act 1986.

Blood sample collection

At 16 weeks, blood was collected by puncture of the retro-orbital sinus under terminal anaesthesia within 1-3 hours of lights on, and collected into EDTA-coated tubes (Kabe Labortechnik GmbH, Numbrecht, Germany) for hematology (Scil Vetabc, Montpellier, France) and into heparinised tubes (Kabe Labortechnik GmbH) for plasma preparation. A total of 26 parameters were determined from plasma using

an Olympus AU400 analyser (Beckman Coulter Ltd, High Wycombe, UK). Insulin and erythropoietin were determined using a Meso Scale Discovery array (Rockville, MD, USA) and IL-6 was measured by ELISA (eBioscience Ltd, Hatfield, UK).

Histopathology

Full necropsy was performed on two male and two female *Kcne2*$^{tm1a/tm1a}$ and two controls of each sex. All tissues were collected, fixed in formalin and embedded in paraffin wax according to standard protocols. Sections were cut and stained with haematoxylin and eosin or Perls' Prussian blue according to standard methods.

Data analysis and statistics

For all data, except transferrin, ferritin and erythropoietin, the impact of genotype was assessed using a mixed model framework as described [13]. For each phenotypic trait tested, the global P-value was adjusted to account for multiple comparisons to control the false discovery rate to 5% (R function: P=0.0163) and is reported in the text, the genotype P value is indicated on the figures and the full details are listed in table 1. Transferrin, ferritin and erythropoietin were analysed using a one-way ANOVA using Sidak's multiple comparisons test and adjusting for multiple testing using Prism v6 (GraphPad, San Diego, CA, USA).

Results and Discussion

Seven hematological parameters were significantly different in male *Kcne2*$^{tm1a/tm1a}$ mutants compared to controls (Table 1 and Supplementary Table 1). There was a decrease in the red blood cell count ($P=4.35x10^{-4}$, Fig 1A), hemoglobin ($P=3.60x10^{-4}$, Fig 1B), hematocrit ($P=7.30x10^{-4}$, Fig 1C) and mean corpuscular hemoglobin ($P=2.96x10^{-3}$, Fig 1D). This was accompanied by increased red blood cell distribution width (P=0, Fig 1E) and decreased mean corpuscular volume ($P=7.50x10^{-4}$, Fig 1F). These altered red blood cell indices are indicative of hypochromic microcytic anemia and are in agreement with a recent report [7]. There was also evidence of reactive thrombocytosis with an increased platelet count in the male *Kcne2*$^{tm1a/tm1a}$ mutants ($P=9.90x10^{-4}$, Fig 1G). Interestingly, no significant hematological differences were detected in females.

We analysed in detail the plasma chemistry parameters with a focus on those that could correlate with anemia (Table 1 and Supplementary Table 1). There was a significant decrease in the plasma iron concentration in both male and female *Kcne2*$^{tm1a/tm1a}$ mutants compared to the controls ($P=1.09x10^{-8}$, Fig 2A) suggestive of iron-deficient anemia. It has previously been demonstrated that Kcne2 is essential for gastric acid secretion and *Kcne2* deficient mice have an increased stomach pH [3]. As it has also been demonstrated that a low gastric pH is required for absorption of dietary iron [9] we hypothesise that the increased gastric pH in *Kcne2*$^{tm1a/tm1a}$ mutants could account for the low plasma iron and iron-deficient anemia. To support this

finding we tested plasma ferritin, transferrin and erythropoietin. We observed a significant decrease in the plasma ferritin concentration in both male and female *Kcne2*$^{tm1a/tm1a}$ mutants compared to the controls (P<0.0001, Fig 2B). There was a trend to increased transferrin although this was not significant (data not shown), however, using the transferrin/$\log_{10}$(ferritin) ratio suggested to be a sensitive indicator of iron-deficient anemia [14], there was a significant increase in male *Kcne2*$^{tm1a/tm1a}$ mutants compared to the controls (P<0.0001, Fig 2C). Erythropoietin was significantly increased in male *Kcne2*$^{tm1a/tm1a}$ mutants compared to controls (P<0.0001, Fig 2D). As erythropoietin and transferrin/$\log_{10}$(ferritin) ratio were only significantly different in the males this could account, in part, for why hematological abnormalities were only observed in males and we hypothesize that this could be linked to the differential effects of sex hormones on regulating iron stores and erythropoiesis. Plasma magnesium was significantly increased in both male and female *Kcne2*$^{tm1a/tm1a}$ mutants compared to controls (P=1.62x10^{-5}, Fig 2E), although the significance of this finding is unclear. In contrast to Hu *et al* [7] there was no significant difference in potassium, and no evidence of dyslipidemia or altered glucose tolerance (data not shown).

To investigate other causal factors we performed a full histological assessment and in agreement with previous reports [3, 6] *Kcne2*$^{tm1a/tm1a}$ mutant mice display gastric hyperplasia, abnormal parietal cell morphology and decreased numbers of chief cells (data not shown). Inflammation and neutrophil infiltration in the gastric

mucosa was also observed in *Kcne2*$^{tm1a/tm1a}$ mutants. The abnormalities were more severe in the two male samples studied compared with the females (data not shown) and could be linked to the more extreme response of the males to high fat diet challenge which has been demonstrated to have a heightened inflammatory response in males [15]. One of the male *Kcne2*$^{tm1a/tm1a}$ mutants presented with a gastric adenoma (Fig 2F, box) previously observed in aged *Kcne2* deficient mice [6] and *Kcnq1* mutants [16]. There was no indication of disruptions to the small intestine villi, and the bone marrow and spleen did not exhibit any gross abnormalities between *Kcne2*$^{tm1a/tm1a}$ mutants and controls (data not shown). The liver of both controls and *Kcne2*$^{tm1a/tm1a}$ mutants exhibited indications of non-alcoholic fatty liver disease (data not shown) consistent with being placed on a high fat diet for 12 weeks [17]. Upon staining with Perls' Prussian blue to assess iron stores, distinct blue staining could be detected in the spleen sections from controls (Fig 2G) but this was virtually undetectable in all four *Kcne2*$^{tm1a/tm1a}$ samples (Fig 2H).

The link between inflammation, in particular pro-inflammatory cytokines, and alterations to iron homeostasis is well established [18]. As we observed inflammation in our histological examination of *Kcne2*$^{tm1a/tm1a}$ mutants we determined the concentration of cytokines in the plasma. We found that IL-6 levels were below 50 pg/ml in all *Kcne2*$^{tm1a/tm1a}$ mutants and controls (data not shown). This further strengthens the view that the hematological abnormalities observed are due to iron deficiency and not the result of systemic inflammation.

In conclusion, this study has provided further evidence for the importance of gastric pH-regulating mechanisms in the absorption of dietary iron, the consequence of which is the development of iron-deficient anemia. Both sexes presented with decreased plasma iron whereas only the males developed anemia, we speculate this effect is linked to the differential effect of sex hormones on iron stores and erythropoiesis [19]. Interestingly these findings could be clinically relevant as it was recently reported that impaired function of KCNQ1 in Jervell and Lange-Nielsen syndrome results in iron-deficient anemia and gastric hyperplasia [15]. Given the similarities in the gastric phenotype of *Kcne2* and *Kcnq1* deficient mice we speculate that impaired function of KCNE2 could result in a similar clinical presentation.

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Author contributions

GS, ELC, ZM, MJA, CI, CS, YH, The Sanger Mouse Genetics Project and AOS generated the data. GS, ELC, ZM, MJA, NAK and AOS analysed the data. RRS managed mouse production and genotyping, The Sanger Mouse Genetics Project generated, genotyped and phenotyped the mice, RRS, DJA, JKW and AOS led the project. GS, ELC, ZM, JKW and AOS wrote the manuscript with contributions from all authors.

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Figure legends

Table 1. Mixed model output for the significant hematology and plasma chemistry parameters as assessed by a significance threshold of <0.0163 on the global test output. This threshold was selected to manage multiple testing and control the false discovery rate to 5%. The global test P value is a test of the genotype impact. The methodology assesses for sexual dimorphism and, when significant (sexual dimorphism P value <0.05), then the model will estimate the genotype effect for each sex separately (see Genotype*Female and Genotype*Male) and based on the significance of the P values for each sex effect, the genotype effect can be classified (eg male only). When sexual dimorphism was not significant, the data from both sexes were combined to assess the overall genotype effect (Effect of genotype).

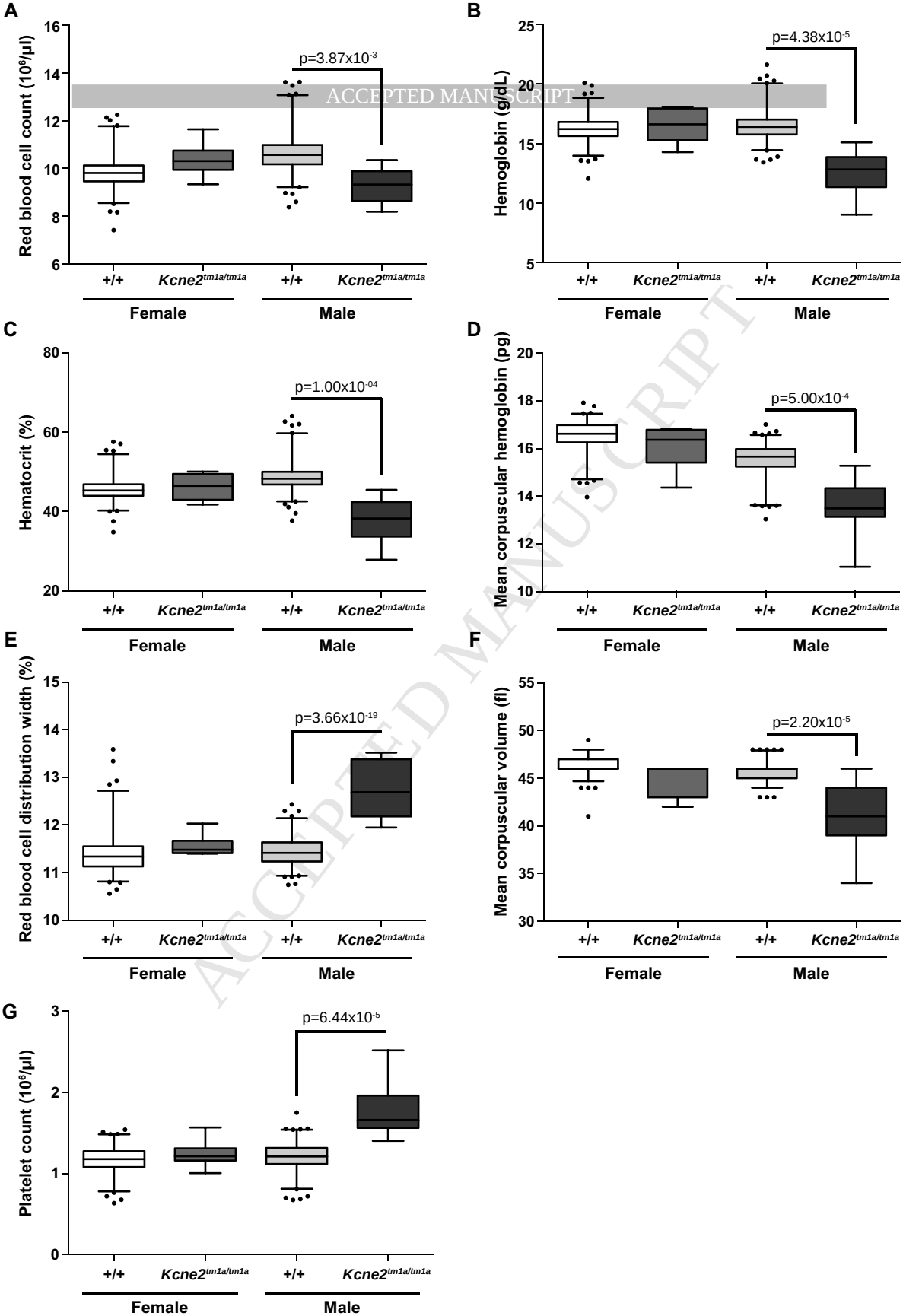
Figure 1. Altered hematological parameters in *Kcne2*$^{tm1a/tm1a}$ mutants. Red blood cell count (A), hemoglobin (B), hematocrit (C), mean corpuscular hemoglobin (D), red blood cell distribution width (E), mean corpuscular volume (F) and platelet count (G) were all determined at 16 weeks of age. P values for male control versus male *Kcne2*$^{tm1a/tm1a}$ are indicated with the boxplots showing the mean interquartile range, with whiskers to the 2.5 and 97.5 percentile and dots for outliers. For all graphs n=7

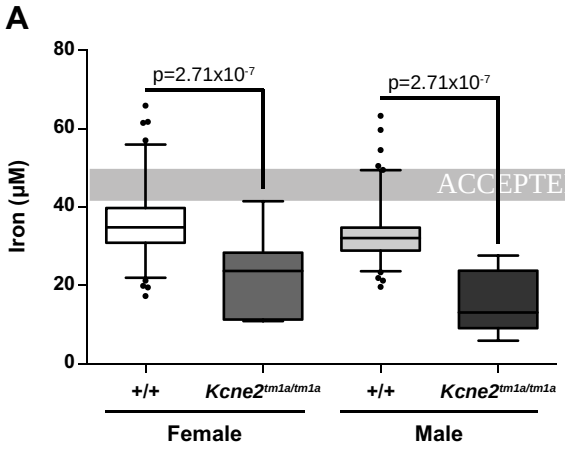
for female *Kcne2*$^{tm1a/tm1a}$ mutants, n=187 for female controls, n=7 for male *Kcne2*$^{tm1a/tm1a}$ mutants and n=202 for male controls.

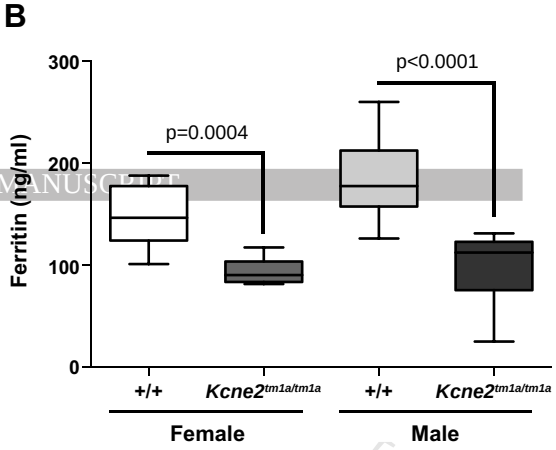
Figure 2. Altered plasma chemistry parameters in *Kcne2*$^{tm1a/tm1a}$ mutants. Iron (A), ferritin (B), transferrin/$\log_{10}$(ferritin) ratio (C), erythropoietin (D) and magnesium (E), were all determined at 16 weeks of age. P values for the genotype effect or male control versus male *Kcne2*$^{tm1a/tm1a}$ are indicated with the boxplots showing the mean interquartile range, with whiskers to the 2.5 and 97.5 percentile and dots for outliers. For iron, magnesium, ferritin and transferrin n=7 for *Kcne2*$^{tm1a/tm1a}$ female and male mutants, erythropoietin n=5 for female and n=6 for male *Kcne2*$^{tm1a/tm1a}$ mutants. For iron and magnesium n=186 female and n=202 male controls. Erythropoietin n=19 female and n=21 male controls, ferritin n=21 female and n=23 male controls and transferrin n=22 for female and n=23 for male controls. Presence of a gastric adenoma (surrounded by the box), with architectural and nuclear atypia typical of a dysplastic adenoma, in a male *Kcne2*$^{tm1a/tm1a}$ mutant (F) and reduced iron content in spleen as detected by Perls' Prussian blue stain representative image from a male control (G) and a male *Kcne2*$^{tm1a/tm1a}$ mutant (H).

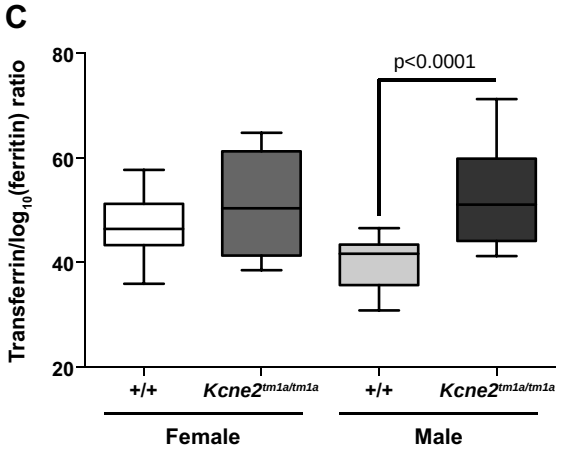
Variable	Global test	Sexual dimorphism	Genotype Effect		Genotype*Female		Genotype*Male		Classification
	P value	P value	Effect size	P value	Effect size	P value	Effect size	P value	
Red blood cell count	$4.35x10^{-4}$	$1.00x10^{-4}$			$0.523x10^{6}$	0.157	$-1.30x10^{6}$	$3.87x10^{-3}$	Males only
Hemoglobin	$3.60x10^{-4}$	$2.00x10^{-4}$			0.305	0.684	-3.635	$4.38x10^{-5}$	Males only
Hematocrit	$7.30x10^{-4}$	$4.00x10^{-4}$			0.482	0.826	-10.117	$1.00x10^{-4}$	Males only
Mean corpuscular hemoglobin	$2.96x10^{-3}$	0.0426			-0.510	0.273	-1.840	$5.00x10^{-4}$	Males only
Red blood cell distribution width	0	$1.37x10^{-8}$			0.174	0.225	1.345	$3.66x10^{-19}$	Males only
Mean corpuscular volume	$7.50x10^{-4}$	0.0413			-1.713	0.136	-5.033	$2.20x10^{-5}$	Males only
Platelet count	$9.90x10^{-4}$	$5.20x10^{-3}$			66250	0.579	$5.392x10^{5}$	$6.44x10^{-5}$	Males only
Iron	$1.09x10^{-8}$	0.236	-14.244	$2.71x10^{-7}$					Both sexes equally
Magnesium	$1.62x10^{-5}$	0.532	0.1257	$1.00x10^{-4}$					Both sexes equally

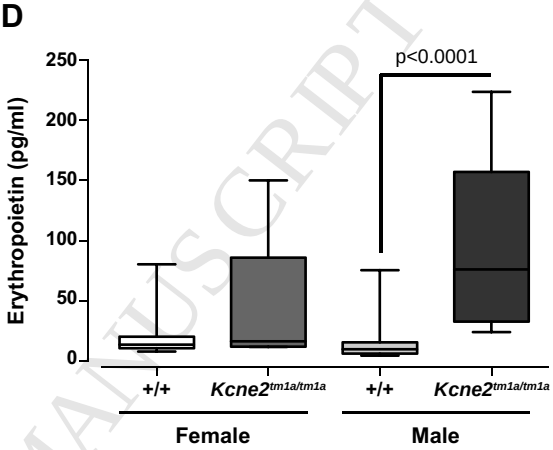
Table 1

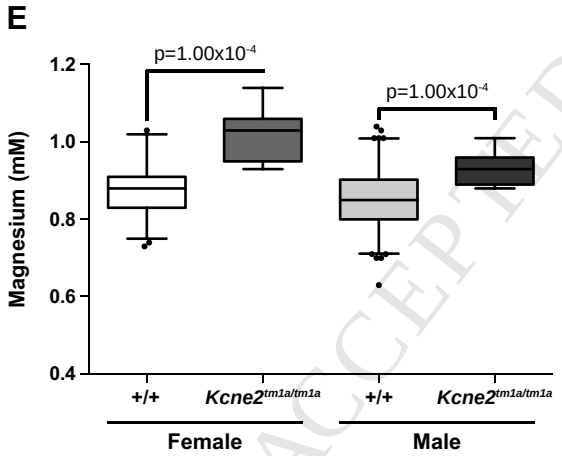


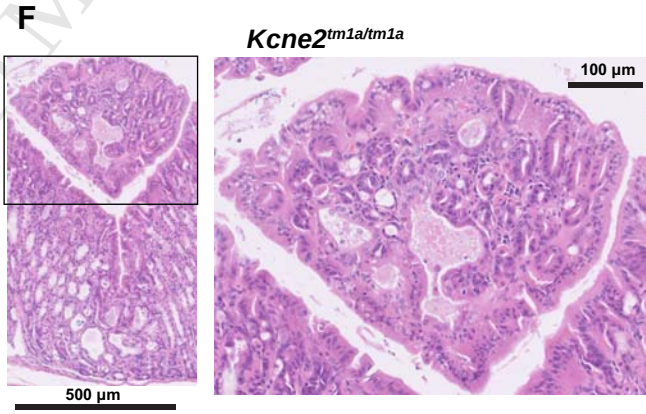


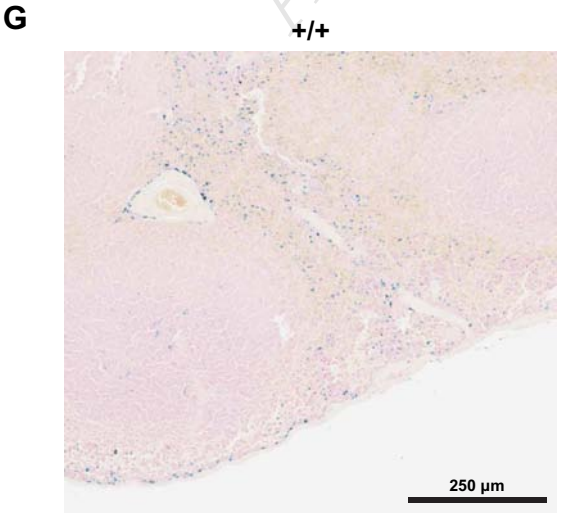


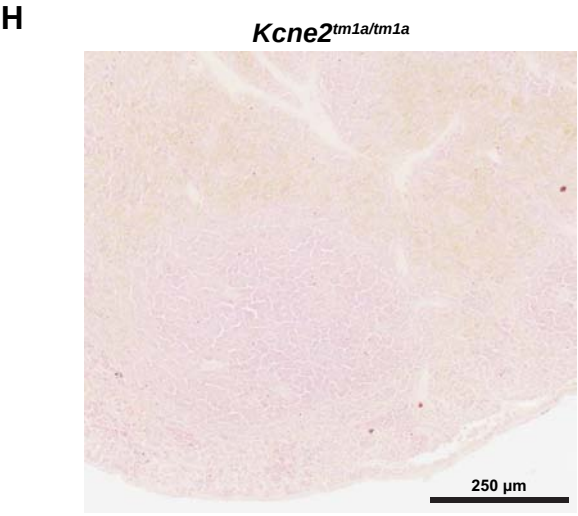












Highlights

- We have generated and phenotyped *Kcne2*$^{tm1/tm1a}$ mice
- *Kcne2*$^{tm1a/tm1a}$ males presented with hypochromic microcytic anemia
- Both *Kcne2*$^{tm1a/tm1a}$ males and females had decreased plasma iron
- Only males had increased erythropoietin and transferrin/ferritin ratio
- We believe this is due to impaired iron absorption resulting from achlorhydia

Variable	Female				Male			
	+/+		*Kcne2*$^{tm1a/tm1a}$		+/+		*Kcne2*$^{tm1a/tm1a}$	
	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD
Red blood cell count (x10^6/µl)	187	9.87 ± 0.68	7	10.35 ± 0.72	202	10.69 ± 0.86	7	9.28 ± 0.74
Hemoglobin (g/dL)	187	16.2 ± 1.13	7	16.5 ± 1.40	202	16.5 ± 1.25	7	12.7 ± 1.93
Hematocrit (%)	187	45.7 ± 3.10	7	46.1 ± 3.18	202	48.9 ± 3.94	7	37.9 ± 5.77
Mean corpuscular hemoglobin (pg)	187	16.5 ± 0.74	7	16.0 ± 0.90	202	15.5 ± 0.76	7	13.5 ± 1.30
Red blood cell distribution width (%)	187	11.4 ± 0.42	7	11.6 ± 0.22	202	11.5 ± 0.31	7	12.8 ± 0.59
Mean corpuscular volume (fl)	187	46.3 ± 0.92	7	44.6 ± 1.81	202	45.8 ± 0.94	7	40.7 ± 3.82
Platelet count (x10^6/µl)	187	1.18 ± 0.16	7	1.24 ± 0.17	202	1.21 ± 0.17	7	1.78 ± 0.37
Iron (µM)	186	35.8 ± 7.7	7	23.7 ± 10.5	202	32.6 ± 5.9	7	15.4 ± 7.9
Ferritin (ng/ml)	21	149.8 ± 28.3	7	93.4 ± 12.9	23	149.8 ± 28.3	7	95.8 ± 37.4
Transferrin (mg/dL)	22	103.1 ± 13.1	7	101.6 ± 17.7	23	90.2 ± 12.69	7	99.8 ± 6.7
Erythropoietin (pg/ml)	19	20.1 ± 17.8	5	42.6 ± 60.3	21	14.2 ± 15.4	6	95.2 ± 78.1
Magnesium (mM)	186	0.88 ± 0.06	7	1.02 ± 0.07	202	0.85 ± 0.07	7	0.94 ± 0.05

Supplementary table 1. Full breakdown of hematology and plasma chemistry parameters showing n, mean and standard deviation.