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Genome-wide Meta-analysis Unravels Novel Interactions between Magnesium Homeostasis and Metabolic Phenotypes

Citation for published version:

Corre, T, Arjona, FJ, Hayward, C, Youhanna, S, de Baaij, J, Belge, H, Nagele, N, Debaix, H, Blanchard, MG, Traglia, M, Harris, S, Ulivi, S, Rueedi, R, Lamparter, D, Mace, A, Sala, C, Lenarduzzi, S, Ponte, B, Pruijm, M, Ackermann, D, Ehret, G, Baptista, D, Polasek, O, Rudan, I, Hurd, T, Hastie, N, Vitart, V, Waeber, G, Kutalik, Z, Bergmann, S, Vargas-Poussou, R, Konrad, M, Gasparini, P, Deary, I, Starr, J, Toniolo, D, Vollenweider, P, Hoenderop, JGJ, Bindels, RJM, Bochud, M & Devuyst, O 2018, 'Genome-wide Meta-analysis Unravels Novel Interactions between Magnesium Homeostasis and Metabolic Phenotypes' Journal of the American Society of Nephrology. DOI: 10.1681/ASN.2017030267

Digital Object Identifier (DOI):

[10.1681/ASN.2017030267](https://doi.org/10.1681/ASN.2017030267)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Journal of the American Society of Nephrology

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JASN

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Journal:	<i>Journal of the American Society of Nephrology</i>
Manuscript ID	JASN-2017-03-0267.R2
Manuscript Type:	Original Article - Meta-analysis
Date Submitted by the Author:	16-Jul-2017
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Keywords:	zebrafish, Genetic determinants, Magnesium homeostasis, Metabolic syndrome, Gene-environment interaction, Tubular transport

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Manuscripts

JASN-2017-03-0267. R2

Genome-wide Meta-analysis Unravels Novel Interactions between Magnesium Homeostasis and Metabolic Phenotypes

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19 * T.C., F.J.A. and C.H. have equally contributed to the study

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21 # O.D., M.B. and R.J.M.B. have co-directed the study

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26 **Running Title:** Genetic determinants of magnesium homeostasis and metabolism

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ABSTRACT

Magnesium (Mg^{2+}) homeostasis is critical for metabolism. The genetic determinants of the renal handling of Mg^{2+} , which is crucial for Mg^{2+} homeostasis, and their potential influence on metabolic traits in the general population are unknown. Plasma and urine parameters were obtained of 9,099 individuals from 7 cohorts for the first genome-wide meta-analysis of Mg^{2+} homeostasis. Two novel loci, *TRPM6* (rs3824347, $P = 4.4 \times 10^{-13}$) and *ARL15* (rs35929, $P = 2.1 \times 10^{-11}$), for urinary Mg^{2+} (uMg) were identified, accounting together for 2.3% of the variation in 24-h uMg excretion. In human kidney cells, *ARL15* was shown to regulate *TRPM6*-mediated currents. In zebrafish, the expression of the highly conserved *ARL15* orthologue, *arl15b*, was regulated by dietary Mg^{2+} and *arl15b*-knockdown resulted in renal Mg^{2+} wasting and metabolic disturbances. The association of uMg with fasting insulin and fat mass was modified by *ARL15* rs35929 in a general population. The combined observational and experimental approach uncovers a novel gene-environment interaction linking Mg^{2+} deficiency to insulin resistance and obesity.

INTRODUCTION

Magnesium (Mg^{2+}) is an essential cation for multiple enzymatic reactions, including those involving DNA and protein synthesis and energy metabolism¹. Abnormal Mg^{2+} levels in serum are associated with common diseases such as diabetes and metabolic disorders². Interventional and longitudinal observational studies in humans show that high dietary Mg^{2+} protects against the risk of developing type 2 diabetes³⁻⁶ and improves glycemic control in diabetic patients⁷ as well as in overweight non-diabetic subjects⁸⁻¹⁰. In young adults, high Mg^{2+} intake is significantly associated with lower incidence of metabolic syndrome¹¹. Furthermore, there is evidence linking Mg^{2+} intake to body weight regulation, with low intake potentially impairing lean body mass growth^{12,13}, whereas Mg^{2+} supplementation may increase lean body mass and decrease fat mass in overweight women¹⁴.

Plasma Mg^{2+} levels are closely regulated and remain constant throughout life, despite the fact that dietary Mg^{2+} intake and intestinal absorption decrease with age¹⁵. The control of Mg^{2+} balance is ensured by a tightly regulated reabsorption of Mg^{2+} in the distal tubular segments of the kidney. In particular, the Mg^{2+} filtered in the glomerulus is reabsorbed in the proximal tubule and thick ascending loop of Henle (TAL) *via* paracellular routes, while downstream, in the distal convoluted tubule (DCT), Mg^{2+} is efficiently reabsorbed through transcellular mechanisms involving the transient receptor potential cation channel, subfamily M, member 6 (TRPM6)². The reabsorption of Mg^{2+} in the DCT determines the final urinary Mg^{2+} (uMg) excretion since no reabsorption of Mg^{2+} occurs in posterior segments. Subjects harboring inherited or acquired dysfunctions of the renal tubular handling of Mg^{2+} show inappropriate urinary loss of Mg^{2+} causing chronic hypomagnesemia and severe, multi-systemic manifestations^{2,16,17}.

The genetic component of Mg^{2+} homeostasis is indicated by a significant heritability (15-39%) of serum Mg^{2+} ^{18,19} and by rare monogenic disorders disturbing renal tubular

1
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3 transport of Mg^{2+} ²⁰⁻²³. Yet, most of the regulatory genes of renal Mg^{2+} channels and
4
5 transporters remain unknown. A genome-wide association study (GWAS) on serum Mg^{2+} in
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7 European ancestry adults identified six loci, one of which included the gene encoding
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9 TRPM6 ²⁴. Two more recent studies in children ²⁵ and in African-Americans ²⁶ did not reveal
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11 additional loci. However, changes in uMg excretion precede changes in circulating serum
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13 Mg^{2+} levels, being thus a more sensitive indicator of any disturbance in Mg^{2+} homeostasis ²⁷.
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15 Furthermore, Mg^{2+} depletion can be found in individuals with apparently normal total serum
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17 Mg^{2+} levels ²⁸, which underscores the limitations of total serum Mg^{2+} as a marker of Mg^{2+}
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19 status. Assessment of uMg in GWAS is therefore crucial to elucidate new genes involved in
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21 Mg^{2+} homeostasis. Furthermore, studying the association between the genetic determinants of
22
23 uMg and metabolic phenotypes linked to disturbed Mg^{2+} balance (e.g. obesity and diabetes)
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25 may provide novel insights on links between Mg^{2+} and common diseases. Indeed, plasma
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27 triglycerides and glucose are major determinants of the Mg^{2+} balance in diabetic patients ²⁹.
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32 In order to shed light on the genetic factors and molecular mechanisms linking Mg^{2+}
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34 handling and metabolic disorders, we performed a meta-analysis of GWAS for the renal
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36 handling of Mg^{2+} by combining genetic isolates and population-based studies and
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38 investigating the biological relevance of the identified loci using cellular systems and model
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40 organisms. Given the known relationships of Mg^{2+} intake with metabolic disorders, we also
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42 explored whether the identified loci modified these relationships. These studies establish a
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44 novel biological control of renal Mg^{2+} handling, and a novel gene-environment interaction
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46 sustaining the link between Mg^{2+} homeostasis, insulin resistance and obesity.
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RESULTS

Meta-analyses of GWAS for magnesium homeostasis

The initial discovery phase consisted of a GWAS (2.5 M markers) performed on the population-based CoLaus cohort (5,150 samples) testing for association with uMg-to-creatinine ratio. The analysis revealed a single genome wide significant signal at rs3824347 ($P = 3.6 \times 10^{-8}$), corresponding to the *TRPM6* locus on chromosome 9, and six suggestive loci with P values below 10^{-5} (Suppl. Fig. 1). These association results were combined with the genome-wide association scans from six additional European cohorts (LBC1936, CROATIA-Split, CROATIA-Vis, Carlantino, CROATIA-Korcula, and Val Borbera) into a single meta-analysis (9,099 samples). As shown on the Manhattan plot (Fig. 1a), two signals showed a P value below 5×10^{-8} . The QQ plot showed no problematic inflation ($\lambda=1.014$, $se=3.16 \times 10^{-5}$) (Suppl. Fig. 2a). Both the forest plots (Fig. 1b) and the low I-square values (Table 1) indicated little heterogeneity across cohorts. No secondary signals were detected in the approximate conditional analysis using the lead single nucleotide polymorphisms (SNPs) as covariates in the regression (Suppl. Fig 2b and 2c). The lowest combined P value (4.4×10^{-13}) was observed for the SNP rs3824347 on chromosome 9, at a locus comprising five genes, *TRPM6*, *C9orf40*, *C9orf41*, *NMRK1* and *OSTF1* (Fig. 1c). The second strongest signal maps to chromosome 5 with a P value of 2.1×10^{-11} for the lead SNP rs35929 (Fig. 1d). This SNP and all other genome-wide significant SNPs at this locus lie in the first intron of the ADP ribosylation factor like GTPase 15 (*ARL15*) gene. None of the two signals showed significant association with urinary creatinine.

Results from the versatile gene-based association study (VEGAS analysis)³⁰ of uMg/creatinine ratio identified *C9orf40*, *C9orf41* and *TRPM6* as the only genes with a statistically significant result ($P < 2.8 \times 10^{-6}$), however these signals are overlapping with the single SNP association in the meta-analysis (rs3824347). None of the discovered SNPs tag

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3 copy number variations (CNVs). A pathway analysis has been performed using the Magenta
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5 algorithm, but no enrichment appeared to be significant after multiple testing correction. Sex-
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7 stratified analyses did not reveal additional genome-wide significant loci. No significant
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9 eQTL is referenced for rs35929 nor rs3824347 in the GTEx portal.
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11 A meta-GWAS of serum Mg^{2+} levels replicated the previously published locus on
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13 chromosome 1²⁴ containing among many others the gene *MUC1* ($P = 4.45 \times 10^{-14}$) (Suppl.
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15 Table 1, Suppl. Fig. 3). Our meta-analysis on fractional excretion of Mg^{2+} (FEMg) yielded 12
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17 suggestive loci with a $P < 10^{-5}$ (Suppl. Fig. 4) but no genome-wide significant association
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19 (the sample size was reduced to cohorts having serum and urine from the same time point,
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21 therefore without LBC1936, Split and Vis; $n = 7,976$). The association of rs35929 ($P =$
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23 2.8×10^{-4}) and rs3824347 ($P = 1.4 \times 10^{-5}$) with FEMg were nominally, but not genome-wide,
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25 significant.
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32 **A genetic score including rs35929 and rs3824347 associates with uMg traits**

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34 Urinary Mg-to-creatinine ratio (Fig. 2a, CoLaus cohort), FEMg in spot urine (Fig. 2b,
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36 CoLaus cohort) and uMg excretion in 24-h urine (Fig. 2c, Swiss Kidney Project on Genes in
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38 Hypertension (SKIPOGH) cohort), but not serum Mg^{2+} (Fig. 2d, CoLaus), were strongly and
39
40 linearly associated with an additive unweighted genetic risk score including rs35929-A
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42 (*ARL15*) and rs3824347-G (*TRPM6*) alleles. The genetic score explained 2.3% of 24-h uMg
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44 excretion in the SKIPOGH study and 1.0% of uMg-to-creatinine ratio in CoLaus.
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49 **ARL15 regulates TRPM6 channel activity**

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51 To validate the association between uMg excretion and *ARL15* and *TRPM6* variants, we first
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53 showed that the highest mRNA expression of *Arll5* was in distal nephron segments including
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55 the TAL and DCT, the DCT being also strongly positive for *Trpm6* (Fig. 3a). At protein
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3 level, ARL15 is localized predominantly in the TAL and the DCT segments (Fig. 3b), which
4 are enriched in Tamm-Horsfall (TH) protein and the Na⁺-Cl⁻-co-transporter (NCC)
5 respectively. Importantly, TRPM6 is also distinctly localized in the DCT segment³¹. The
6
7 spatial correlation between TRPM6 and ARL15 expression in the DCT prompted us to
8 postulate a functional interaction between these two proteins. In addition, functional protein
9 association network analyses for ARL15 (Suppl. Fig. 5) showed that ARL15 interacts with
10 key proteins for endocytic trafficking (RAB11 and ARFGEF families), a process that may
11 increase the abundance and channel activity of TRPM6 in the plasma membrane.
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21 To assess the potential effect of ARL15 on TRPM6 channel activity, human
22 embryonic kidney 293 (HEK293) cells were transfected with wild type (WT) TRPM6 in
23 parallel with a mock transfect, WT ARL15 or a dominant negative (T46N) ARL15 mutant.
24 The T46N ARL15 mutant was predicted to be of dominant negative nature by comparative
25 analysis with T27N ARF6, a dominant negative mutant affecting a protein (ARF6) that
26 belongs to the same protein family as ARL15³². Patch clamp recordings showed
27 characteristic slowly developing outwardly-rectifying currents in all conditions (Fig. 3c-f). A
28 significant increase in TRPM6 channel activity was observed in the presence of WT ARL15,
29 but not with the T46N ARL15 mutant (Fig. 3e). HEK293 cells express endogenous TRPM7
30 channels³³. Here, these currents were small (< 50 pA/pF) and were not significantly
31 increased by expression of WT ARL15 (Fig. 3f).
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48 **Physiological relevance of ARL15 in model organisms**

49 To support the important role of ARL15 in Mg²⁺ homeostasis, we investigated the regulation
50 of TRPM6 and ARL15 gene expression in mice upon exposure to variable Mg²⁺ diets, known
51 to affect the expression of critical genes in the mouse kidney and intestine³⁴, where *Arll5* is
52 expressed (Suppl. Fig. 6a). Metabolic profiling (Suppl. Tables 6-7) revealed a remarkable
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3 adaptation after exposure to high and low Mg^{2+} diets, reflected by significant changes in the
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5 renal mRNA expression of *Trpm6* and parvalbumin (*Pvalb*), contrasting with the stable
6
7 expression of *Arl15* and other segmental markers of the TAL and DCT (Suppl. Fig. 6b). A
8
9 similar effect was observed in the ileum and the caecum, also involved in Mg^{2+} handling^{2, 34}
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11 (Suppl. Fig. 6c-d).

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14 We further tested the biological relevance of ARL15 by using a zebrafish model,
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16 which shows tubular segmentation patterns that are equivalent to the mammalian kidney³⁵.
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18 Two distinct orthologs of mammalian ARL15 were identified in zebrafish: *ar115a* and
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20 *ar115b*, presenting 68% and 83% of AA identity respectively when aligned to the human
21
22 counterpart (Suppl. Fig. 7a). Given the higher AA conservation of *ar115b* compared to
23
24 *ar115a*, *ar115b* was selected to study the function of its mammalian ortholog ARL15. As
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26 mammalian *ARL15*, *ar115b* was ubiquitously expressed in all adult zebrafish tissues tested
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28 (Suppl. Fig. 7b). When adult zebrafish were fed different Mg^{2+} diets for 3 weeks, *ar115b*
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30 expression patterns reflected compensatory mechanisms to cope with Mg^{2+} deficiency
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32 (kidney and gills) or Mg^{2+} surplus (gut) (Suppl. Fig. 7c-e).

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36 A loss-of-function approach using morpholinos (MOs) against *ar115b* in zebrafish
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38 larvae, where *ar115b* is expressed in the pronephros (Suppl. Fig. 8), was used to relate
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40 ARL15 function to renal Mg^{2+} wasting. Since intestinal Mg^{2+} absorption does not take place
41
42 during the time frame where the knockdown is applied, a change in zebrafish larvae Mg
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44 content reflects a change in the renal and/or skin (renal-like tissue³⁶) uptake of Mg^{2+} . Total
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46 Mg^{2+} content decreased consistently in *ar115b* morphants compared with controls, indicating
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48 renal Mg^{2+} wasting since the pronephric kidney is the only extrusion route for Mg^{2+} (Fig. 4a-
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50 b). In parallel, cardiovascular impairments and morphological phenotypes characterized by a
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52 poorly metabolized yolk in comparison with controls, indicative of metabolic disturbances,
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54 were also observed (Fig. 4c-g). Co-injection of *ar115b*-MO with human WT *ARL15* cRNA
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3 induced a rescue of all phenotypes observed, whereas co-injection with T46N *ARL15* cRNA
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5 did not result in any rescue (Fig. 4h-k). These rescue experiments demonstrated the
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7 specificity of the renal Mg^{2+} wasting and metabolic defects associated to dysfunctional
8
9 *ARL15*.
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14 **Genetic background influences the link between uMg and metabolism phenotypes**

16 The functional relevance of *ARL15* for Mg^{2+} homeostasis, as demonstrated in this study, in
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18 combination with the association between *ARL15* and obesity and insulin biology³⁷ urged us
19
20 to investigate a possible link between genetic background, renal Mg^{2+} handling and metabolic
21
22 traits in general population cohorts.
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25 Urinary Mg-to-creatinine ratio was associated positively with circulating fasting
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27 adiponectin ($P < 0.001$) and glucose levels ($P = 0.001$) as well as the presence of type 2
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29 diabetes ($P = 0.03$), and negatively with body mass index (BMI) ($P = 0.001$), fasting
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31 triglycerides ($P = 0.012$), fasting insulin ($P = 0.03$), homocysteine ($P < 0.001$) and γ -glutamyl
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33 transeptidase (GGT) ($P < 0.001$), independently of serum Mg^{2+} , in CoLaus (the largest cohort
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35 available). In CoLaus, the positive associations with adiponectin and fasting glucose, as well
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37 as the negative associations with BMI, GGT and homocystein, remained significant ($P <$
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39 0.05) upon adjustment for all the other metabolic phenotypes. In SKIPOGH, 24-h uMg
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41 excretion was negatively associated with fasting serum insulin ($P < 0.001$), even after
42
43 adjustment for the all other metabolic covariates ($P = 0.003$). The negative associations of 24-
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45 h uMg excretion with fasting triglycerides or glucose disappeared upon adjustment for fasting
46
47 insulin; we found no association of 24-h uMg excretion with BMI nor with fat mass ($P >$
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49 0.40).
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54 In CoLaus, the negative association of the *ARL15* rs35929 variant with uMg excretion
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56 was stronger in overweight people ($P = 0.005$ for interaction between BMI and rs35929 for
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3 their effect on uMg concentration in spot urine), in people with high fat mass (P interaction =
4 0.024) (Fig. 5a), and in people with high fasting insulin levels (P interaction = 0.012) (Fig.
5 5b), highlighting a SNP-by-environment interaction. In SKIPOGH, the negative association
6 of daytime uMg excretion with fasting insulin was stronger in carriers of the *ARL15* rs35929
7 A allele (P interaction = 0.035), which is consistent with results in CoLaus in that rs35929
8 appears to modify the association between uMG excretion and fasting insulin. The
9 corresponding P values for interaction were 0.140 and 0.143 when replacing fasting insulin
10 by BMI or fat mass, respectively.
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DISCUSSION

In this multi-step study (Fig. 6), we conducted the first genome-wide meta-analysis for uMg levels using data from seven population-based studies of European descent. We identified two genome-wide significant loci associated with uMg-to-creatinine ratio: *i*) rs3824347 located on chromosome 9, near the *TRPM6* gene coding for a Mg^{2+} channel; *ii*) rs35929 located on chromosome 5, an intronic variant of the *ARL15* gene known for its association with adiponectin³⁸ and lipid levels^{39,40}, type 2 diabetes⁴⁰, fasting insulin levels⁴¹ and coronary heart disease³⁸, but without a prior physiological link to Mg^{2+} homeostasis. Using a multi-level approach, we provide robust evidence that *ARL15* influences renal Mg^{2+} reabsorption through regulation of the Mg^{2+} channel *TRPM6*. An additive genetic score including the rs35929 A (*ARL15*) and rs3824347 G (*TRPM6*) alleles is strongly associated with 24-h uMg²⁺ excretion, a physiological test of Mg^{2+} status, which integrates both intestinal absorption and renal wasting⁴². In addition, the association of *ARL15* rs35929 with uMg levels is modified by the metabolic status, and the association of uMg excretion with metabolic phenotypes is modified by *ARL15* rs35929 in the general adult population. Altogether, these findings suggest a gene-diet interaction relating Mg^{2+} homeostasis to metabolic disorders in the general population. If replicated in independent populations, the observed genetic effect sizes are sufficiently large to be of clinical and public health relevance.

In contrast to *TRPM6*, little is known about the function of the *ARL15* gene, except its association with adiponectin and lipid levels, type 2 diabetes and higher fasting insulin levels in humans^{38-40,43}. We found that *ARL15* is highly expressed in the TAL and DCT, where Mg^{2+} reabsorption is mostly regulated and where the main bulk of Mg^{2+} is reabsorbed. Particularly, we show that *ARL15* is enriched along with the well-characterized Mg^{2+} channel *TRPM6* in the DCT, and that *ARL15* increases *TRPM6*-mediated currents in renal

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3 epithelial cells. Since TRPM6 is the gatekeeper in transepithelial Mg^{2+} transport, its
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5 regulation by ARL15 supports a key role of the latter in the transepithelial Mg^{2+} transport in
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7 the distal part of the nephron. ARL15 is structurally similar to Ras-related GTP-binding
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9 proteins, which regulate intracellular vesicle trafficking⁴⁴. It interacts with proteins of the
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11 RAB11 and ARFGEF families, which are also involved in endocytic traffic, suggesting that
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13 ARL15 regulates the trafficking of vesicular TRPM6 to the plasma membrane, thereby
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15 influencing Mg^{2+} reabsorption in the kidney. In this sense, our *in vivo* data substantiated
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17 ARL15 as a key protein for the maintenance of Mg^{2+} homeostasis. This conclusion is
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19 supported by the high sensitivity of *ARL15* expression to dietary Mg^{2+} , as demonstrated by
20
21 the regulation of the highly conserved zebrafish ortholog of *ARL15*, *arl15b*, in the gut, kidney
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23 and gills of fish exposed to different Mg^{2+} diets – as it was already observed for the zebrafish
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25 ortholog of *TRPM6*⁴⁵. Conversely, *Arll5* expression in intestine and kidney remained
26
27 unchanged in mice fed different Mg^{2+} diets. This discrepancy between the mouse and the
28
29 zebrafish model might be due to that in zebrafish, and freshwater fish in general, active
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31 transcellular Mg^{2+} reabsorption is more prominent than in terrestrial vertebrates, as
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33 freshwater fish are hyperosmotic respect to the surrounding aquatic medium^{46,47}. Thus, the
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35 dietary Mg^{2+} challenge performed with mice in the present study is not sufficiently severe to
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37 evoke regulation of *Arll5* expression.
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43 The association of the *ARL15* rs35929 variant with uMg excretion in the present
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45 GWAS predicted a physiological relevance of ARL15 in Mg^{2+} homeostasis. In agreement
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47 with these findings, knockdown experiments in the zebrafish recognized ARL15 as a relevant
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49 key protein that determines renal Mg^{2+} excretion. In agreement with previous GWAS linking
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51 *ARL15* variants with risk of congestive heart disease³⁸, cardiovascular impairments, i.e.
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53 deficient tail blood circulation, was recognized in zebrafish *arl15b* morphants. Furthermore,
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55 previous links between *ARL15* and lipid metabolism^{37-39, 43} were functionally supported in
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3 zebrafish where *arl15b* morphants showed a poor mobilization of yolk metabolite reserves. In
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5 zebrafish larvae, the yolk serves mainly as a lipid store^{48,49}, therefore the deficiency in yolk
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7 absorption observed in *arl15b* morphants revealed poor lipid mobilization. The zebrafish
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9 *arl15b* morphants also accumulated fluid in the pericardial cavity and in the pronephros in the
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11 form of cysts showing kidney dysfunction. However, when lowering the intensity of the
12
13 *arl15b*-knockdown (by using doses of 1 and 2 ng exon 4 skipping *arl15b*-MO/embryo), renal
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15 Mg^{2+} wasting was the only phenotype observed in the fish morphants. Therefore, the
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17 disturbances in Mg^{2+} balance observed in *arl15b* morphants are a primary effect of Arl15b
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19 dysfunction and not a secondary disturbance to the kidney/metabolic/cardiovascular
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21 phenotypes observed. The phenotype rescue experiments performed with normal or mutant
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23 versions of human ARL15 confirmed that renal Mg^{2+} wasting and metabolic defects were
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25 directly due to ARL15 knockdown. Altogether, the ARL15 functions unraveled in the present
26
27 study illustrate an unprecedented case of shared pathophysiological genetics of renal Mg^{2+}
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29 wasting, cardiovascular disease and metabolic disorders, and demonstrate the pleiotropic
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31 nature of ARL15, that was inferred from the present and published GWAS. The importance
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33 of *ARL15* is also supported by recent genetic evidence for its positive selection among
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35 adaptive loci identified in cattle⁵⁰. Thus far, no mutations in *ARL15* have been associated
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37 with a monogenic disorder, including unidentified forms of renal Mg^{2+} wasting (Data not
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39 shown).
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46 Could the metabolic traits linked to *ARL15* variants be connected? By integrating
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48 intestinal absorption and renal wasting, uMg^{2+} excretion represents a physiological test of
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50 Mg^{2+} status, more suitable than total serum Mg^{2+} levels⁴². Urinary Mg-to-creatinine ratio was
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52 associated with multiple metabolic phenotypes, independently of serum Mg^{2+} , in the
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54 population-based CoLaus study, providing novel insights into the metabolic consequences of
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56 Mg^{2+} deficiencies. Our results are in line with longitudinal studies in humans suggesting that
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3 self-reported dietary Mg^{2+} intake may protect against type 2 diabetes, insulin resistance⁵¹ and
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5 metabolic syndrome⁵². Growing experimental evidence in favor of a beneficial role of Mg^{2+}
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7 intake on type 2 diabetes is accumulating in humans. Mg^{2+} supplements were found to
8
9 significantly improve glucose tolerance and insulin sensitivity in diabetic and non-diabetic
10
11 people⁵³. Whether such benefit would persist over years is currently unknown. The public
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13 health relevance of dietary Mg^{2+} intake is further underscored by an association with all-
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15 cause mortality and risk of cardiovascular disease⁵¹. The novelty of our results is that we
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17 benefit from an objective measure of Mg^{2+} intake, namely uMg excretion, which, unlike
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19 circulating Mg^{2+} , was found to be associated with the risk of ischemic heart disease⁵⁴.
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23 In population-based studies, the association between uMg and metabolic phenotypes
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25 (i.e. BMI, fat mass, fasting insulin levels) tended to differ by *ARL15* rs35929 variants. These
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27 results are in line with human studies showing that Mg^{2+} intake exerts more protective effects
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29 on type 2 diabetes in overweight compared to non-overweight people³. Such gene-diet
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31 interaction has already been observed for type 2 diabetes, for which the increased risk
32
33 conferred by the rs7903146 *TCF7L2* genotype was only observed in the absence of lifestyle
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35 intervention⁵⁵. Therefore, the *ARL15* gene may modulate the beneficial effect of Mg^{2+} intake
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37 on insulin resistance in humans, offering a novel paradigm in nutrigenetics. Furthermore, the
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39 beneficial effect of high Mg^{2+} intake on metabolic disorders, such as type 2 diabetes and
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41 obesity, may not be uniform across individuals.
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45 In conclusion, the translational data described in this study (Fig. 6) offer novel
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47 insights for the understanding of Mg^{2+} homeostasis and its relation with metabolic
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49 phenotypes, and identify *ARL15* as a key and central player for these processes.
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CONCISE METHODS

Genome-wide meta-analyses

Seven European population-based cohorts with Caucasian ethnicity (CoLaus, CROATIA-Vis, CROATIA-Korcula, CROATIA-Split, Lothian Birth Cohort 1936, INGI-Val Borbera, INGI-Carlantino, described in the Supplementary Information) participated in the study, with a total of 9,099 individuals. A variety of electrolytes including Mg^{2+} as well as creatinine, were measured in both urine and serum (methods and mean values described in the Supplementary Information).

All cohorts were whole genome genotyped before imputation using HapMap CEU panel as reference (Supplementary Information). Serum Mg^{2+} measurements and FEMg were subjected to quantile-quantile normalization in order to reach normality prior to performing linear regression. Urinary Mg^{2+} measurements expressed in mg/dl were first standardized to urinary creatinine, then corrected for age, sex and study specific covariates (such as ancestry principal components, study center...) before undergoing a quantile-quantile normalization. The approximately 2.5M SNPs passing quality control checks were subjected to a linear regression of the residuals for each phenotype, and association summary statistics were collected.

Even though quantile-quantile plots of the individual GWAS showed minimal inflation, ruling out the presence of population substructure, a genomic-control (GC) correction based on the lambda factor was applied to the association P values. Subsequently, a meta-analysis was conducted using the inverse variant weighting method implemented in METAL⁵⁶. A new GC correction was applied to combined statistics.

SKIPOGH cohort

SKIPOGH is a population-based family-based multicentric study focusing on blood pressure regulation and renal function⁵⁷. Each participant collected 24-h urine. The study was approved by the institutional ethical committees of each participating university hospital and participants signed written informed consent.

VEGAS analysis

The SNP association P values from the meta-analysis of uMg were analysed using VEGAS³⁰, a program for performing gene-based tests for association using the summary statistics from genetic association studies. VEGAS assigns SNPs to genes and combines their association P values into a gene-based test statistic. Permutations are used to calculate the null distribution of the test statistic for each gene in order to derive an empirical gene-wise P value. The gene-based approach also reduces the multiple-testing problem of GWAS by only considering statistical tests for 17,787 genes giving a Bonferroni-corrected threshold of $P < 2.8E-6$.

Pathway analysis

We used Magenta⁵⁸ to check enrichment for pathways in either the BIOCARTA KEGG REACTOME databases which were contained in the msigdb version 4.0 (<http://www.broadinstitute.org/gsea/msigdb/collections.jsp#C2>).

Metabolome analysis

Nuclear magnetic resonance spectroscopy data obtained from urine samples was binned and normalized to produce metabolome features⁵⁹. These features were then associated with the

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3 SNPs of interest, including relevant covariates among standard covariates (age, sex, genotype
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5 principal components) and lifestyle factors (smoking, caffeine use, dietary intake, etc.).
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8 9 **CNV analysis**

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11 In house datasets have been used to call CNVs and to check their correlation with the SNPs
12
13 of interest. The CNVs call has been done using pennCNV software⁶⁰. A SNP by sample
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15 matrix with the copy number status has been created. Then the square correlation (Pearson's
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17 correlation) between value of each SNP of interest and the SNPs copy number status in a +/-
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19 100 kb region has been calculated. For the SNPs of interest for which no correspondence has
20
21 been found in the datasets, they have been replaced by the closest SNPs in high linkage
22
23 disequilibrium (LD) and present in the datasets. LD between the SNPs of interest and a list of
24
25 SNPs tagging CNVs from the Genetic Investigation of ANthropometric Traits (GIANT)
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27 consortium has also been calculated. The SNPs from the GIANT list are in LD higher than
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29 0.8 with their corresponding CNV⁶⁰.
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36 **Laboratory measurements**

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38 Electrolytes, haematology parameters and glycaemia were dosed in the biochemical platform
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40 of the University of Zürich using standard clinical laboratory methods. Creatinine was
41
42 measured using Jaffe kinetic compensated method (Roche Diagnostics, Switzerland, intra-
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44 assay variability 0.7-2.9%). The Chronic Kidney Disease Epidemiology Collaboration (CKD-
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46 EPI) formula was used to calculate the estimated glomerular filtration rate (eGFR). In
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48 CoLaus, urinary uromodulin, creatinine, electrolytes and osmolality concentrations were
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50 measured in morning spot urine samples.
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54 All urinary biochemical parameters were measured from samples stored at -80°C,
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56 using the same biochemical platform UniCel® DxC 800 Synchron® Clinical System
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3 (Beckman Coulter, Nyon, Switzerland) at the University of Zürich. Appropriate controls and
4
5 sets of calibration standards were used before running each sample batch. All cohorts were
6
7 subjected to the same measurement protocol, in the same laboratory.
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10 11 **Tissue distribution and localization of ARL15**

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13 The tissue distribution of *ARL15* and of its highly conserved zebrafish ortholog *arl15b* was
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15 studied in adult mouse, adult zebrafish and zebrafish larvae, as described in the
16
17 Supplementary Information. Segmental expression of ARL15 in the mouse (C57BL/6J)
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19 kidney was studied at both protein and mRNA level. Five- μm sections of fixed frozen kidney
20
21 samples were co-stained for ARL15 and specific segment markers: breast cancer resistance
22
23 protein (BCRP) for the proximal tubule (PT), TH for the TAL, NCC for the DCT, and
24
25 aquaporin-2 (AQP2) for the collecting duct (CD). For mRNA expression, kidneys were
26
27 dissected and minced, before isolation of well-characterized nephron segments. For more
28
29 details of the immunohistochemistry, micro-dissection studies and mRNA analyses in the
30
31 nephron segments, see the [Supplementary Information](#).
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39 **Functional studies in cells and animal models**

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41 To study the regulation of TRPM6 channel activity by ARL15, human embryonic kidney 293
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43 (HEK293) cells were transfected with 1 μg of human TRPM6 cDNA³³ and 250 ng of empty
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45 vector (mock), human WT ARL15 or a human dominant negative (T46N) ARL15 mutant as
46
47 negative control. Whole-cell patch clamp recordings were performed to determine the
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49 electrophysiological properties of the cells transfected with the constructs mentioned above
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51 (see details in the [Supplementary Information](#)).
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55 The regulation of *ARL15* gene expression by dietary Mg^{2+} was studied in C57BL/6J
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57 mice and Tupfel long-fin zebrafish (details in the Supplementary Information). These studies
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3 were followed by a loss-of-function approach in zebrafish larvae using two non-overlapping
4 splice-site blocking morpholinos (MOs): 5'-AAACACTGAAAGACGGGACAAAGAC-3'
5 and 5'-GTTAAGCGAGTATTAGGTTACCTCT-3' (Gene Tools, Philomath, OR, USA),
6
7 designated as exon skipping 3 and 4 *arl15b*-MO respectively. These antisense
8
9 oligonucleotides were used to knockdown the highly conserved *ARL15* ortholog in the
10
11 zebrafish, *arl15b*. Phenotype rescue experiments were performed by co-injecting *arl15b*-
12
13 MOs with human *ARL15* cRNA as previously described⁶¹ and detailed in the [Supplementary](#)
14
15 [Information](#). The use of two non-overlapping *arl15b*-MOs and the combination with rescue
16
17 experiments with human *ARL15* cRNA served to rule out potential off-target effects in our
18
19 knockdown approach.
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27 **Statistical analyses in experiments performed in cellular and animal models**

28 All results are depicted as mean \pm standard error of the mean (SEM). Statistical analyses were
29
30 conducted by one-way ANOVA followed by the Tukey's multiple comparison post-test.
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32 When only two experimental groups were affected by the factor of variance, an unpaired
33
34 Student's *t*-test was used. Statistical significance was set at $P < 0.05$.
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41 **Metabolic factors associated with uMg excretion**

42 We used multiple linear regression to explore the association between square-root-
43
44 transformed uMg-to-creatinine ratio in spot urine and several metabolic markers (i.e. fasting
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46 triglycerides, total cholesterol, HDL-cholesterol, glucose, insulin, homocysteine, adiponectin,
47
48 GGT and BMI), taken one-at-a-time as the dependent variable, while adjusting for serum
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50 Mg^{2+} and other potential confounders in SKIPOGH and CoLaus cohorts. In SKIPOGH, we
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52 used multivariate linear mixed effect regression to explore the association of fasting insulin
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54 with 24-h uMg excretion, while adjusting for potential confounders. We used a conservative
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3 *P* value ($0.05/9=0.0056$) threshold to consider the associations of urinary Mg with metabolic
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5 phenotypes as statistically significant.
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8 9 **SNP-by-environment interaction on uMg levels**

10 We explored the role of metabolic phenotypes on the association of *ARL15* rs35929 with
11
12 uMg levels in spot urine among CoLaus participants. We conducted multiple linear
13
14 regression to explore the modifying effect of BMI, fat mass and fasting insulin levels on the
15
16 association of the *ARL15* rs35929 with square-root transformed uMg concentration, while
17
18 including age, sex, height, lean mass, CKD-EPI, urinary creatinine (square-root), serum
19
20 Mg^{2+} , serum and urine Ca^{2+} (square-root) and menopausal status. The *P* values for
21
22 interaction were not corrected for multiple testing, as we tested a single global hypothesis
23
24 (“does the association between rs35929 and urinary Mg differ by metabolic status?”) guided
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26 by prior knowledge.
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34 **Note:** The datasets and summary statistics are available in the IUMSP Research Data
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36 Repository, under the link <https://data.iumsp.ch/index.php/catalog/6>.
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38 ID Number: CHE-GWAS-V01. Created on July 12, 2017.
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ACKNOWLEDGEMENTS

We thank Dr. Sjoerd Verkaart, Anke Hoefnagels and Sami G. Mohammed for their excellent technical support during the performance of the functional analyses and acknowledge Drs. Erwin van Wijk and Gert Flik for providing the facilities for zebrafish experimentation.

FUNDING

This work was supported by the Long-Term Fellowship from the European Renal Association - European Dialysis and Transplant Association (ERA LTF 175-2014) and the Impulsion Grant from the ERA-EDTA Working Group on Inherited Kidney Disorders (WGIKD) to FJA; and by the grant from the Netherlands Organization for Scientific Research (NWO VICI 016.130.668) to JGJH. JHFdB is supported by the grant from NWO (Rubicon 825.14.021) and RJMB by the EUREnOmics project from the European Union Seventh Framework Programme (FP7/2007–2013, agreement no 305608). ZK received financial support from the Leenaards Foundation, the Swiss National Science Foundation (31003A-143914) and SystemsX.ch (51RTP0_151019). MBO and TC are supported by the Swiss National Centre of Competence in Research Kidney Control of Homeostasis (NCCR Kidney.CH) program. OD is supported by grants from the European Community's Seventh Framework Programme (305608 EUREnOmics), the Swiss National Centre of Competence in Research Kidney Control of Homeostasis (NCCR Kidney.CH) program, the Swiss National Science Foundation (31003A_169850) and the Rare Disease Initiative Zurich (radiz), a clinical research priority program of the University of Zurich, Switzerland.

The SKIPOGH study is supported by a grant from the Swiss national science foundation (FN 33CM30-124087). The CoLaus study is supported by research grants from GlaxoSmithKline, the Faculty of Biology and Medicine of Lausanne, and the Swiss National Science Foundation (grants 33CSCO-122661, 33CS30-139468 and 33CS30-148401). The

1
2
3 CROATIA-Korcula and CROATIA-Split studies were funded by grants from the Medical
4
5 Research Council (UK), European Commission Framework 6 project EUROSPAN (Contract
6
7 No. LSHG-CT-2006-018947) and Republic of Croatia Ministry of Science, Education and
8
9 Sports research grants to I.R. (108-1080315-0302). We would like to acknowledge the
10
11 invaluable contributions of the recruitment team in Korcula and Split, the administrative
12
13 teams in Croatia and Edinburgh and the people of Korcula and Split. The SNP genotyping for
14
15 the CROATIA-Korcula cohort was performed in Helmholtz Zentrum München, Neuherberg,
16
17 Germany. The SNP genotyping for the CROATIA-Split cohort was performed by AROS
18
19 Applied Biotechnology, Aarhus, Denmark. INGI-Carlantino : We thank Anna Morgan and
20
21 Angela D'Eustacchio for technical support; the municipal administrators for their
22
23 collaboration on the project and for logistic support; and all participants to this study. For the
24
25 INGI-VALBORBERA study, the research was supported by funds from Compagnia di San
26
27 Paolo, Torino, Italy; Fondazione Cariplo, Italy and Ministry of Health, Ricerca Finalizzata
28
29 2008 to DT. Phenotype collection in LBC1936 was supported by Age UK (The Disconnected
30
31 Mind project). Genotyping was funded by the BBSRC (BB/F019394/1). The work was
32
33 undertaken by the University of Edinburgh Centre for Cognitive Ageing and Cognitive
34
35 Epidemiology, part of the cross council Lifelong Health and Wellbeing Initiative
36
37 (MR/K026992/1). Funding from the BBSRC and Medical Research Council (MRC) is
38
39 gratefully acknowledged. We thank the LBC1936 participants, the LBC1936 team for data
40
41 collection and collation, and the staff at the Wellcome Trust Clinical Research Facility for
42
43 bio-sample collection and genotyping.
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52 **COMPETING FINANCIAL INTERESTS:** None.
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Table 1: Summary statistics for the meta-analysis of uMg-to-creatinine ratio.

SNP	Effect allele	Other allele	Mean effect allele frequency	Effect size discovery (SE)	p-value discovery	Effect size replication (SE)	p-value replication	Effect size Combined analysis (SE)	p-value combined analysis	Direction of effects of individual cohorts	I ²
rs35929	a	g	0.21	-0.112 (-0.025)	5.57×10^{-6}	-0.135 (-0.027)	5.95×10^{-7}	-0.1227 (-0.183)	2.11×10^{-11}	-----+	35.7
rs3824347	g	a	0.41	-0.109 (-0.02)	3.61×10^{-8}	-0.1125 (-0.024)	2.02×10^{-6}	-0.1103 (-0.152)	4.38×10^{-13}	+++++++	7

The effect size sign for each individual cohort on the last but one column is represented by the sign – or +. The cohort displaying a “-” for rs32929 is LBC1936, having anyhow an effect close to 0. The last column shows the I² value, representing the heterogeneity across cohorts.

LEGENDS TO FIGURES

Figure 1. Genome-wide meta-analysis results for uMg-to-creatinin Urinary Mg-to-creatinine ratio.

(a) Manhattan plot showing $-\log_{10}(P)$ values for all SNPs in the genome-wide meta-analysis for normalized uMg-to-creatinine ratio in Europeans ($n = 9,099$), ordered by chromosomal position. The values correspond to the association of normalized uMg-to-creatinine ratio, including age and sex as covariates in the model as well as study-specific covariates if needed. The gene closest to the SNP with the lowest P value is listed at each locus. Two loci reached genome-wide significance ($P < 5 \times 10^{-8}$) at combined analysis (*TRPM6*, rs3824347 and *ARL15*, rs35929). (b) Forest plot for rs35929 (*ARL15*) and rs3824347 (*TRPM6*) showing effect sizes and 95% confidence intervals across studies as well as the summary meta-analysis results. (c) Regional association plot at the rs3824347 (*TRPM6*) locus. Regional association plot showing $-\log_{10} P$ values for the association of SNPs at the locus of interest ordered by their chromosomal position with normalized uMg-to-creatinine ratio. The $-\log_{10} P$ value for each SNP is colored according to the correlation of the corresponding SNP with the SNP showing the lowest P value (index SNP) within the locus using different colors for selected levels of linkage disequilibrium (r^2). Correlation structures correspond to HapMap 2 CEU. The blue line represents the recombination according to the scale shown on the right-side Y axis. (d) Regional association plot at the rs35929 (*ARL15*) locus. See Panel b legend.

Figure 2. External population-based validation using 24-h urine data.

(a-d) Associations of uMg excretion and fractional excretion with an unweighted genetic risk score including rs35929 (*ARL15* locus) and rs3824347 (*TRPM6* locus). Data are geometric

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3 means and whiskers are 95% confidence intervals for Mg-related phenotypes in the CoLaus
4 (panels **a,b,d**) and SKIPOGH (panel **c**) studies. The X axes represent the unweighted genetic
5 score generated from rs35929 (*ARL15* locus) and rs3824347 (*TRPM6* locus), using as effect
6 allele the one associated with lower uMg-to-creatinine ratio, i.e. the A allele for rs35929 and the
7 G allele for rs3824347. *P* values are from non-parametric trend tests across genetic score. The
8 numbers in each genetic score category are listed in all panels. The Y axis represents uMg-to-
9 creatinine ratio (mg/g) (**a**), FEMg (%) (**b**), uMg excretion (mg/24h) (**c**) and serum Mg²⁺ (mg/dl)
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Figure 3. ARL15 localizes in renal DCT regulating TRPM6 channel activity.

(a) Gene expression analyses of *Trpm6*, *Arl15*, *Podocin*, *Sglt2*, *Snat3*, *Nkcc2*, *Ncc* and *Aqp2* in micro-dissected mouse nephron segments showed co-expression of *Arl15* and *Trpm6* in DCT.
(b) Double immunofluorescence staining of mouse kidney cortex sections for ARL15 (in green) and BCRP (red); TH (red); NCC (red); or AQP2 (red) as markers of the PT, TAL, DCT or CD respectively.
(c) Typical current-voltage curves obtained from transfected HEK293 cells 200 s after break-in. Outwardly-rectifying currents are observed in response to a 500 ms voltage ramp (from -100 to +100 mV) applied 200 s after break-in.
(d) The average time development of the current density measured at +80 mV is shown (n ≥ 10). The mock + ARL15 condition is not shown for clarity reasons.
(e) WT ARL15 (red, WT, n = 47) but not the T46N ARL15 mutant (black, T46N, n = 18) increased the whole-cell current density of TRPM6. Asterisks indicate significant differences respect to the cells transfected with *TRPM6* only (blue, '-', n = 44). One-way ANOVA followed by Tukey's multiple comparisons post-test, *P* < 0.05.
(f) Transfection of

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3 HEK293 cells with WT *ARL15* did not evoke a significant increase in whole-cell current density
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5 when compared with mock-transfected of cells ($n \geq 10$, unpaired Student's *t*-test).
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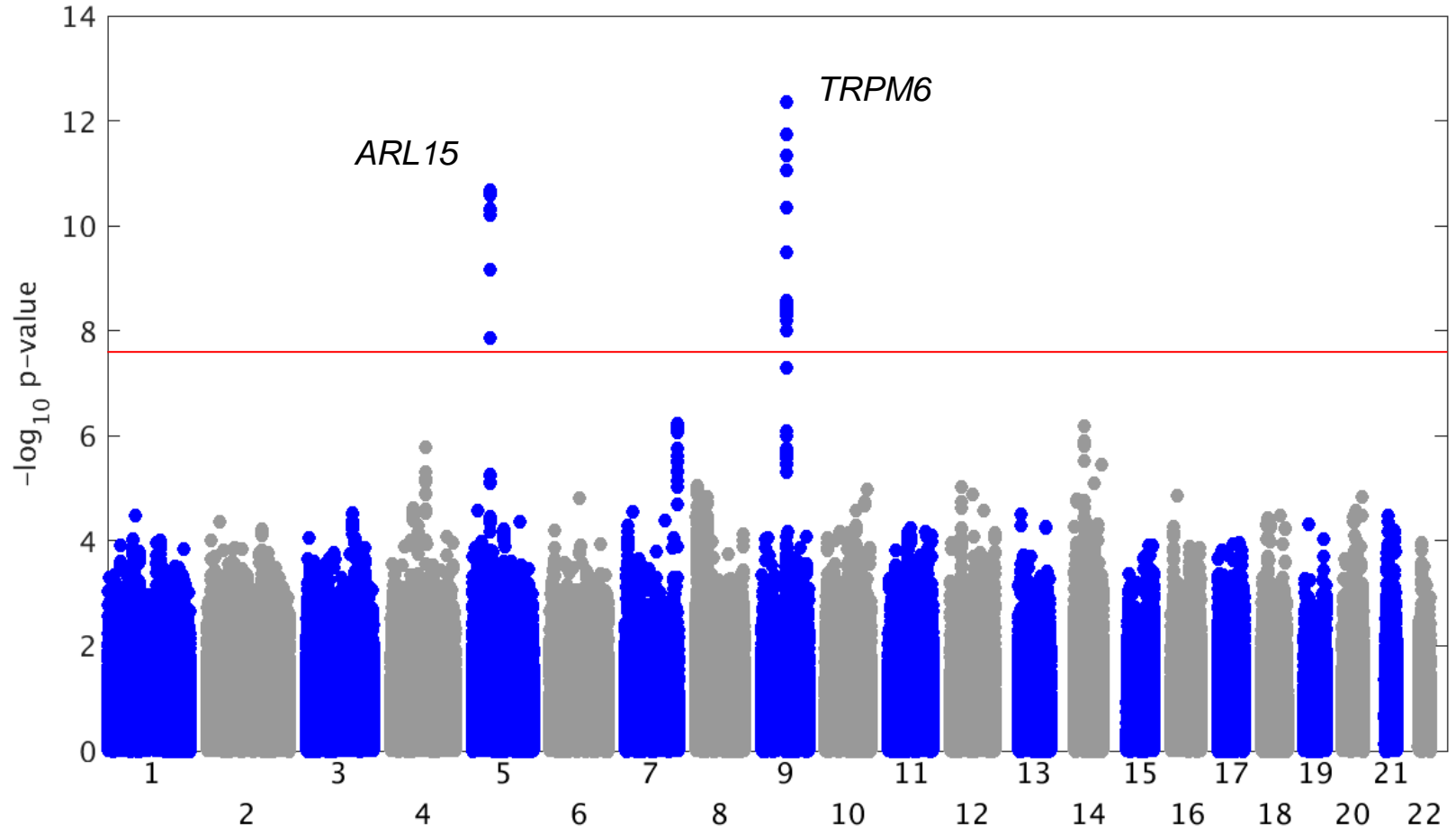
10 **Figure 4. Physiological relevance of ARL15 in kidney.**

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12 **(a-b)** Knockdown of *arl15b* by 0.5-2 **(a)** and 2-8 **(b)** ng/embryo of the two *arl15b*-MOs used
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14 (exon skipping 3 and 4 *arl15b*-MOs) resulted in a dose-dependent decrease of the total Mg
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16 content of zebrafish *arl15b* morphants, reflecting renal Mg^{2+} wasting. The zero dose represents
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18 injection with control-MO (2 **(a)** and 8 **(b)** ng MO/embryo). **(c)** Morphological phenotypes
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20 distinguished in zebrafish larvae (5 days post-fertilization (dpf)) following treatment with
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22 *arl15b*-MO or control-MO. A complete description of each phenotype is detailed in the
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24 Supplementary Information. Metabolic defects (poor metabolization of the yolk) are indicated by
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26 arrows. **(d-g)** Distribution of morphological phenotypes in zebrafish larvae injected with 0.5-2
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28 **(d, f)** and 2-8 **(e, g)** ng/embryo of exon skipping 3 **(d, e)**, exon skipping 4 **(f, g)** *arl15b*-MO or
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30 control-MO (2 **(d, f)** and 8 **(e, g)** ng MO/embryo). **(h-k)** Rescue of renal Mg^{2+} wasting **(h, j)** and
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32 of metabolism defects **(i, k)** in morphant zebrafish by co-injection of exon skipping 3 *arl15b*-MO
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34 (0.5 ng MO/embryo, **h-i**) or exon skipping 4 *arl15b*-MO (8 ng MO/embryo, **j-k**) with cRNA
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36 encoding human WT ARL15 (50 pg cRNA/embryo). Co-injection with cRNA encoding human
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38 T46N ARL15 mutant (50 pg cRNA/embryo) did not rescue renal Mg^{2+} wasting or the defects in
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40 metabolism. **(d-g, i, k)** Numbers on top of the bars indicate the number of animals in each
41
42 experiment. **(a-b, h, j)** Data are presented as mean \pm SEM ($n = 10$, except for **b**, where $n = 6-10$).
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44 **(a-b, h, j)** Asterisks indicate significant differences respect to the control condition (One-way
45
46 ANOVA followed by Tukey's multiple comparisons post-test, $P < 0.05$).
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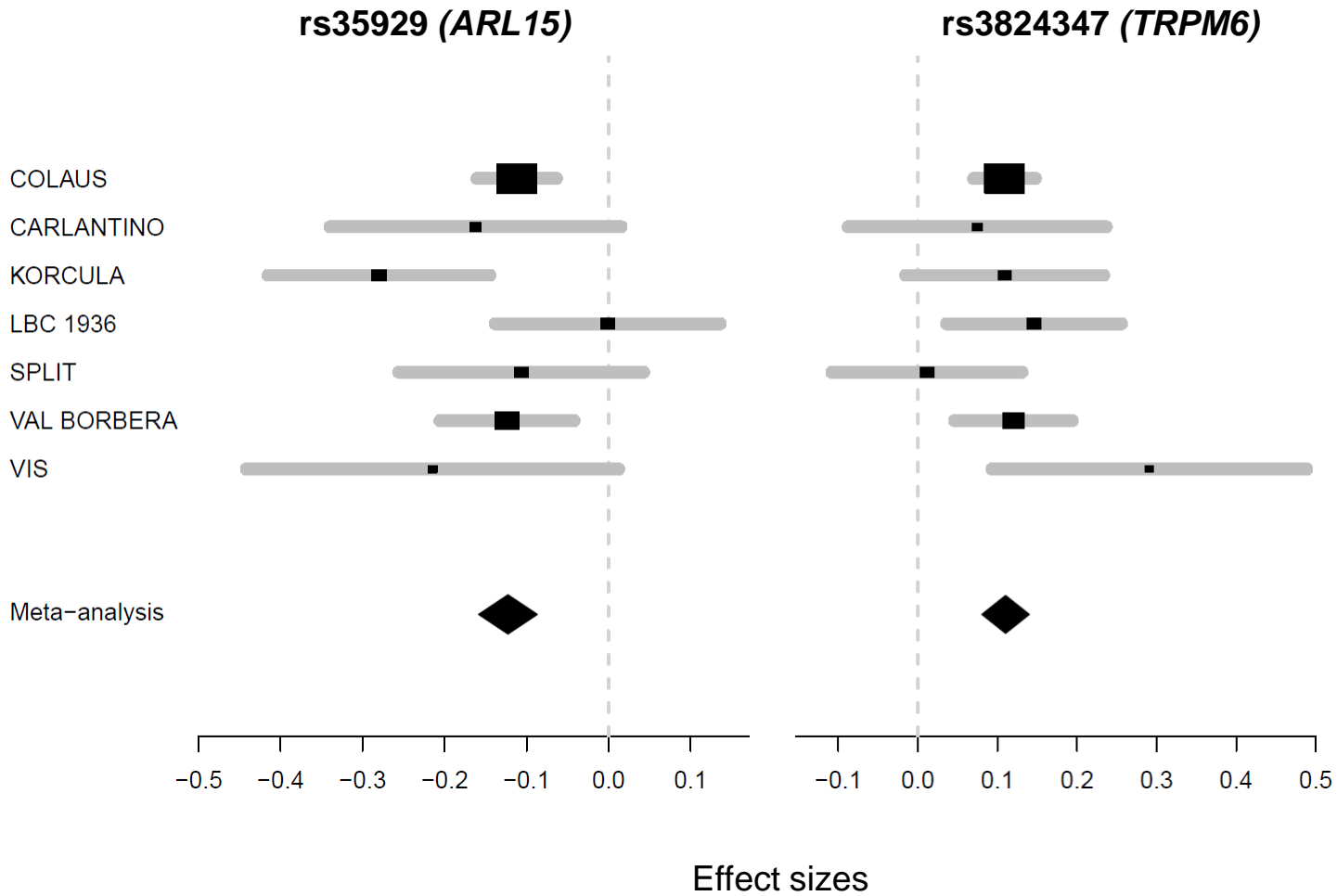
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3 **Figure 5. Effect modification of metabolic phenotypes on the association of uMg with the**
4 ***ARL15* locus.**
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8 (a) Effect modification of fat mass on the association of uMg concentration with rs35929
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10 (*ARL15*) genotypes. Data represent adjusted square-root transformed uMg levels by rs35929
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12 genotypes and fat mass strata in CoLaus. The model was adjusted for age, sex, height, lean mass,
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14 CKD-EPI, urinary creatinine (square-root), serum Mg^{2+} , serum and urinary Ca^{2+} (square-root)
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16 and menopausal status. *P* interaction = 0.024. Fat mass strata are cut by sex-specific medians. n
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18 = 4,729. (b) Effect modification of fasting insulin on the association of uMg levels with rs35929
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20 (*ARL15*) genotypes. Data represent adjusted square-root transformed uMg levels by rs35929
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22 genotypes and fasting insulin strata in CoLaus. The model was adjusted for age, sex, height, lean
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24 mass, CKD-EPI, urinary creatinine (square-root), serum Mg^{2+} , serum and urinary Ca^{2+} (square-
25
26 root) and menopausal status. *P* interaction = 0.012. Fat mass strata are cut by sex-specific
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28 medians (n = 4,729).
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37 **Figure 6. Multi-step approach used to investigate the genetic determinants of the renal**
38 **handling of Mg^{2+} and their influence on metabolic traits in the general population.**
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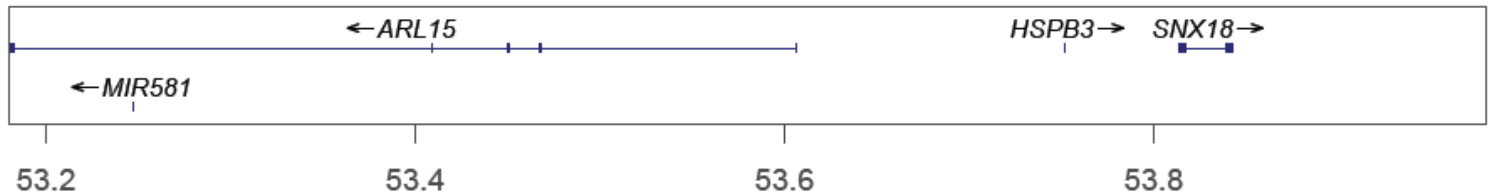
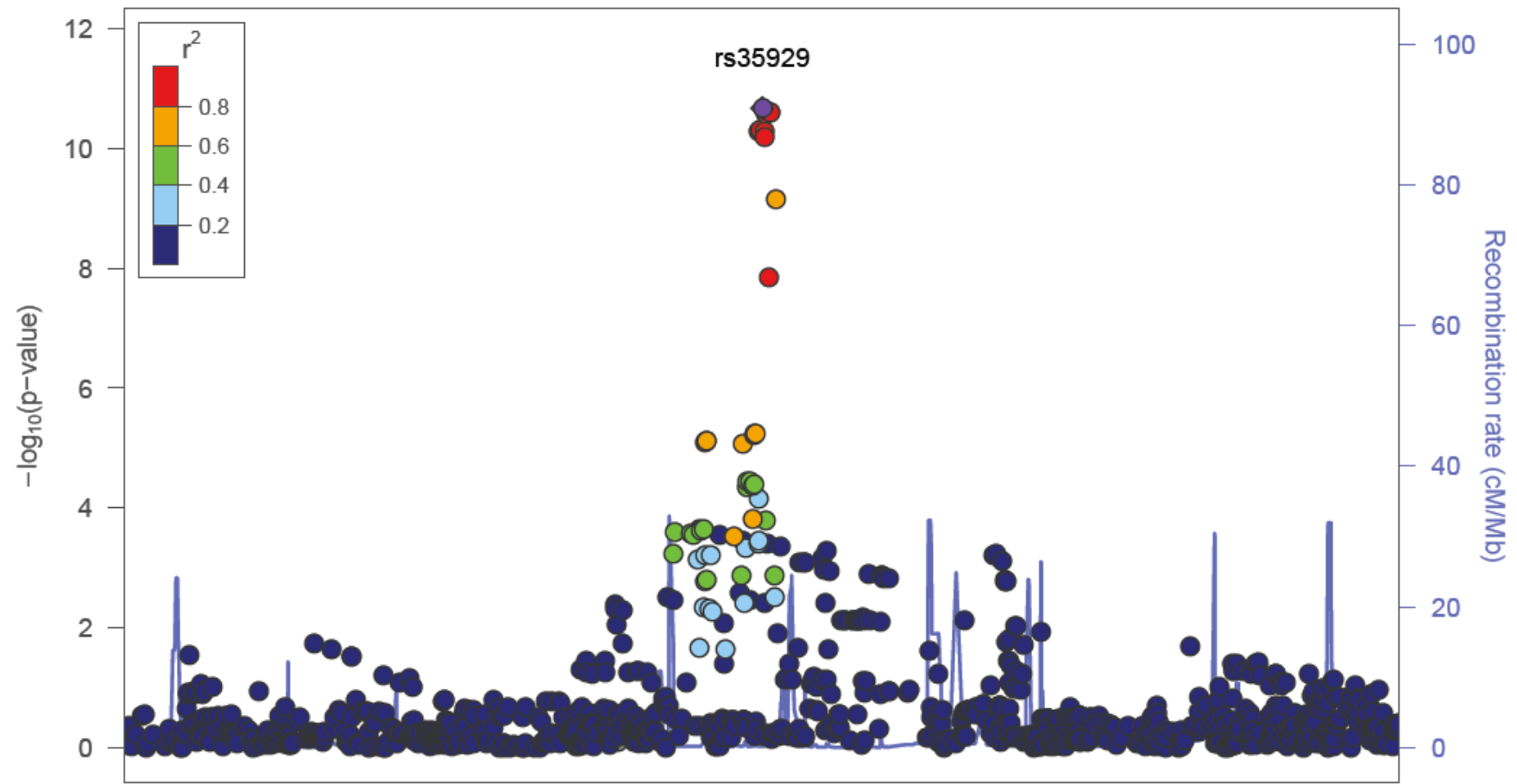


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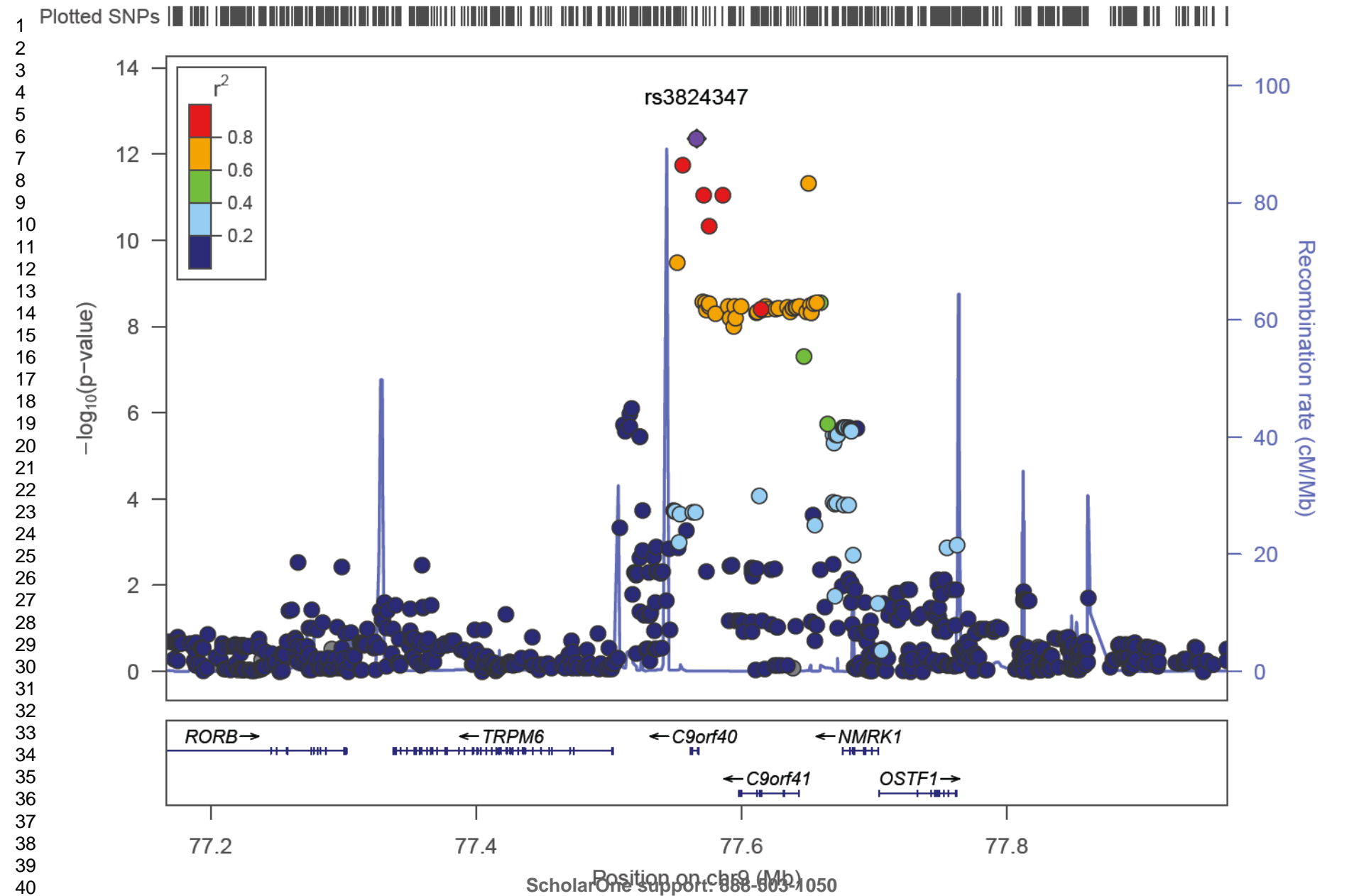


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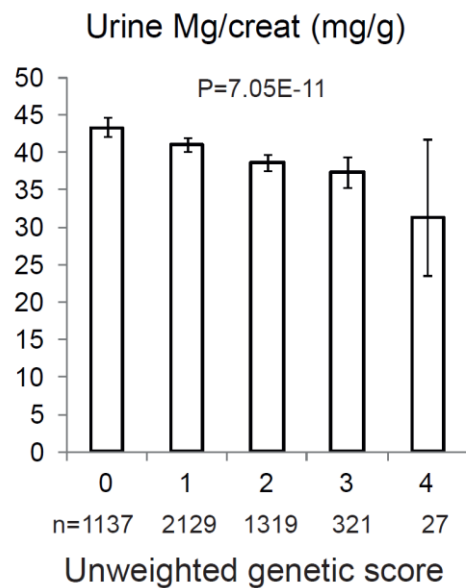
Plotted SNPs



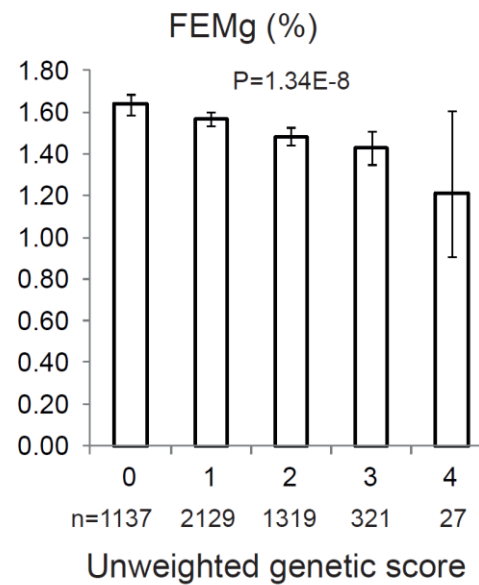
53.2 53.4 53.6 53.8
Position on chr5 (Mb)



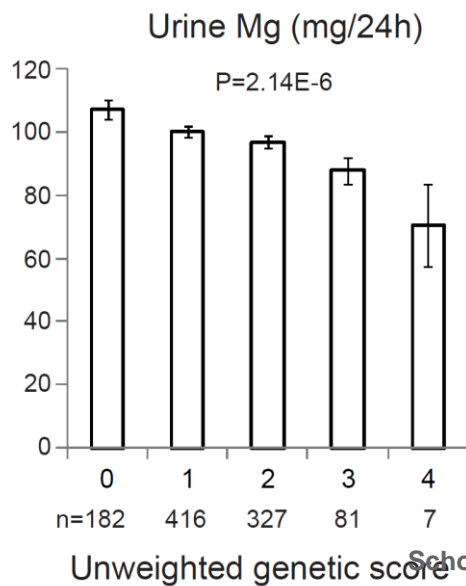
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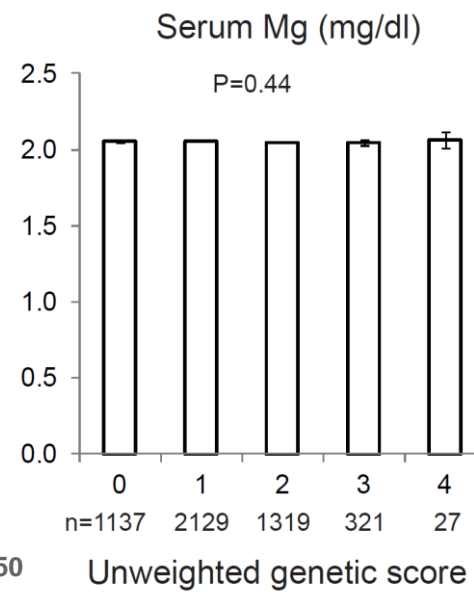
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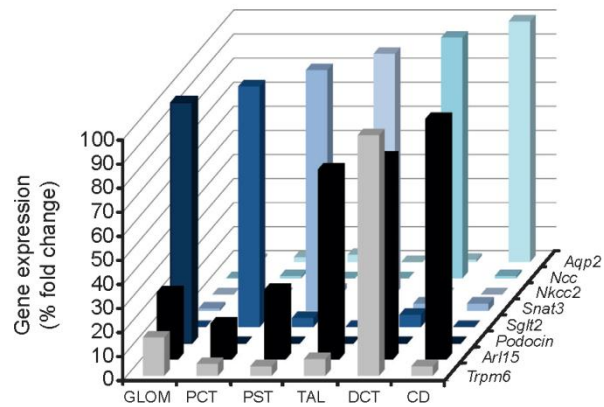


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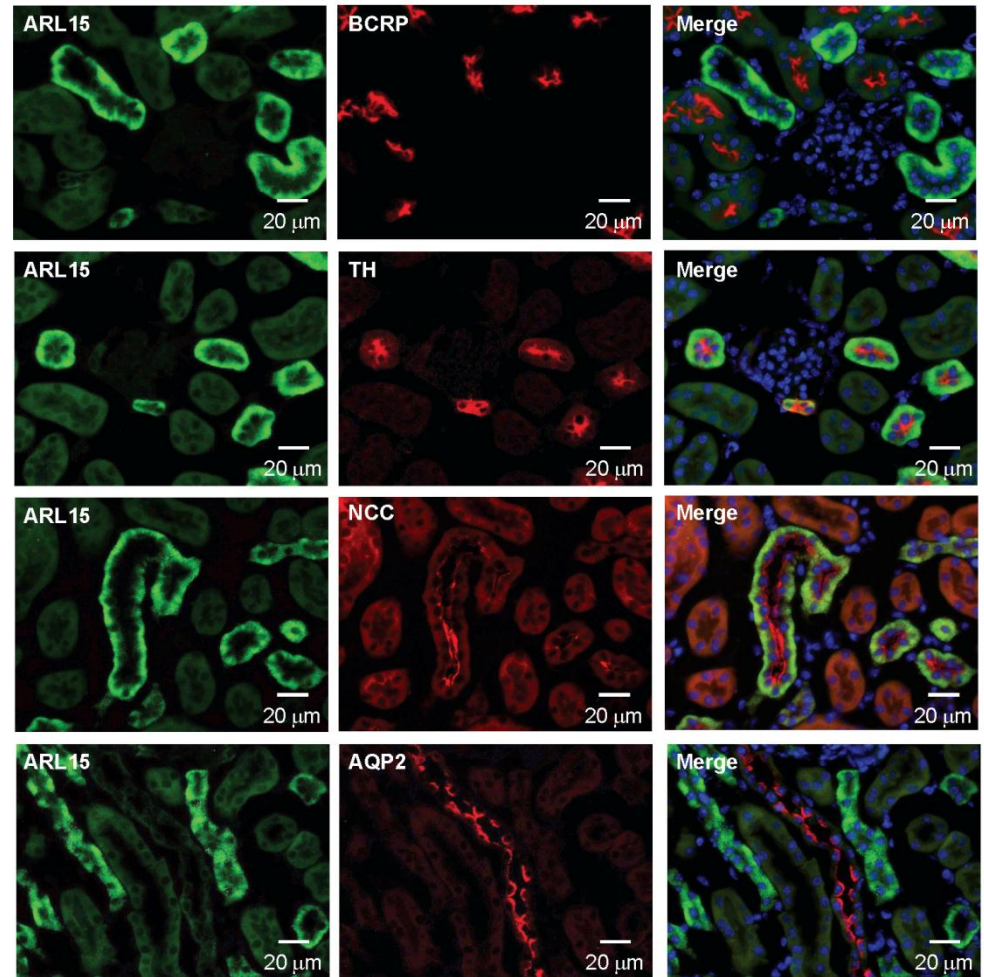


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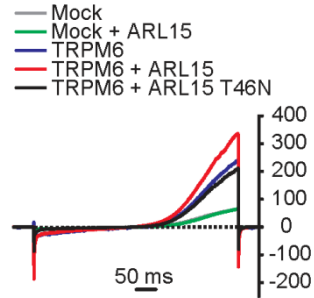
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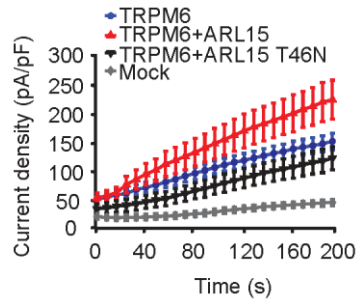
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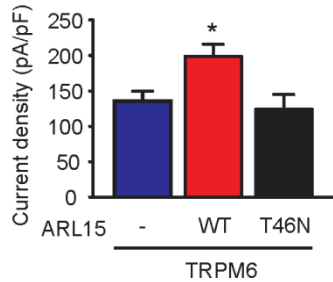
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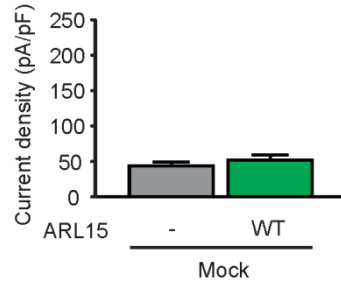
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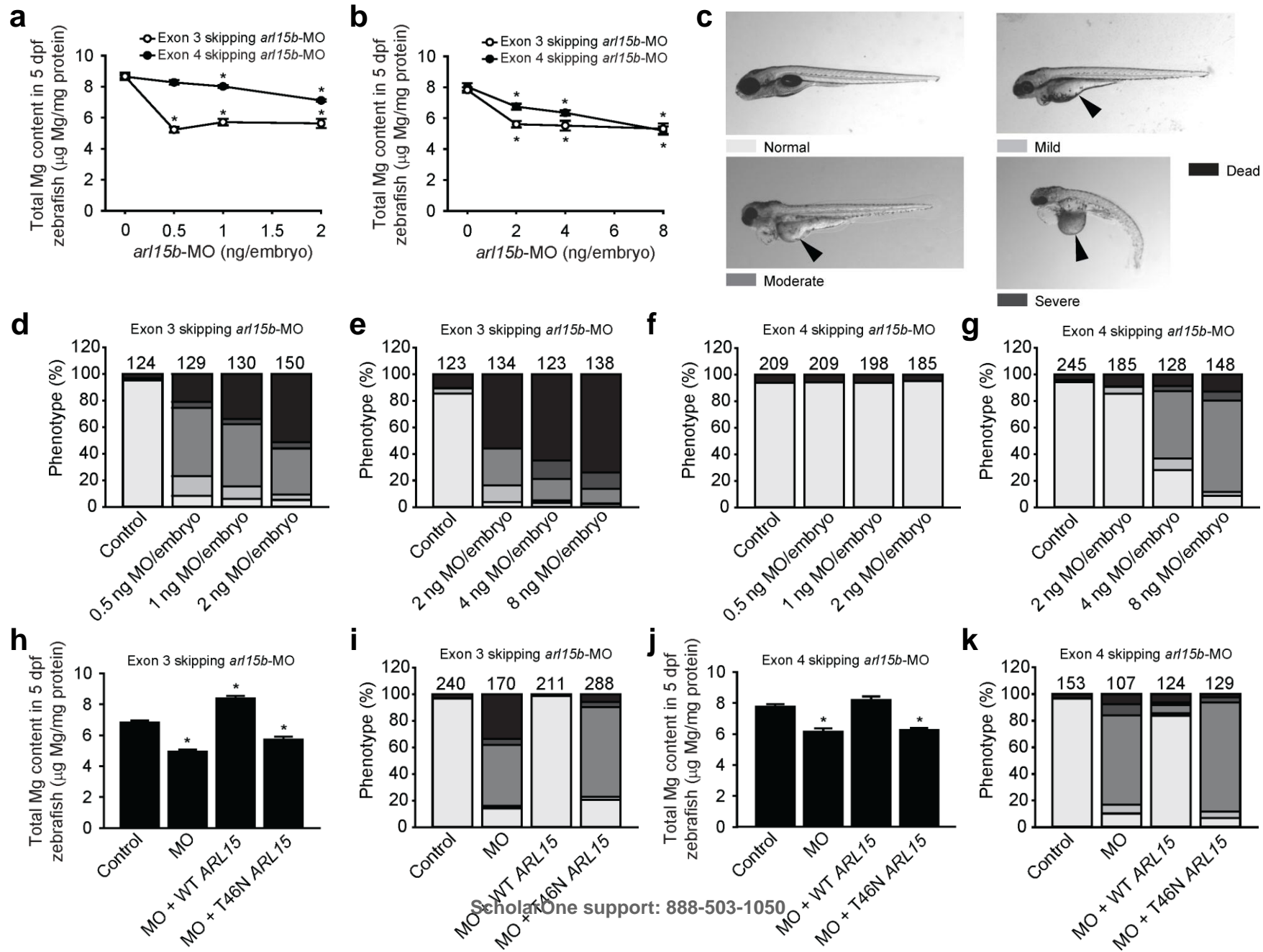


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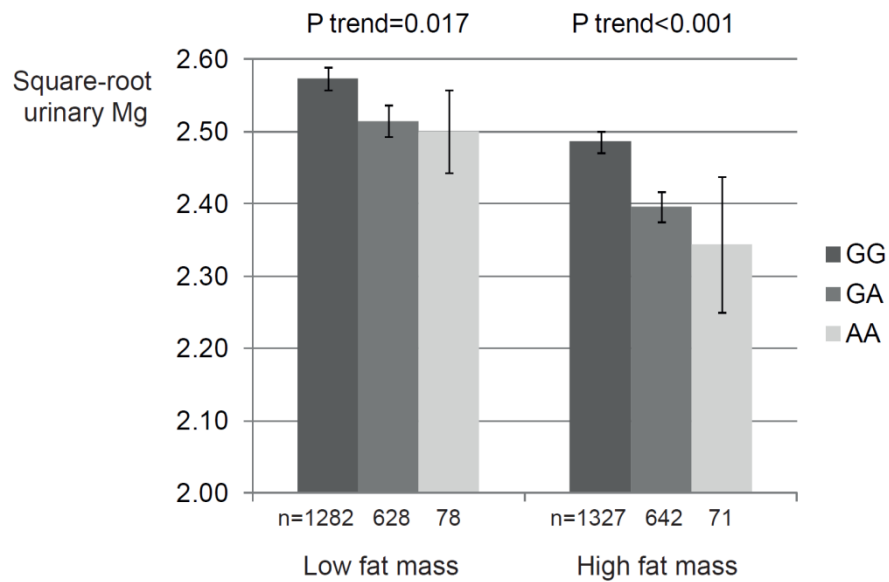
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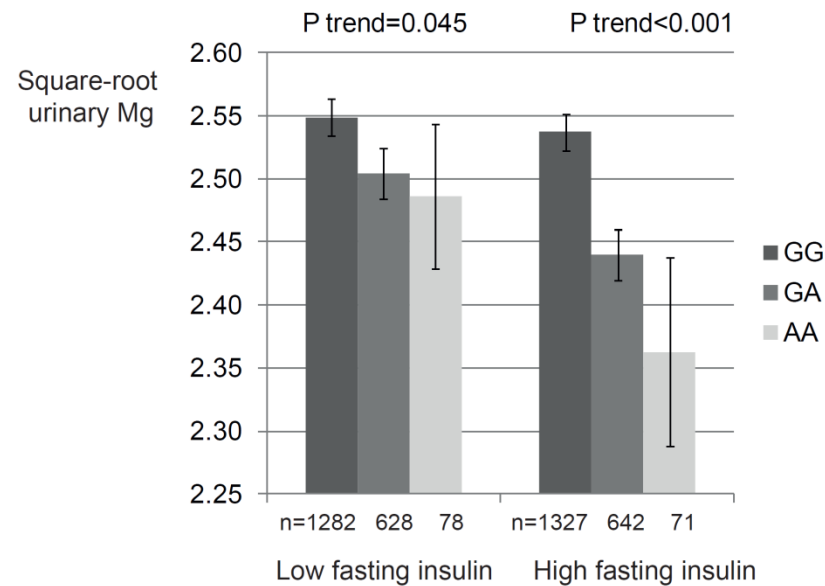


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2 **Discovery GWAS:** uMg-to-creat (CoLaus; N=5,150)
3 → rs3824347 (chr 9, *TRPM6*)
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5 **Meta-GWAS:** uMg-to-creat (CoLaus + LBC1936, LBC1936, CROATIA-Split, CROATIA-Vis, Carlantino, CROATIA-Korcula, Val Borbera;
6 N=9,099)
7 → rs3824347 (chr 9, *TRPM6*) + rs35929 (chr 5, *ARL15*)
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11 **Additive genetic risk score:** rs3824347 + rs35929
12 → uMg-to-creat and FEMg (CoLaus); uMg 24h (SKIPOGH)
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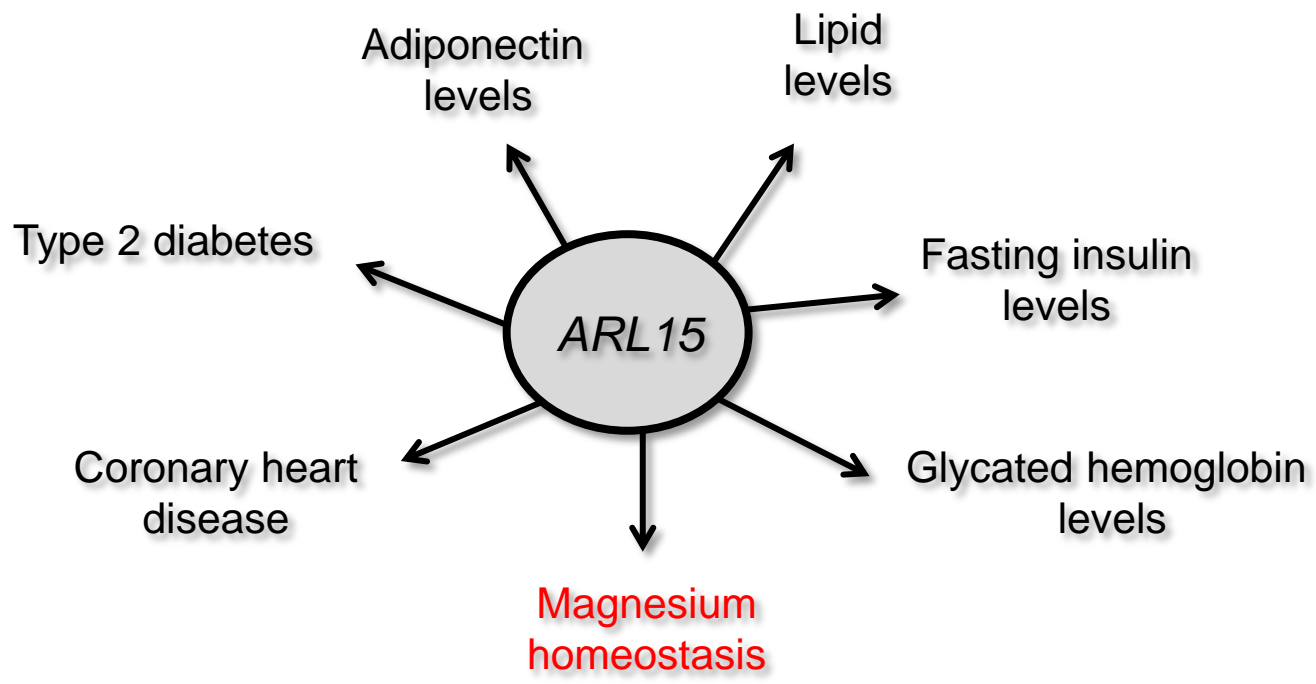
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16 **Expression studies of ARL15 and TRPM6 in mouse kidney**
17 → mRNA (RT-qPCR) and protein (immunofluorescence)
18

19 **Functional studies:** Influence of ARL15 on channel activity of TRPM6
20 → Patch-clamp recordings on HEK293 cells transfected with ARL15 and TRPM6
21

22 **In vivo studies: Mouse** - Effects of magnesium diets (kidney, ileum, caecum)
23 → Differential mRNA expression patterns (*Arl15*, *Trpm6*, other genes)
24

25 **In vivo studies: Zebrafish**
26 → Identification of ARL15 orthologs
27 → Expression of *arl15b* in adult fish
28 → Effect of magnesium diets on *arl15b* expression patterns (kidneys, gills, gut – adult fish)
29 → Loss-of-function and rescue (larvae): Magnesium balance and metabolic phenotypes
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34 **Link genetic background (*ARL15* rs35929) with renal magnesium handling and metabolic traits**
35 → CoLaus: Association of uMg with metabolic traits
36 → SKIPOGH: Association of 24h uMg with metabolic traits
37 → CoLaus: Effect of body weight, BMI and fat mass on the association of rs35929 with uMg excretion
38 → SKIPOGH: Effect of rs35929 on the association of uMg excretion with fasting insulin
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Supplementary Information

**Genome-wide Meta-analysis Unravels Novel Interactions between
Magnesium Homeostasis and Metabolic Phenotypes**

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- Supplementary Methods
- Supplementary Figures 1-10
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For Peer Review

SUPPLEMENTARY METHODS

GWAS Cohorts

CoLaus is a population-based cohort with baseline examination conducted between 2003 and 2006. It includes 6,184 individuals of European descent aged 35-75 years randomly selected from the registry of the city of Lausanne ¹. The *CROATIA-Vis* study, Croatia, is a family-based, cross-sectional study in the isolated island of Vis that included 1,056 examinees aged 18-93. Blood samples were collected in 2003 and 2004 ². The *CROATIA-Korcula* study, Croatia, is a family-based, cross-sectional study in the isolated island of Korcula that included 965 examinees aged 18-95. Blood samples were collected in 2007 ³. The *CROATIA-Split* study, Croatia, is population-based, cross-sectional study in the Dalmatian city of Split that so far includes 1,012 examinees aged 18-95. Blood samples were collected in 2009 -2011 ⁴. The *Lothian Birth Cohort 1936* (LBC1936) consists of 1,091 relatively healthy older participants, most of whom took part in the Scottish Mental Survey of 1947 at the age of about 11 years. At a mean age of 69.5 years (SD 0.8) they were recruited to a study investigating influences on cognitive ageing ⁵. A second wave of cognitive and physical testing occurred at approximately 73 years of age at which time a urine sample was collected ^{5,6}. The *INGI-Val Borbera* population is a collection of 1,785 genotyped samples (18-102 years) collected in the Val Borbera Valley, a geographically isolated valley located within the Appennine Mountains in Northwest Italy ⁷. The *INGI-Carlantino* study is a population-based, cross-sectional study in a village situated in the Southeastern part of the Apennines in a hilly area of the Puglia region. Main study characteristics are summarized in Suppl. Table 4, genotyping details on Suppl. Table 5.

Micro-dissection studies in mouse kidney

Well-characterized tubular segments were microdissected from mouse kidneys as described previously ⁸. Kidneys from male C57BL/6J mice were dissected and minced before incubation with 0.1% (w/v) type 2 collagenase solution that contained 100 µg/ml soybean trypsin inhibitor for 30 min at 37°C. After digestion, the supernatant was sieved through 250- and 80-µm nylon filters. Nephron fragments remained in the 80-µm sieve and were resuspended by flushing. Distinct segments [glomeruli (GLOM), proximal convoluted and straight tubules (PCT and PST respectively), thick ascending loop of Henle (TAL), distal convoluted tubule (DCT) and collecting duct (CD)] were isolated upon their morphologic features. Three collections were snap-frozen in liquid nitrogen and conserved at -80°C.

Immunohistochemistry

Nephron immunohistochemistry was performed as described previously^{9,10}. In short, co-staining for ARL15, breast cancer resistance protein (BCRP), Tamm-Horsfall protein (TH), thiazide sensitive Na⁺-Cl⁻ cotransporter (NCC) and aquaporin-2 (AQP2) was performed on 5- μ m sections of fixed frozen mouse (C57BL/6J) kidney samples. The sections were incubated for 16 h at 4°C with the following primary antibodies: rabbit anti-ARL15 (1:100, Sigma Chemical Co., St Louis, USA), rat anti-BCRP (1:250¹⁰), sheep anti-TH (1:750¹⁰), rabbit anti-NCC (1:50¹¹) or rabbit anti-AQP2 (1:1000, kindly provided by Dr. Deen, Nijmegen, The Netherlands). For detection, kidney sections were incubated with Alexa Fluor-conjugated secondary antibodies. Images were taken with a Zeiss Axio Imager 1 microscope (Oberkochen, Germany) equipped with a HXP120 Kubler Codix fluorescence lamp and a Zeiss AxioCam MRm digital camera.

Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells were grown at 37°C in DMEM (Biowhittaker Europe, Vervier, Belgium) supplemented with 10% (v/v) FCS (PAA Laboratories, Linz, Austria), non-essential amino acids (AAs), and 2 mM L-glutamine in a humidified 5% (v/v) CO₂ atmosphere.

Cells were seeded in 12-well plates and subsequently transfected with a total of 1.25 μ g of cDNA (per well in a 12 wells plate) using Lipofectamine 2000 (Invitrogen, Breda, The Netherlands). HA-tagged human TRPM6 was in the pCINeo-IRES-GFP mammalian expression vector¹². A human ARL15 clone was obtained from Source BioScience (Berlin, Germany) and subcloned into the pCINeo-IRES-mCherry vector, used for transfections. The T46N mutation was inserted in the ARL15 construct using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. All constructs were verified by sequence analysis. Mock conditions were obtained by transfecting the empty pCINeo-IRES-mCherry vector. Approximately 36 h after transfection, cells were seeded on glass coverslips coated with 50 μ g/ml fibronectin (Roche Diagnostics, Mannheim, Germany). Experiments were started 2 h after seeding the cells. Cells displaying both GFP and mCherry fluorescence were chosen for recording.

Electrophysiology

All experiments were performed at room temperature. Whole-cell recordings were undertaken and analyzed using an EPC-9 amplifier and the Patchmaster software (HEKA electronics,

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3 Lambrecht, Germany). The sampling interval was set to 100 μ s (10 kHz) with a low-pass
4 filter set at 2.9 kHz. Pipettes were pulled from thin wall borosilicate glass (Harvard
5 Apparatus, March-Hugstetten, Germany) and had resistance between 1 and 3 M Ω when filled
6 with the pipette solution. Series resistance compensation was set to 75-95% in all
7 experiments. Currents were elicited by a series of 500 ms voltage ramps (from -100 to +100
8 mV) applied every two seconds from a holding voltage of 0 mV. Current densities were
9 obtained by normalizing the current amplitude to the cell capacitance.

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16 The extracellular solution contained (in mM): 150 NaCl, 1 CaCl₂, 10 HEPES and pH
17 adjusted to 7.4 using NaOH. The pipette solution was made of (in mM): 150 NaCl, 10
18 Na₂EDTA, 10 HEPES and pH adjusted to 7.2 using NaOH.

22 23 **Conservation of ARL15 proteins**

24 TBLASTN searches were performed on the mouse, cow and zebrafish genome database at
25 NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) using the human ARL15 as query AA
26 sequence. Sequences with a significant homology with human ARL15 were selected. Multiple
27 sequence alignments were carried out using ClustalW (<http://www.ebi.ac.uk/Tools/clustalw/>)
28 and the degree of AA conservation across species was discerned.

34 35 **Distribution of *Arl15* gene expression and dietary Mg²⁺ challenge in mice**

36 To study the tissue distribution of *Arl15* gene expression, three C57BL/6J mice were
37 sacrificed; lung, bone, kidney, peritoneum, stomach, brain, heart, liver and muscle tissues
38 were collected. The dietary Mg²⁺ challenge was performed on age- and gender-matched
39 C57BL/6J wild type (WT) littermates. Mice were housed in a temperature- and light-
40 controlled room with *ad libitum* access to standard pellet chow (SSNIFF Spezialdiäten, Soest,
41 Germany) and deionized drinking water for 4 weeks until the start of the experiment. Three
42 groups of mice were next fed a control diet (0.19% (w/w) Mg; n = 10), a Mg²⁺-deficient diet
43 (0.0005% (w/w) Mg; n = 10), or a Mg²⁺-enriched diet (0.48% (w/w) Mg; n = 10) (SSNIFF
44 Spezialdiäten, Soest, Germany) for 10 days. Mice were housed individually in metabolic
45 cages overnight at baseline, day 5 and day 10 for urine collection (16 h sampling). Venous
46 blood samples were obtained from the inferior vena cava during sacrifice at day 10, and
47 kidneys and intestine (ileum and caecum) were harvested as previously described¹³. All
48 protocols were conducted in accordance with the National Research Council Guide for the
49 Care and Use of Laboratory animals and were approved by the local Ethics Committee.

Distribution of *arl15b* gene expression and dietary Mg^{2+} challenge in zebrafish

Zebrafish from the Tupfel long-fin (TLF) strain were used for experimentation. For the study of the tissue distribution of the highly conserved ARL15 zebrafish ortholog (*arl15b*) in adult zebrafish tissues, 3 females and 3 males were dissected following anaesthesia (0.1% (v/v) 2-phenoxyethanol (Sigma Chemical Co., St Louis, USA)) and brain, ovary, gills, testis, heart, spleen, kidney, gut, operculum, scales and liver tissues were collected and stored at -80°C until analysis. In 5 days post-fertilization (dpf) larvae, animals were anaesthetised with tricaine/Tris pH 7.0 solution and pronephric tissue was isolated with fine-point needles under the microscope (Leica Microsystems Ltd). After removal of the yolk and swim bladder, the pronephros could be distinguished by the pigments that surround the pronephric tubules (Suppl. Fig. 8). Then, pronephric-enriched tissue was scratched and subsequently aspirated with a Pasteur pipette. Samples were constituted by 10 pronephros each, which were stored at -80°C until further analysis. Control genes were used to verify the purity of the pronephric tissue isolated: *ncc*, uniquely expressed in the pronephros¹⁴, served as positive control; and *ncc-like*, whose gene expression is restricted to the skin¹⁵, served as negative control.

To study the regulation of *arl15b* gene expression by the Mg^{2+} status in zebrafish ionoregulatory tissues (gills, kidney and gut), fish Mg^{2+} balance was challenged by different Mg^{2+} diets as previously reported¹⁶. Briefly, 27 adult zebrafish were weighed and randomly divided into 3 groups of 9 animals each and kept in 3 separate 2-liter tanks. During 2 weeks (acclimation to control conditions), all fish were fed a Mg^{2+} -control diet (Hope Farms, Woerden, The Netherlands; 0.07% (w/w) Mg) at a daily ration of 2% (w/w) of the total body weight. After this period, for 2 groups, the control diet was replaced by a Mg^{2+} -deficient diet (Hope Farms; 0.01% (w/w) Mg) or a Mg^{2+} -enriched diet (Hope Farms; 0.7% (w/w) Mg). These 2 groups were kept under these feeding regimes for 3 weeks, while the remaining group (fed a Mg^{2+} -control diet) served as a control. At the end of this period, all groups were sampled. Sampling took place 24 h after the last feeding. Fish were anaesthetised in 0.1% (v/v) 2-phenoxyethanol (Sigma). After anaesthesia, death of animals was induced by spinal transaction and organs were collected, immediately frozen in liquid nitrogen and stored at -80°C until analysis. All animal procedures detailed here were performed in accordance with national and international legislation and were approved by the ethical review committee of the Radboud University Nijmegen.

Knockdown of the zebrafish ortholog of human ARL15 and rescue experiments

WT TLF zebrafish were bred and raised under standard conditions (28.5°C and 14 h of light: 10 h of dark cycle) in accordance with international and institutional guidelines. Zebrafish eggs were obtained from natural spawning. The following splice-site blocking morpholinos (MOs) were designed to knockdown *arl15b* expression: 5'-AAACACTGAAAGACGGGACAAAGAC-3' and GTTAAGCGAGTATTAGGTTACCTCT-3' (Gene Tools, Philomath, OR, USA), designated as exon 3 and 4 skipping *arl15b*-MO respectively. A standard mismatch MO, directed against a human β -globin intron mutation, 5'-CCTCTTACCTCAGTTACAATTTATA-3', was also used in the experiments to control for toxic effects of the MO molecule. MOs were diluted in deionized, sterile water supplemented with 0.5% (w/v) phenol red and injected in a volume of 1 nl into the yolk of one- to two-cell stage embryos using a Pneumatic PicoPump pv280 (World Precision Instruments, Sarasota, FL, USA). WT embryos (uninjected) were also included in the experiments to control for the effects of the injection procedure *per se*. To determine the most effective dose of the *arl15b*-MO, 0.5, 1 and 2 ng *arl15b*-MO; and 2, 4 and 8 ng *arl15b*-MO were injected in two sets of experiments. In these experiments, control embryos were injected with 2 or 8 ng of the standard mismatch control-MO (the highest dose for each set of experiments respectively). After injection, embryos from the same experimental condition were placed in 3 Petri dishes and cultured at 28.5°C in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄), which was refreshed daily.

Morphological phenotypes characterizing kidney function and yolk metabolization were analyzed in larvae at 5 dpf. Larvae were classified into different classes of phenotypes on the basis of comparisons with stage-matched control embryos (injected with the control-MO) of the same clutch. In *arl15b* morphant larvae (5 dpf), 4 different phenotypes were distinguished: normal; mild, larvae with mild-moderate pericardial edema (indicative of kidney dysfunction) and metabolic defects (poor metabolization of the yolk); moderate, moderate pericardial edema accompanied by kidney cysts in approximately 75% of the cases, metabolic defects (poor metabolization of the yolk) and cardiovascular defects (poor blood circulation in the tail); severe, severe pericardial edema accompanied by kidney cysts in approximately 80% of the cases, metabolic defects (poor metabolization of the yolk) and cardiovascular defects (poor blood circulation or absence in the tail and severe bradychardia (heart beat rate below 100 beats per minute)). Representative images were obtained with a DFC450C camera (Leica Microsystems Ltd) after anaesthetising larvae with tricaine/Tris pH 7.0 solution.

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3 For electrolyte measurements or RNA isolation, 7-10 zebrafish larvae were pooled as
4 one sample. Samples were then snap frozen in liquid nitrogen and stored at -80°C in order to
5 ensure euthanasia of animals.
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8 *In vivo* cRNA rescue experiments were performed with human WT ARL15 and
9 dominant negative mutant (T46N) ARL15 cRNAs. Constructs were sub-cloned into the
10 pT7Ts expression vector, suitable for rescue experiments in zebrafish¹⁷, and cRNAs were
11 prepared using the mMMESSAGE mMACHINE Kit (Ambion, Austin, TX, USA) according to
12 the manufacturer's instructions. The cRNAs, in an amount of 50 pg, as based on previous
13 studies^{18,19}, were (co)injected together with MOs as described above. Zebrafish larvae were
14 phenotyped at 5 dpf.
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23 **Electrolyte measurements in animal studies**

24 In mice, urinary creatinine and electrolytes as well as plasma urea and creatinine (enzymatic
25 determination) were measured on a Synchron Unicel DxC 800 analyzer (Beckman Coulter,
26 Brea, USA). In zebrafish, sample processing started by washing twice the samples with
27 nanopure water in order to avoid contamination of remaining waterborne Mg²⁺. Fish were
28 then dried and digested as described previously¹⁹. The total Mg content in each sample was
29 determined with a colorimetric assay (Roche). Within-run precision and accuracy was
30 controlled by means of an internal control Precinorm (CV = 0.8%). Furthermore, samples
31 were normalized by protein content, which was determined with the Pierce BCA protein assay
32 kit (Pierce Biotechnology, Rockford, IL, USA).
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42 **RNA isolation and cDNA synthesis**

43 In mouse, total RNA was extracted from different tissues using the AurumTM Total RNA
44 Fatty and Fibrous Tissue Kit (Bio-Rad, Hercules, CA), following the manufacturer's protocol.
45 In zebrafish, RNA was isolated from zebrafish tissues and larvae using TRIzol reagent
46 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions in which
47 glycogen (Fermentas GmbH, St. Leon-Rot, Germany) was used in order to maximize the
48 RNA recovery. This method allowed the isolation of more than 3 µg RNA per tissue or
49 whole-larvae sample (n = 7-10 larvae/sample), and of more than 200 ng RNA per pronephric
50 samples (10 pronephros/sample). In both, mouse and zebrafish RNA samples, one µg of
51 RNA, or 200 ng in the case of pronephric samples, was subjected to DNase treatment to
52 prevent genomic DNA contamination and subsequently used to perform the reverse
53 transcriptase reaction.
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Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

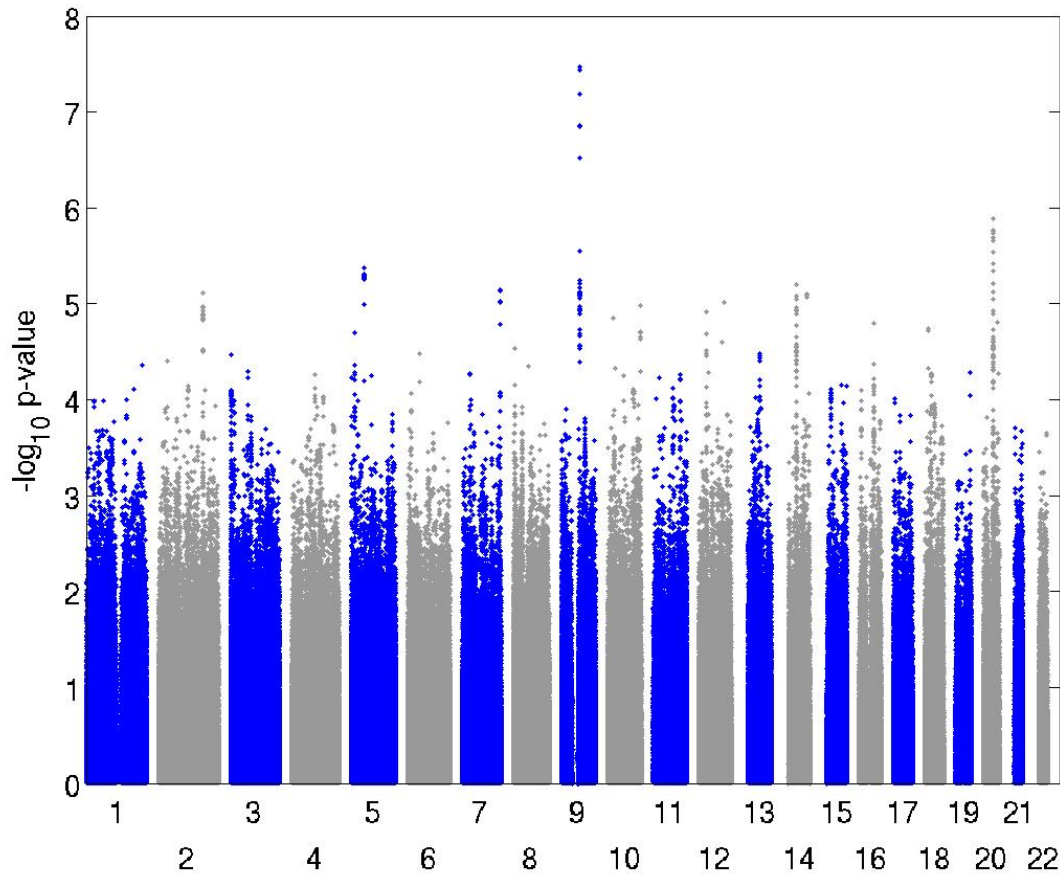
Changes in target genes mRNA levels were determined by relative RT-qPCR following the MIQUE guidelines²⁰ with a CFX96TM Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using iQTM SYBR Green Supermix (Bio-Rad) detection of single PCR product accumulation. To study the effect of dietary Mg²⁺ on gene expression in mouse kidney, ileum and caecum, the geNorm algorithm was used with 5 reference genes to calculate the normalization factor (software geNorm version 3.4). The reference genes used encoded for β -actin (*Actb*), glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), peptidylprolyl isomerase A (*Ppia*), attachment region binding protein (*Arbp*) and hypoxanthine phosphoribosyltransferase 1 (*Hprt1*). In the rest of procedures with mice (tissue distribution and gene expression profile in different mouse nephron segments) and in the zebrafish studies, gene expression levels were normalized to the expression levels of the standard species-specific reference genes *Gapdh* (for mice^{21,22}) and elongation factor-1 α (*elf1 α* , for zebrafish^{16,23}). Here, relative mRNA expression was analysed using the Livak method ($2^{-\Delta\Delta C_t}$). Primer sequences are shown in Suppl. Table 8.

Efficacy of the splice-site blocking *arl15b*-morpholinos. To determine the efficacy of the splice blocking induced by the exon 3 skipping *arl15b*-MO, cDNA from 5 dpf control (injected with 0.5 ng control-MO) and morphant (injected with 0.5 ng *arl15b*-MO) larvae was used for PCR analysis. The primers used were 5'- CGAGGTCACAGGGGTGTTTC-3' as forward primer and 5'- GACGAAGCGCTGTCCAAAAC-3' as reverse primer. Fragments thus obtained were extracted from a 1.5% agarose gel and Sanger-sequenced (Suppl. Fig. 9) to confirm the splice blocking induced.

In the case of the exon 4 skipping *arl15b*-MO, cDNA was generated from fish injected with 2 ng control or *arl15b*-MO. Primers used were 5'- CTGACGGGTCTGGGAAGAC-3' as forward primer and 5'- ACCGTGCTTCCCTCTAGGAT-3' as reverse primer. Given the absence of a spliced-*arl15b* transcript in 2-5 dpf fish (as a result of nonsense-mediated decay of *arl15b* mRNA by the exon 4 skipping *arl15b*-MO, Suppl. Fig. 10), the efficacy of the approach was further studied by RT-qPCR using specific primers that discriminate for the functional (non-spliced) *arl15b* transcript: the forward primer targets the exon 3-4 junction and the reverse primer targets exon 4 (Suppl. Table 8).

SUPPLEMENTARY FIGURES

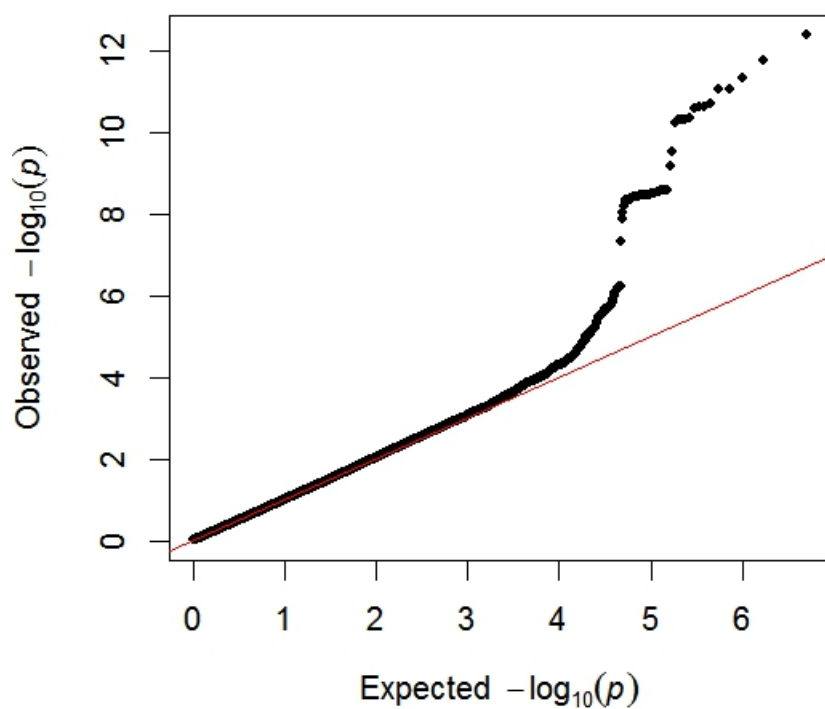
Supplementary Figure 1. Manhattan plot from the GWAS on uMg-to-creatinine ratio for the CoLaus cohort.



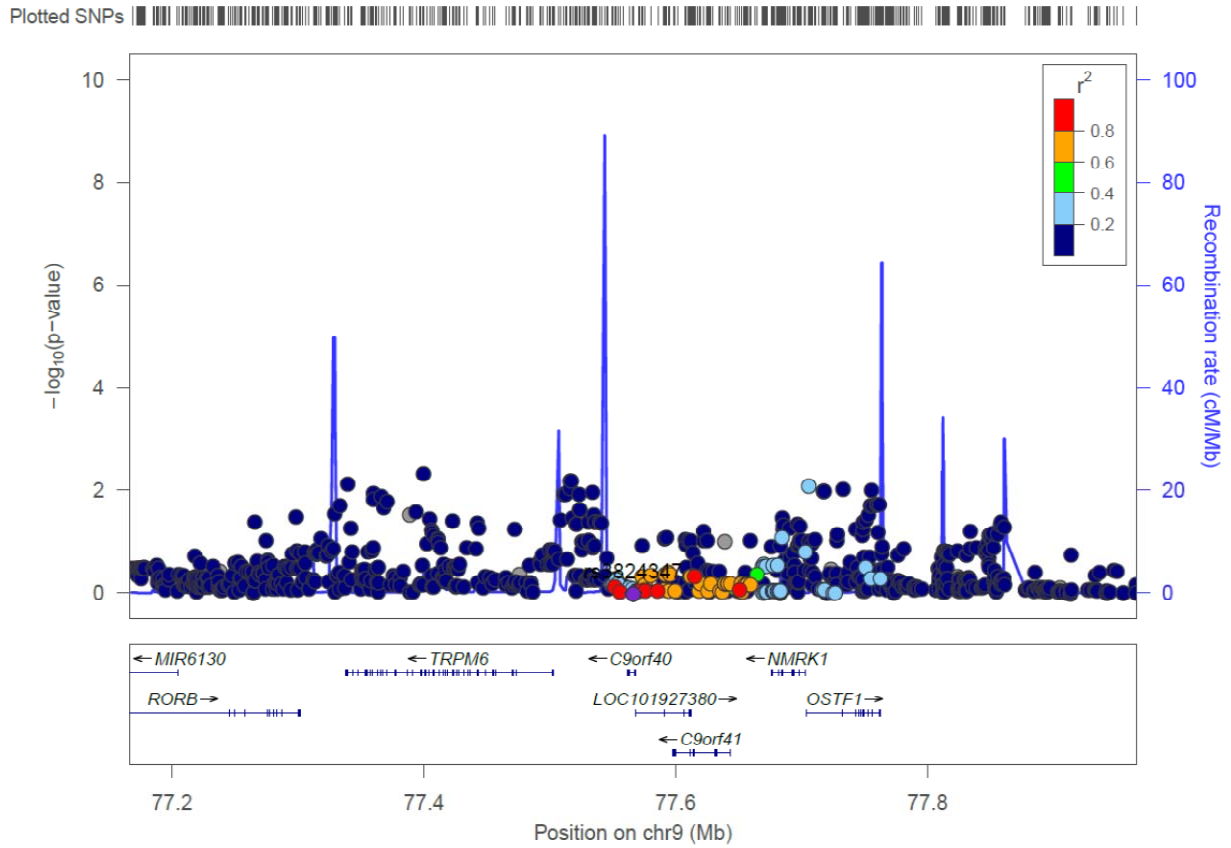
Manhattan plot showing $-\log_{10}(P$ values) for all SNPs in the genome-wide association for normalized uMg-to-creatinine ratio in CoLaus ($n=5,150$), ordered by chromosomal position. The values correspond to the association of normalized uMg-to-creatinine ratio, including as covariates age, sex and the first principal components generated from all genotypes to take population structure into account. One locus (*TRPM6*) reached genome-wide significance ($P < 5 \times 10^{-8}$).

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3 **Supplementary Figure 2. QQ plot and conditional analyses for the uMg-to-creatinine**
4 **ratio.**
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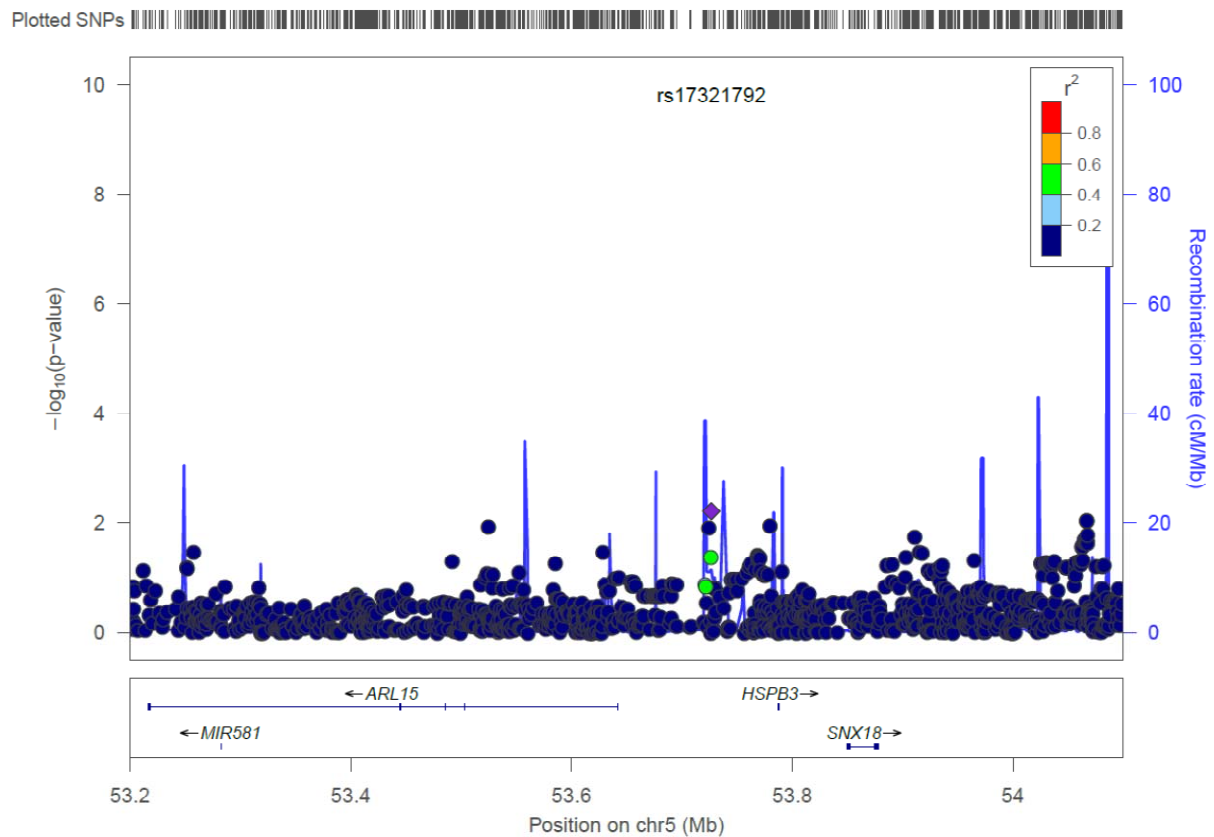
6
7 (a) QQ plot from the meta-analysis of uMg-to-creatinine ratio.
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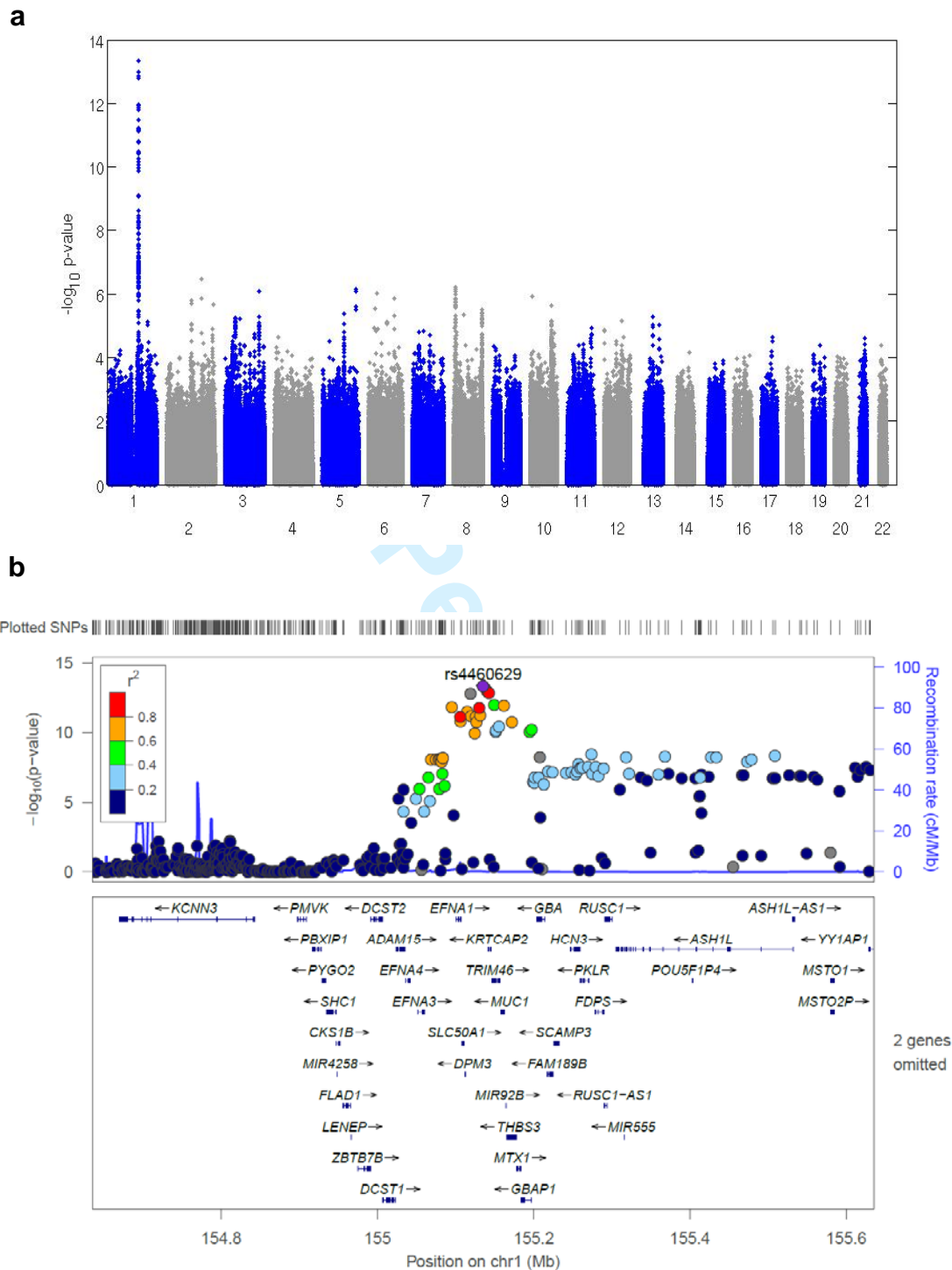
(b) Zoom plot showing the *TRPM6* region's associations to uMg-to-creatinine ratio after conditioning the analysis for rs3824347.



(c) Zoom plot showing the *ARL15* region's associations to uMg-to-creatinine ratio after conditioning the analysis for rs35929.

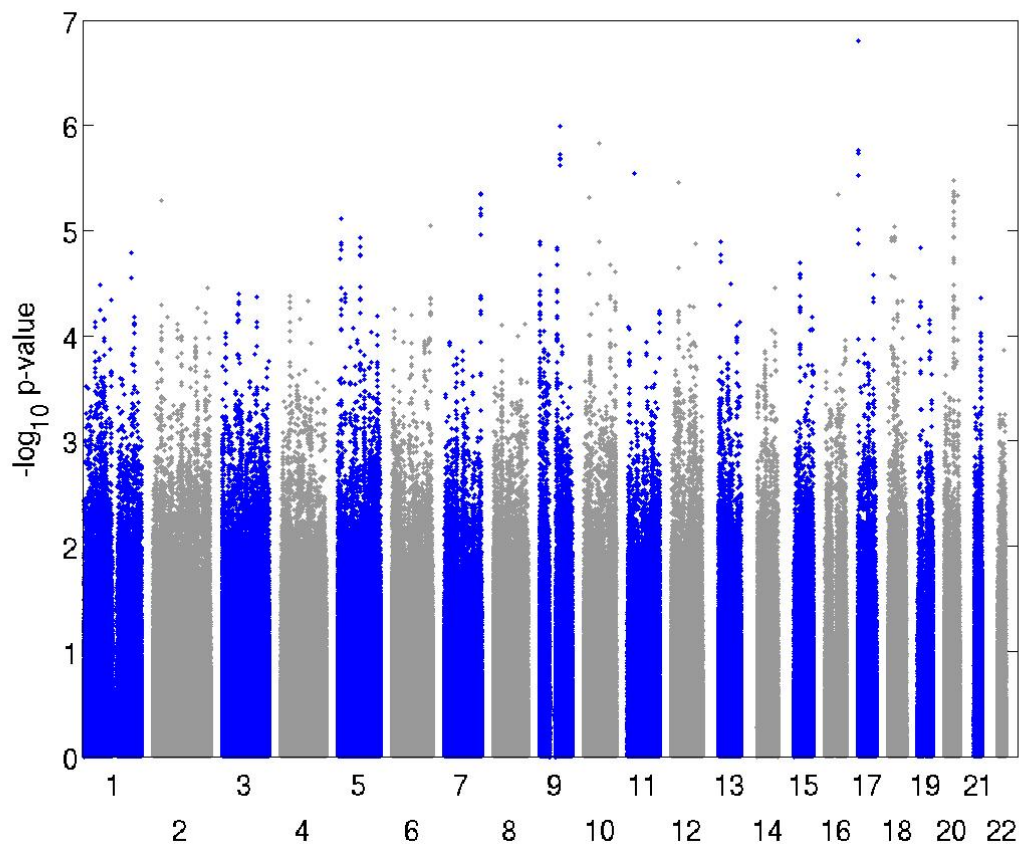


Supplementary Figure 3. Manhattan plot from the GWAS on serum Mg^{2+} levels for the meta-analysis.



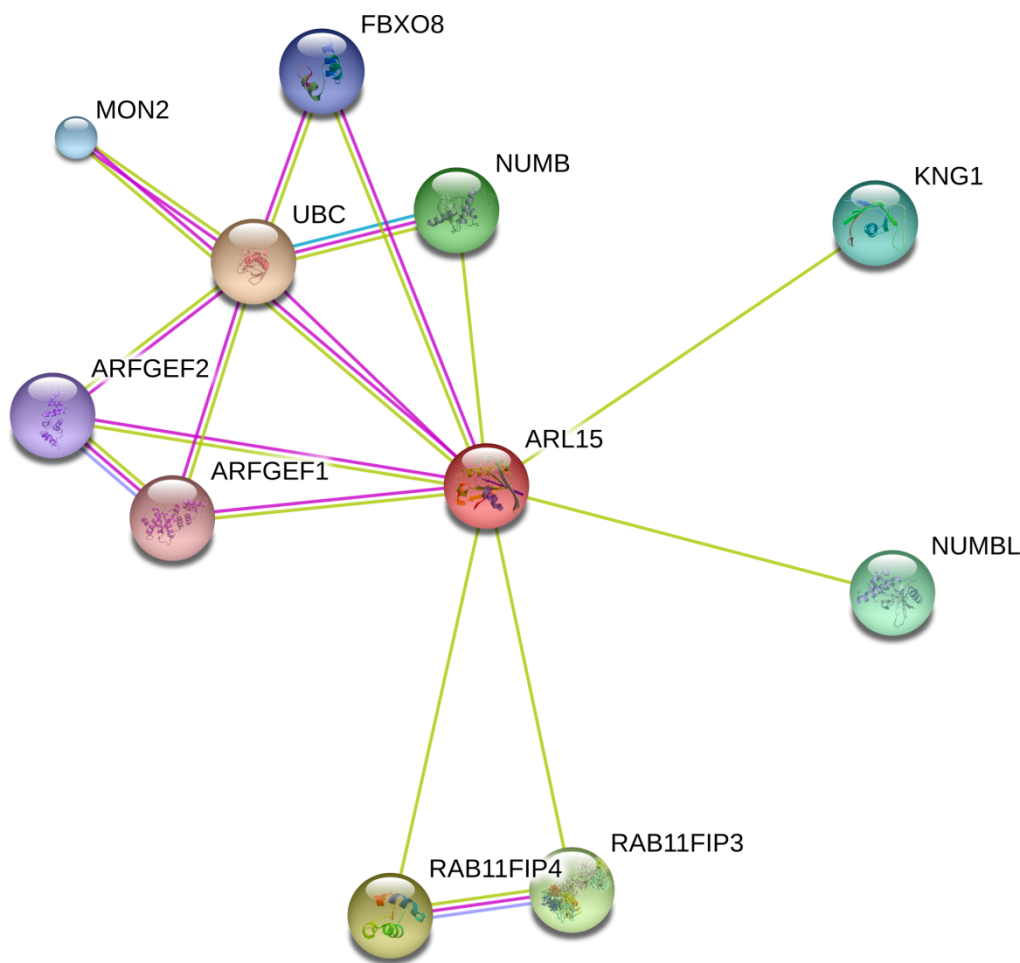
(a) Manhattan plot showing all $-\log_{10}(P$ values) for the combined analysis of the GWAS on serum Mg^{2+} , ordered by chromosome position. A locus on chromosome one reaches genome-wide significance. (b) Regional association plot of the region on chromosome one identified on the Manhattan plot on panel A. It shows a gene-rich locus, with no obvious candidate for being the gene involved.

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3 **Supplementary Figure 4. Manhattan plot from the meta-analysis of fractional excretion**
4 **of Mg²⁺ (FEMg) GWASs.**
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Manhattan plot showing all $-\log_{10}(P \text{ values})$ for the combined analysis of the GWAS on FEMg, ordered by chromosome position. No signal reaches genome-wide significance.

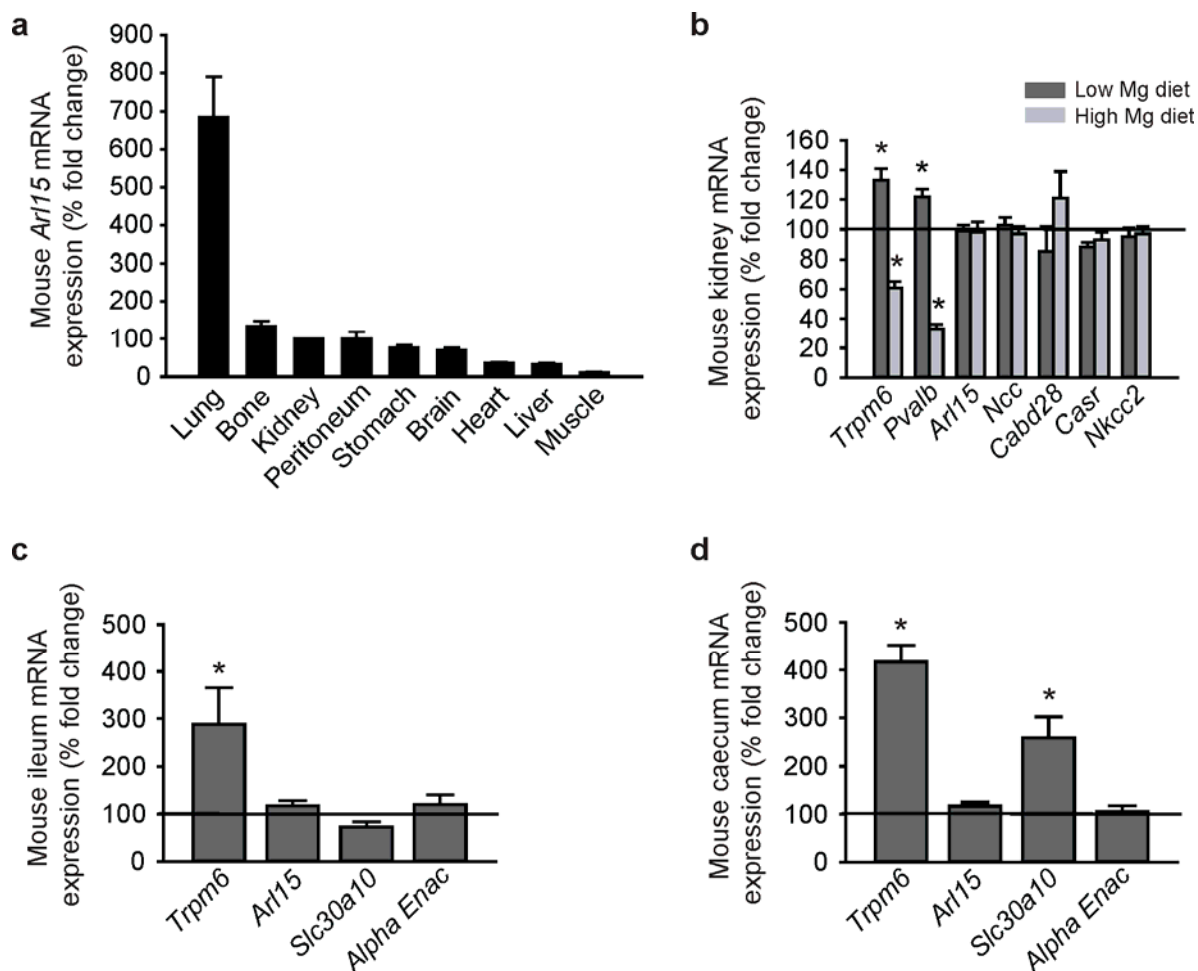
Supplementary Figure 5. Functional protein association network analyses for ARL15.



ARL15 interacts with regulators of endocytic (RAB11FIP4, UBC) and vesicular (ARFGEF1) trafficking, factors involved in maintenance of cell polarity (ARFGEF1), factors influencing sodium handling (KNG1) and regulators of ubiquitination (UBC).

(<http://string-db.org/cgi/network.pl?taskId=NPTdrqbgkFZS>). Network nodes represent proteins, whereas different colored nodes refer to different proteins. Small nodes (MON2) refer to proteins of unknown 3D structure while large nodes represent proteins whose 3D structure is known or predicted (ARL15, KNG1, NUMBL, RAB11FIP3, RAB11FIP4, ARFGEF1, ARFGEF2, UBC, NUMB, FBXO8). Edges represent protein-protein associations: blue, interactions from curated databases (e.g. UBC-NUMB); magenta, experimentally determined interactions (e.g. ARL15-ARFGEF1, ARL15-ARFGEF2, ARL15-UBC, ARL15-MON2, ARL15-FBXO8); green, interactions derived from text mining (e.g. ARL15-RAB11FIP3, ARL15-RAB11FIP4, ARL15-NUMBL, ARL15-KNG1, ARL15-NUMB); purple, interactions deduced by protein homology (e.g. ARFGEF1-ARFGEF2).

Supplementary Figure 6. Tissue distribution of *Arl15* gene expression in mouse tissues and regulation of gene expression by dietary Mg^{2+} in mouse tissues relevant for Mg^{2+} handling.



(a) Tissue distribution of *Arl15* gene expression in mouse tissues. Relative gene expression in mouse tissues and organs was analysed using the Livak method ($2^{-\Delta\Delta C_t}$), where results are normalized against the reference genes *Gapdh* and expressed relative (%) to the gene expression in the kidney, chosen as calibrator. Data are presented as mean \pm SEM (n = 3). (b-d) Gene expression levels of *Trpm6*, parvalbumin (*Pvalb*), *Arl15*, the Na^+ - Cl^- cotransporter (*Ncc*), calbindin D-28k (*Cabd28*), the calcium-sensing receptor (*Casr*) and the Na^+ - K^+ - Cl^- cotransporter isoform 2 (*Nkcc2*) in mouse kidney (b); of *Trpm6*, *Arl15*, the zinc transporter 10 (*Slc30a10*) and the alpha subunit of the epithelial sodium channel (*Alpha Enac*) in mouse ileum (c); and *Trpm6*, *Arl15*, *Slc30a10* and *Alpha Enac* in mouse caecum (d). Mice were fed a control diet (0.19% (w/w) Mg), a Mg^{2+} -deficient diet (0.0005% (w/w) Mg), or a Mg^{2+} -enriched diet (0.48% (w/w) Mg) for 10 days. Gene expression data were calculated using the geNorm algorithm and represent the mean fold difference (mean \pm SEM, n = 10 for kidney and n = 5 for ileum and caecum) from the control group (mice on the control diet). * $P < 0.05$ compared with the control group.

Supplementary Figure 7. The zebrafish *Arl15b* protein is a highly conserved ortholog of human *ARL15* being its gene expression is magnesiotropic in kidney, gills and gut.

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Human ARL15      1 --MSDLRITTEAFLYMDYLCFRALCCKGPPPARPEYDLVCIGLTGSGKTSLLSKLCSESPD
Mouse ARL15     1 --MSDLRITTEAFLYMDYLCFRALCCKGPPPARPEYDLVCIGLTGSGKTSLLSELCSESPD
Cow ARL15      1 --MSDLRISEAFLYMDYLCFRALCCKGPPPARPEYDLVCIGLTGSGKTSLLSKLCSESPD
Zebrafish Arl15b 1 -----MLDYCFRTLCCKGPPPPRPEYDVVCIGLTGSGKTSLLSRCLCEATD
Zebrafish Arl15a 1 MSEHIQLSVALCRIGFYTCLQKLCCKAPPPRPEYDVVCIGLSGAGKTSLLSRCLCEISD

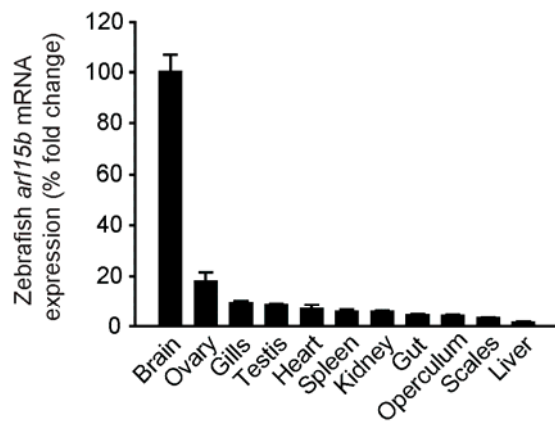
Human ARL15     59 NVVSTTGFSIKAVPFQNAILNVKELGGADNIRKYWSRYQGSQGVIFVLDSASSEDDLEA
Mouse ARL15     59 NVVSTTGFSIKAVPFQNAVLNVKELGGADNIRKYWSRYQGSQGVIFVLDSASSEDDLET
Cow ARL15      59 SVVSTTGFSIKAVPFQNAILNVKELGGADNIRKYWSRYQGSQGVIFVLDSASSEDDLET
Zebrafish Arl15b 47 NIVPTTGFSIKAVPFQNAILNVKELGGADSIKKYWSRYQGSQGVIFVLDSASSEDDLEV
Zebrafish Arl15a 61 GTVPTTGFSIKAVPFQNAILNVKELGGAETIKKYWSRYQGSQGVIFVLNSAASDEEMEA

Human ARL15     119 ARNELHSALQHPQLCTLPFLILANHQDKPAARSVQEIKKYFELEPLARGKRWILQPCSLD
Mouse ARL15     119 ARNELHSALQHPQLCTLPFLILANHQDKPAARSVQEIKKYFELEPLARGKRWILQPCSLD
Cow ARL15      119 ARNELHSALQHPQLCTLPFLILANHQDKPAARSVQEIKKYFELEPLARGKRWILQPCSLD
Zebrafish Arl15b 107 ARTELHSALQHPQLCTLPFLILANHQDKPAARSVQEIKKYFELEPLARGKSWIILEGSTVD
Zebrafish Arl15a 121 SRSELHIALQHPQLCTLPFLVLANHQDSPAARSVSEIRKFFFELEPLARGKRWILAGTSTN

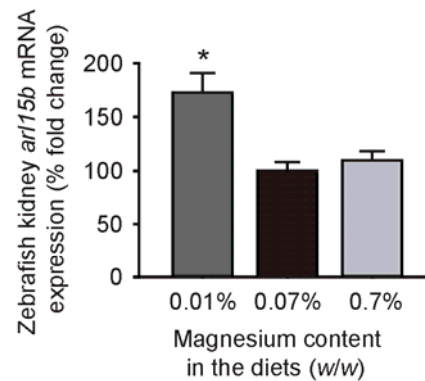
Human ARL15     179 DMDALKDSFSQILINLLEEKDHEAVRM
Mouse ARL15     179 DVDTLKDSFSQILINLLEEKDHEAVRM
Cow ARL15      179 DMEALKDSFSQILINLLEEDHEAVRM--
Zebrafish Arl15b 167 NMTAVKESFAQLIHLEEKDQETGRIT
Zebrafish Arl15a 181 NMEDVKESFSQILISLLEEKDQETGRIT

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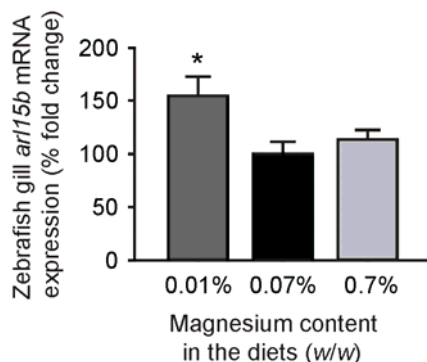
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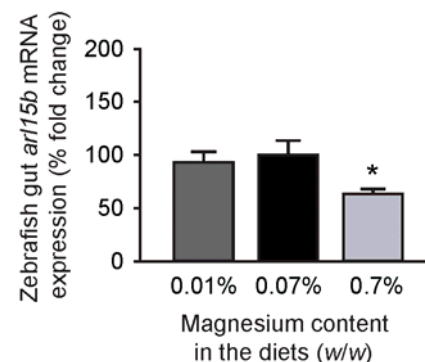
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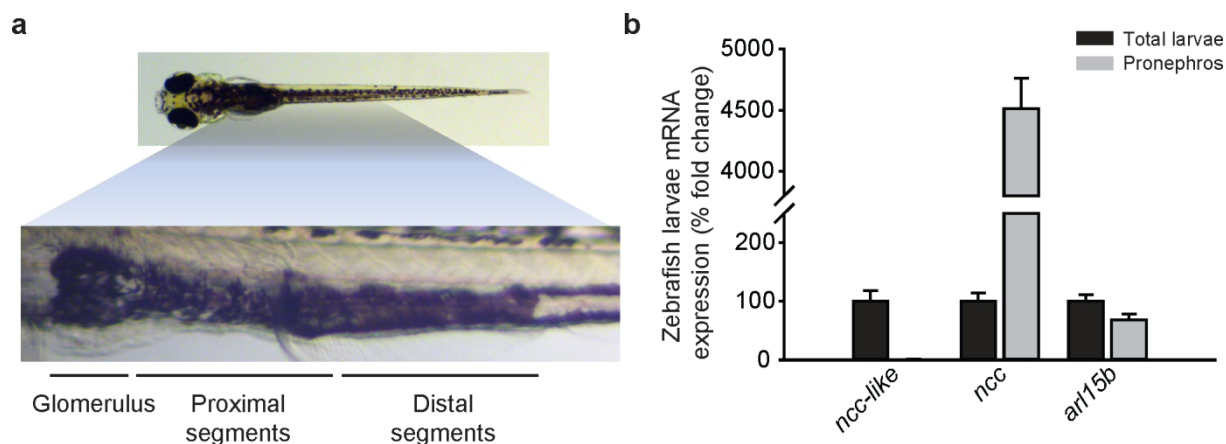
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(a) Alignment of the human, mouse and cow ARL15 proteins (GenBank accession no. NP_061960, NP_766183 and NP_001014943 respectively) and zebrafish Arl15 paralogs: Arl15a and Arl15b (GenBank accession no. NP_001093503 and XP_001923547 respectively). Identical AA are boxed in black, conservative substitutions in gray. Sequence identity of zebrafish Arl15a with human, mouse and cow ARL15 is 68, 66 and 67% respectively. Zebrafish Arl15b displayed a strikingly high degree of AA conservation with its mammalian counterparts: 83, 81 and 79% with human, mouse and cow ARL15 respectively.

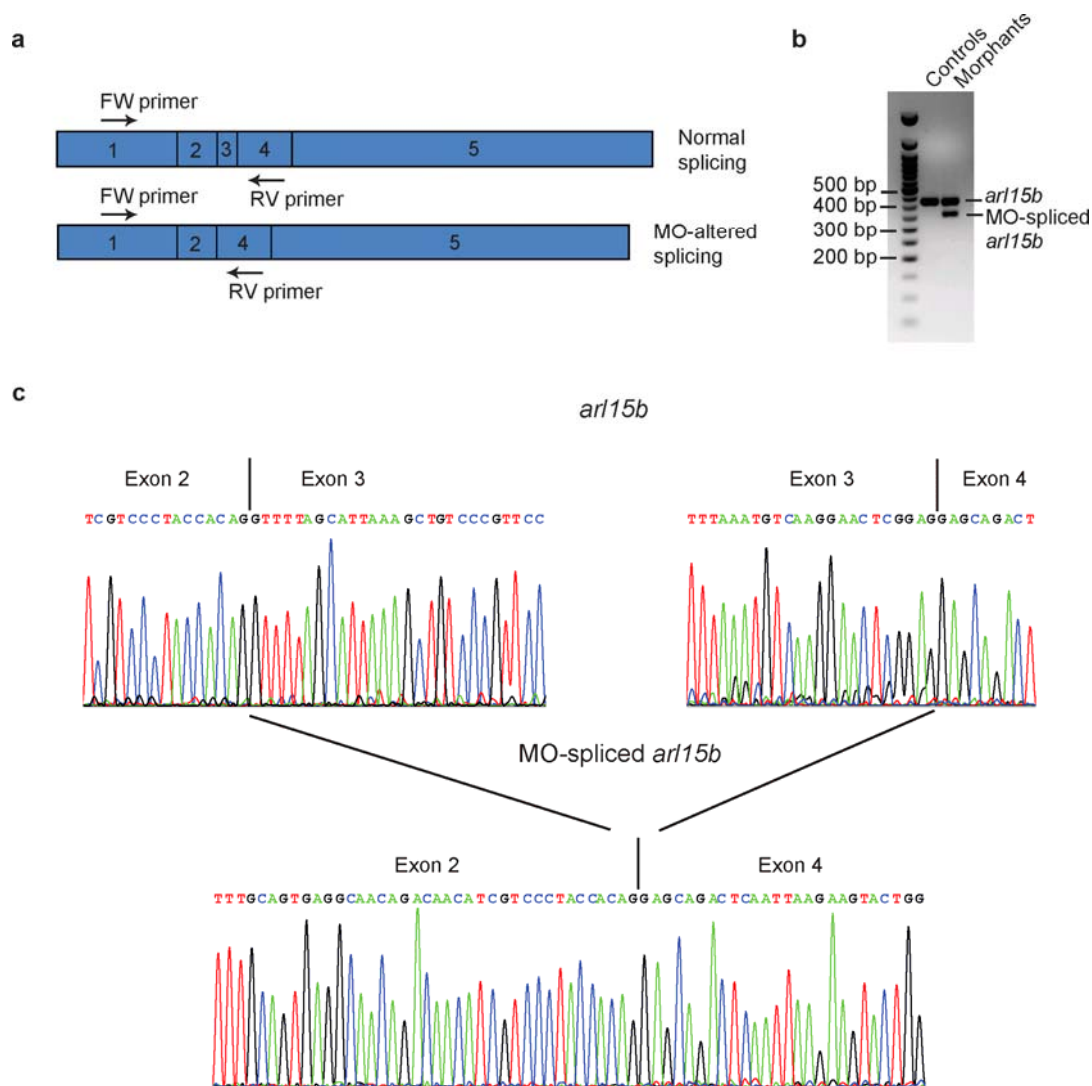
(b) Tissue distribution of *arl15b* gene expression in zebrafish tissues. Relative gene expression in mouse and zebrafish tissues and organs was analysed using the Livak method ($2^{-\Delta\Delta C_t}$), where results are normalized against the translational *elf1a* and expressed relative (%) to the gene expression in the brain, chosen as calibrator. Data are presented as mean \pm SEM (n = 6, except for the ovary and testis where n = 3).

(c-e) Gene expression levels of *arl15b* in kidney (c), gills (d) and gut (e) of zebrafish on a Mg^{2+} -deficient diet (0.01% (w/w) Mg), Mg^{2+} -control diet (0.07% (w/w) Mg) and Mg^{2+} -enriched diet (0.7% (w/w) Mg) for 21 days. Data were calculated using the Livak method ($2^{-\Delta\Delta C_t}$) and they represent the mean fold difference (mean \pm SEM, n = 8-9) from the control group (fish on the control diet). * $P < 0.05$ was considered statistically significant when compared with the control group.

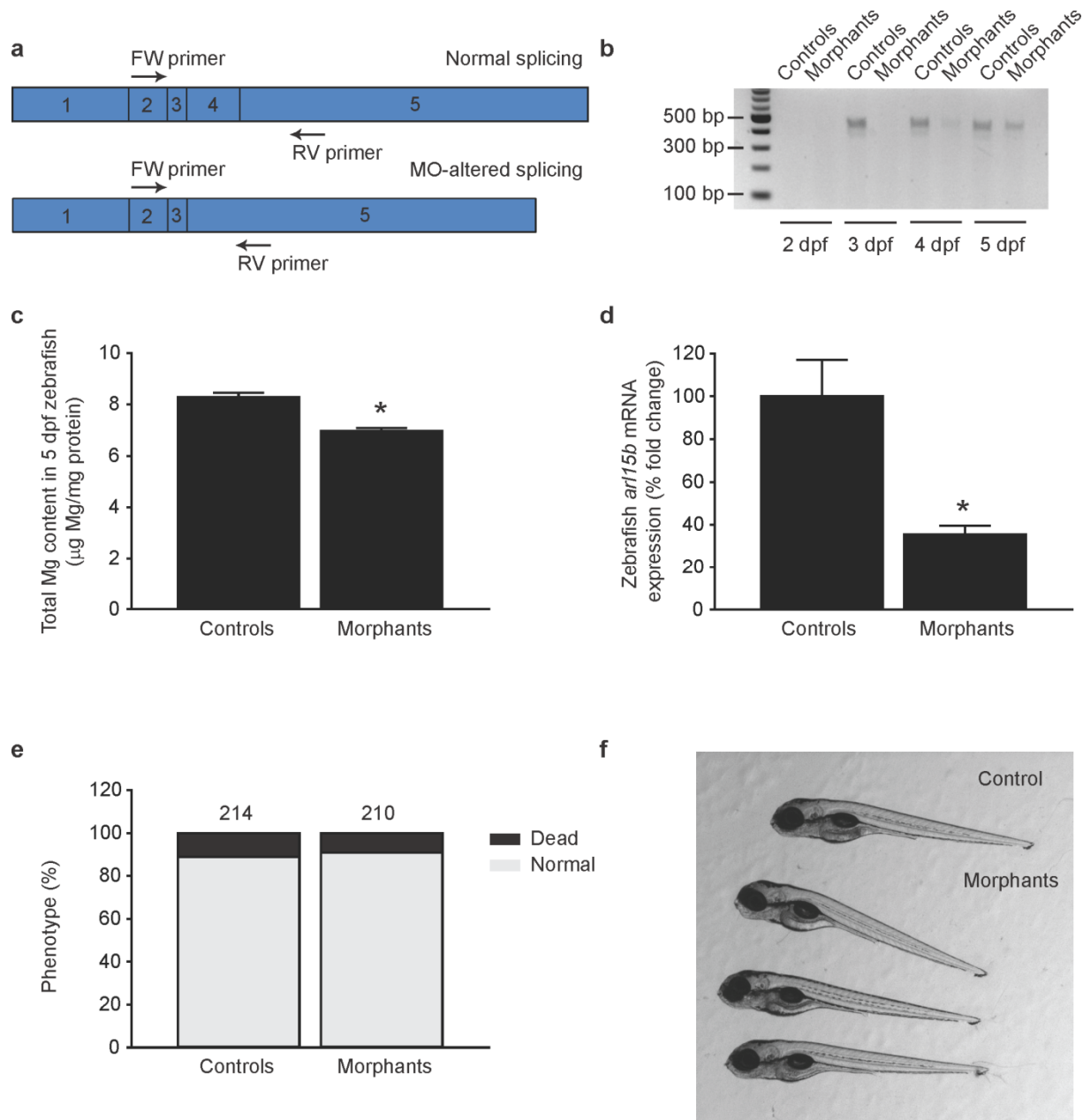
Supplementary Figure 8. The *arl15b* gene is expressed in the pronephric kidney of zebrafish larvae.



(a) The pronephros of 5 dpf zebrafish larva after dissection for collection of pronephric-enriched tissue. Pronephric tubules can be distinguished by the black pigments that develop around the tubuli. (b) Gene expression of *arl15b* and the control genes *ncc* (uniquely expressed in the pronephros, as positive control) and *ncc-like* (distinctly expressed in the skin, as negative control) in the pronephros and total larvae. Relative gene expression was analysed using the Livak method ($2^{-\Delta\Delta C_t}$), where results are normalized against the reference gene *elf1a* and expressed relative (%) to the gene expression in total larvae. Data are presented as mean \pm SEM (n = 3).

Supplementary Figure 9. Efficacy of the exon 3 skipping *arl15b*-MO used.

(a) Expected PCR products using forward (FW) and reverse (RV) primers located in exon 1 and 4 respectively on *arl15b* mRNA with normal splicing or altered splicing evoked by the *arl15b*-MO. (b) Gel electrophoresis image showing defects in the splicing process following injection of *arl15b*-MO (morphants, dose of 0.5 ng *arl15b*-MO/embryo) or control-MO (controls, dose of 0.5 ng control-MO/embryo). Bands showing correctly spliced *arl15b* mRNA and MO-spliced *arl15b* mRNA are shown. PCR was performed on cDNA prepared from larvae 5 days after injection. Shown on the left are the sizes (in bp) of the major bands of the DNA ladder. The expected amplicon size of the correctly spliced *arl15b* mRNA and the MO-spliced *arl15b* mRNA using FW and RV primers is of 411 bp and 351 bp respectively. (c) Chromatograms obtained from Sanger sequencing of the amplicons in the correctly spliced *arl15b* mRNA and the MO-spliced *arl15b* mRNA. The *arl15b*-MO evoked exon 3 skipping yielding a knockdown of Arl15b function. By skipping exon 3 with the *arl15b*-MO, the resulting protein lacks active domains such as the complete switch I region and part of the switch II region (switch regions are surface loops that undergo conformational changes upon GTP binding) and G3 box.

Supplementary Figure 10. Efficacy of the exon 4 skipping *arl15b*-MO used.

(a) Expected PCR products using forward (FW) and reverse (RV) primers located in exon 2 and 5 respectively on *arl15b* mRNA with normal splicing or altered splicing evoked by the *arl15b*-MO. (b) Gel electrophoresis image showing knockdown of *arl15b* gene expression following injection of *arl15b*-MO (morphants, dose of 2 ng *arl15b*-MO/embryo) or control-MO (controls, dose of 2 ng control-MO/embryo) in 2-5 dpf zebrafish larvae. Bands showing correctly spliced *arl15b* mRNA are shown, while bands showing MO-spliced *arl15b* mRNA could not be detected so that the exon 4 skipping *arl15b*-MO evoked nonsense-mediated decay of *arl15b* transcripts. Shown on the left are the sizes (in bp) of the major bands of the DNA ladder. The expected amplicon size of the correctly spliced *arl15b* mRNA and the MO-spliced *arl15b* mRNA (not detected) using FW and RV primers is of 413 bp and 204 bp respectively. (c-f) Characterization of the knockdown evoked by injection of 2 ng of exon 4 skipping *arl15b*-MO. Controls were injected with 2 ng of control-MO. C, total Mg content in

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3 5 dpf morphant and control zebrafish larvae. Data are presented as mean \pm SEM (n = 10). **P*
4 < 0.05 was considered statistically significant when compared with the control group. (d)
5 Quantification of the knockdown evoked by the exon 4 skipping *arl15b*-MO by RT-qPCR
6 using specific primers that discriminate for the correctly spliced (functional) *arl15b* mRNA.
7 Data were calculated using the Livak method ($2^{-\Delta\Delta C_t}$) and they represent the mean fold
8 difference (mean \pm SEM, n = 10) from the control group (fish injected with the control-MO).
9 **P* < 0.05 was considered statistically significant when compared with the control group. (e)
10 Distribution of morphological phenotypes in the zebrafish larvae (5 dpf) used for the
11 quantification of the *arl15b* knockdown by RT-qPCR. Numbers on top of the bars indicate the
12 number of animals in each experimental condition. (f) Illustrative picture showing the
13 characteristic phenotype (normal) evoked by knockdown of *arl15b* (2 ng/embryo of exon 4
14 skipping *arl15b*-MO).
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SUPPLEMENTARY TABLES

Supplementary Table 1. Summary statistics for the genome-wide meta-analysis of serum Mg²⁺ levels.

	Effect allele	Other allele	Mean effect allele frequency	Effect size discovery (SE)	<i>P</i> value discovery	Effect size replica (SE)	<i>P</i> value replica	Effect size combined analysis (SE)	<i>P</i> value combined analysis	Direction of effects of individual cohorts	I ²
rs4460629	T	C	0.56	-0.144 (0.02)	4.89 x10 ⁻¹³	-0.100 (0.04)	1.15 x10 ⁻²	-0.136 (0.018)	4.45 x10 ⁻¹⁴	--?-	14.6

The effect size sign for each individual cohort on the last but one column is represented by the sign – or +. The cohort displaying a “?” is for the Carlantino cohort, not having data for rs4460629. The last column shows the I² value, representing the heterogeneity across cohorts.

Supplementary Table 2. Imputation status and quality for rs35929 and rs3824347.

SNP	COLAUS	CARLANTINO	KORCULA	LBC1936	SPLIT	VAL BORBERA	VIS
rs35929	I(0.95)	I (0.81)	I (1)	I(1)	I(1)	I(1)	I(1)
rs3824347	I(0.97)	I(0.76)	I(0.95)	I(0.97)	I(0.98)	I(0.97)	I(0.98)

Imputation status (G= genotyped, I=imputed) for both SNPs of interest in each cohort. Between brackets is the r^2 value representing imputation quality.

Supplementary Table 3. Association of *ARL15* variants with metabolic phenotypes.

Source	Trait	SNP	p-value of association in published GWAS	Chr	Position	Gene Region	Context	PubMed	reference allele	p-value for uMg/uCreat in Meta-analysis	Beta for uMg/uCreat in Meta-analysis	Standard error of Beta for uMg/uCreat in Meta-analysis
dbGaP	Body Weight	rs2042313	2.45x10 ⁻⁷	5	53212417	ARL15	Intron	17903300	NA	NA	NA	NA
dbGaP	Body Weight	rs2042313	5.15x10 ⁻⁷	5	53212417	ARL15	Intron	17903300	NA	NA	NA	NA
NHGRI GWAS Catalog	Adiponectin	rs6450176	6.00x10 ⁻⁸	5	53298025	ARL15	Intron	22479202	a	0.59	0.0091	0.0169
NHGRI GWAS Catalog	Cholesterol, HDL	rs6450176	5.00x10 ⁻⁸	5	53298025	ARL15	Intron	20686565	a	0.59	0.0091	0.0169
NHGRI GWAS Catalog	Adiponectin	rs4311394	3.00x10 ⁻⁸	5	53300662	ARL15	Intron	20011104	a	0.63	-0.0079	0.0165
dbGaP	Uric Acid	rs10513040	3.55x10 ⁻⁶	5	53502295	ARL15	Intron	17903292	a	0.052	-0.0663	0.0341

Summary of previously associated SNPs in *ARL15* in published GWAS. The SNP associated with body weight is not present in our study. The 3 other SNPs are not significantly associated in the present uMg-to-creatinine meta-analysis, as shown in the last 3 columns.

Data were exported from dbGAP browser: <http://www.ncbi.nlm.nih.gov/gap>.

Supplementary Table 4. Study characteristics.

Study	CoLaus	INGI-Val Borbera	INGI-Carlantino	CROATIA-Vis	CROATIA-Korcula	CROATIA-Split	LBC1936
Sample size	5265	1541	281	195	889	489	660
Female % (N)	53%	56%	56.80%	58%	63.80%	56.50%	47.90%
Age, years mean (SD)	53.4 (10.7)	55 (18)	48 (19.7)	56.36 (15.5)	56.3 (13.9)	49.25 (14.7)	72.74 (0.75)
Serum Mg (mg/dl) mean (SD)	2.06 (0.166)	2.2 (0.3)	2.1 (0.2)	NA	2.04 (0.21)	NA	NA
Serum creatinine (mg/dl) mean (SD)	1.05 (0.24)	0.87 (0.25)	0.8 (0.2)	0.99 (0.32)	0.92 (0.18)	0.93 (0.15)	NA
Urine Mg (mg/dl) mean (SD)	6.9 (4.0)	8.4 (4.8)	8.1 (5)	5.93 (4.52)	7.6 (4)	8.76 (4.9)	8.70 (5.61)
Urine creatinine (mg/dl) mean (SD)	161.9 (81.4)	105.4 (59.1)	80.5 (42.1)	103.18 (65.8)	133.8 (63.8)	154.72 (72.98)	115.75 (62.20)
Urine Mg/creat ratio (mg/gr) mean (SD)	46.51 (25.98)	88.6 (36.5)	104.6 (44.8)	62.55 (33.37)	63.7 (32.5)	60.6 (26.8)	82.03 (42.23)
FEMg mean (SD)	3.27 (1.65)	5.2 (2.5)	6.0 (2.6)	NA	2.55 (1.33)	NA	NA
eGFR (mean, SD)	89.39 (20.03)	89.23 (23.28)	NA	89.41 (21.18)	87.72 (21.16)	94.92 (23.34)	NA

Supplementary Table 5. Study genotyping characteristics.

Study	CoLaus	INGI-Val Borbera	INGI-Carlantino	CROATIA-Vis	CROATIA-Korcula	CROATIA-Split	LBC1936
Array type	Affymetrix 500K	Illumina 370k	Illumina 370k	Illumina HumanHap300v1	Illumina HumanHap370CNV	Illumina HumanHap370CNV	Illumina 610
Genotype calling	BRLMM	BeadStudio analysis software	BeadStudio analysis software	Genome Studio	Genome Studio	Genome Studio	Genome studio
QC filters for genotyped SNPs used for imputation	pHWE<1e-7; individual call rate <90%; SNP call rate<70%; MAF<0.01	call rate >= 90%; MAF >= 1%; pHWE p > 0.0001	call rate >= 90%; MAF >= 1%; pHWE p > 0.0001	SNP Call rate<=0.98, MAF<0.01, pHWE<1e-6, Individual Call rate<0.95	SNP Call rate<=0.98, MAF<0.01, pHWE<1e-6, Individual Call rate<0.95	SNP Call rate<=0.98, MAF<0.01, pHWE<1e-6, Individual Call rate<0.95	pHWE<p < 1x10-3 individual call rate <95%; SNP call rate<98%; MAF<0.01
No of SNPs used for imputation	390'631	332'887	309'430	289'827	307'625	321'456	~500,000
Imputation	IMPUTE v0.2	MACH	MACH	MACH 1.0.16	MACH 1.0.16	MACH 1.0.16	MACH
Imputation Backbone for phased CEU haplotypes (NCBI build)	HapMap release 21 (build 35)	HapMap release 22 (build 36)	HapMap release 22 (build 36)	HapMap release 22 (build 36)	HapMap release 22 (build 36)	HapMap release 22 (build 36)	HapMap II (build 36)
Filtering of imputed genotypes	None	Rsq>0.3	Rsq>0.3	None	None	None	none
Data management and statistical	QUICKTEST	R, GenABEL, ProbABEL (mmscore function was used to	R, GenABEL, ProbABEL (mmscore	R, GenABEL, ProbABEL (mmscore	R, GenABEL, ProbABEL (mmscore function	R, GenABEL, ProbABEL (mmscore function	mach2qtl

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Supplementary Table 6. Urine (U) parameters in mice fed different Mg²⁺-containing diets.

Baseline	Control diet (n=10)	Mg ²⁺ deficient diet (n= 11)	Mg ²⁺ excess diet (n=10)
Body weight (g)	23.7 ± 0.4	23.9 ± 0.8	25.6 ± 0.4*
Diuresis (μl/16h)	1307 ± 162	1467 ± 244	1985 ± 285
Diuresis (μl/min.gBW)	0.058 ± 0.007	0.066 ± 0.012	0.080 ± 0.012
U Na ⁺ (nmol/min)	164 ± 16	170 ± 19	177 ± 15
U K ⁺ (nmol/min)	413 ± 40	479 ± 42	501 ± 46
U Cl ⁻ (nmol/min)	229 ± 21	263 ± 29	265 ± 26
U Mg ²⁺ (nmol/min)	586 ± 59	621 ± 54	656 ± 78
Day 5	Control diet (n=10)	Mg ²⁺ deficient diet (n= 11)	Mg ²⁺ excess diet (n=10)
Body weight (g)	24.3 ± 0.5	24.3 ± 0.8	25.8 ± 0.5*
Diuresis (μl/16h)	1064 ± 49	1390 ± 168	1126 ± 132 [‡]
Diuresis (μl/min.gBW)	0.046 ± 0.002	0.060 ± 0.01	0.045 ± 0.005 [‡]
U Na ⁺ (nmol/min)	156 ± 14	171 ± 20	123 ± 20
U K ⁺ (nmol/min)	382 ± 36	393 ± 45	327 ± 52 [‡]
U Cl ⁻ (nmol/min)	210 ± 20	239 ± 28	168 ± 30 [‡]
U Mg ²⁺ (nmol/min)	478 ± 42	13 ± 3 ^{*.‡}	1269 ± 184 ^{*.‡}
Day 10	Control diet (n=10)	Mg ²⁺ deficient diet (n= 11)	Mg ²⁺ excess diet (n=10)
Body weight (g)	24.1 ± 0.4	24.3 ± 0.9	25.9 ± 0.5*
Diuresis (μl/16h)	1516 ± 136 [§]	1447 ± 149	1334 ± 124 [‡]
Diuresis (μl/min.gBW)	0.066 ± 0.006 [§]	0.062 ± 0.005	0.053 ± 0.005 [‡]
U Na ⁺ (nmol/min)	189 ± 16 [§]	176 ± 23	140 ± 18
U K ⁺ (nmol/min)	532 ± 46 [§]	465 ± 43	409 ± 51
U Cl ⁻ (nmol/min)	287 ± 26 ^{‡.§}	263 ± 33	195 ± 28 ^{*.‡}
U Mg ²⁺ (nmol/min)	723 ± 49 [§]	8.1 ± 1.7 ^{*.‡.§}	1399 ± 149 ^{*.‡}

*P < 0.05 versus control diet; [‡]P < 0.05 versus baseline; [§]P < 0.05 versus day 5

Supplementary Table 7. Plasma (P) parameters in mice fed different Mg²⁺-containing diets.

Day 10	Control diet (n=10)	Mg ²⁺ deficient diet (n= 11)	Mg ²⁺ excess diet (n=10)
Body weight (g)	23.4 ± 0.5	23.3 ± 0.8	24.5 ± 0.6
P urea (mg/dl)	51.4 ± 1	64.7 ± 3.9*	52.0 ± 2.6 [‡]
P creatinine (mg/dl)	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01
P Na ⁺ (mmol/l)	154 ± 1	157 ± 1*	152 ± 1* [‡]
P K ⁺ (mmol/l)	4.0 ± 0.2	4.4 ± 0.1*	4.7 ± 0.2*
P Cl ⁻ (mmol/l)	110 ± 1	111 ± 1	108 ± 1
P Ca ²⁺ (mmol/l)	2.26 ± 0.03	2.06 ± 0.03*	2.29 ± 0.03 [‡]
P Mg ²⁺ (mg/dl)	2.95 ± 0.10	1.08 ± 0.11*	3.13 ± 0.13 [‡]

**P* < 0.05 versus control diet; [‡]*P* < 0.05 versus Mg²⁺ deficient diet

Supplementary Table 8. Primer oligonucleotide sequences used in the present study during RT-qPCR measurements.

	Primer sequence 5' → 3'	Species	Amplicon size (bp)	Efficiency (%)
<i>Actb</i>		Mouse	102	102
Forward	TGCCCATCTATGAGGGCTAC			
Reverse	CCCGTTCAGTCAGGATCTTC			
<i>Arl15</i>		Mouse	133	98
Forward	TCATCAAGACAAGCCAGCAG			
Reverse	GCTGTCCTTCAGTGTGTCCA			
<i>Gapdh</i>		Mouse	176	104
Forward	TGCACCACCAACTGCTTAGC			
Reverse	GGATGCAGGGATGGGGGAGA			
<i>Podocin</i>		Mouse	162	103
Forward	GTCTAGCCCATGTGTCCAAA			
Reverse	CCACTTTGATGCCCAAATA			
<i>Pvalb</i>		Mouse	136	97
Forward	GACGCCATTCTTCTGGAAAT			
Reverse	ATACCCCACTGCCCTAAAA			
<i>Arbp</i>		Mouse	150	99
Forward	CTTCATTGTGGGAGCAGACA			
Reverse	TTCTCCAGAGCTGGGTTGTT			
<i>Ncc</i>		Mouse	148	101
Forward	CATGGTCTCCTTTGCCAACT			

Reverse	TGCCAAAGAAGCTACCATCA			
<i>Nkcc2</i>		Mouse	154	99
Forward	CCGTGGCCTACATAGGTGTT			
Reverse	GGCTCGTGTTGACATCTTGA			
<i>Aqp2</i>		Mouse	147	103
Forward	TCACTGGGTCTTCTGGATCG			
Reverse	CGTTCCTCCCAGTCAGTGT			
<i>Ppia</i>		Mouse	139	102
Forward	CGTCTCCTTCGAGCTGTTTG			
Reverse	CCACCCTGGCACATGAATC			
<i>Hprt1</i>		Mouse	162	99
Forward	ACATTGTGGCCCTCTGTGTG			
Reverse	TTATGTCCCCCGTTGACTGA			
<i>Sgt2</i>		Mouse	164	101
Forward	TTGGGCATCACCATGATTTA			
Reverse	GCTCCAGGTATTTGTCGAA			
<i>Trpm6</i>		Mouse	156	100
Forward	CACAAGCCAGTGACCACCTA			
Reverse	GAGGCTCTTGAGGGCTTTTT			
<i>Snat3</i>		Mouse	109	99
Forward	GTTATCTTCGCCCCAACAT			
Reverse	TGGGCATGATTCGGAAGTAG			
<i>Alpha Enac</i>		Mouse	151	96
Forward	CCTGGGCAGCTTCATCTTTA			

Reverse	GACTCCAGGCATGGAAGACA			
<i>Casr</i>		Mouse	150	97
Forward	CTCTGCTGCTTCTCCAGCT			
Reverse	GGCCTCAAATACCAGGAGGA			
<i>Cabd28</i>		Mouse	154	101
Forward	CTGACAGAGATGGCCAGGTT			
Reverse	AGCAAAGCATCCAGCTCATT			
<i>arl15b</i>		Zebrafish	133	104
Forward	AAGGAACTCGGAGGAGCAGACTCA			
Reverse	AGTGGAGCTCTGTACGCGCC			
<i>elf1a</i>		Zebrafish	89	100
Forward	GAGGCCAGCTCAAACATGGGC			
Reverse	AGGGCATCAAGAAGAGTAGTACCGC			

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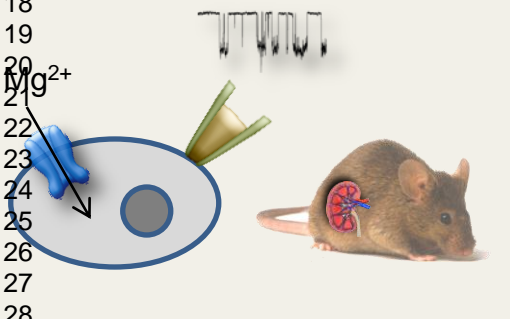
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Genome-wide Meta-analysis Unravels Novel Interactions Between Magnesium Homeostasis and Metabolic Phenotypes

METHODS

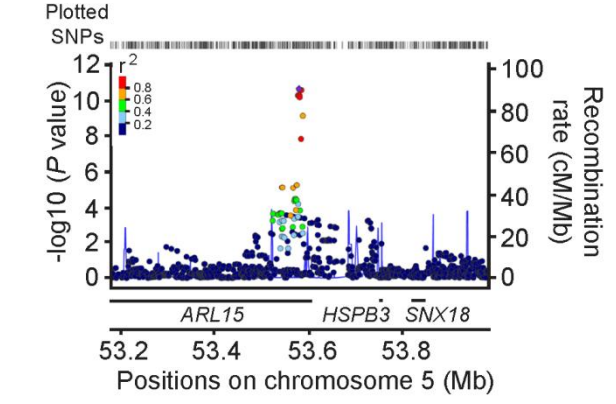


Meta-GWAS of urinary Mg²⁺ in European cohorts (n = 9,099)

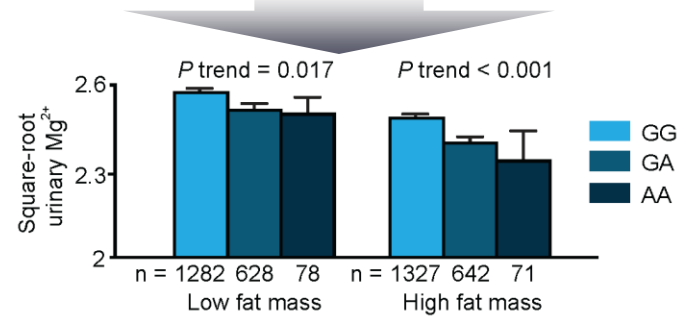


In vitro and in vivo studies of renal Mg²⁺ handling

RESULTS

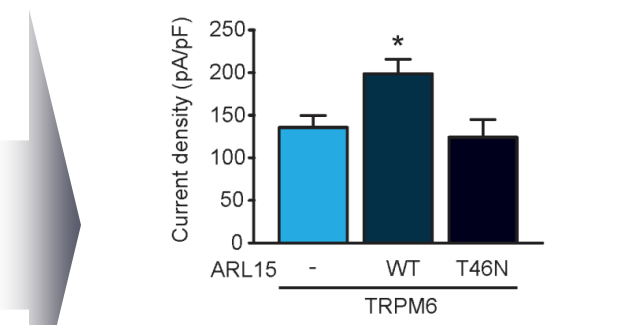


ARL15 is associated with urinary Mg²⁺ in the population

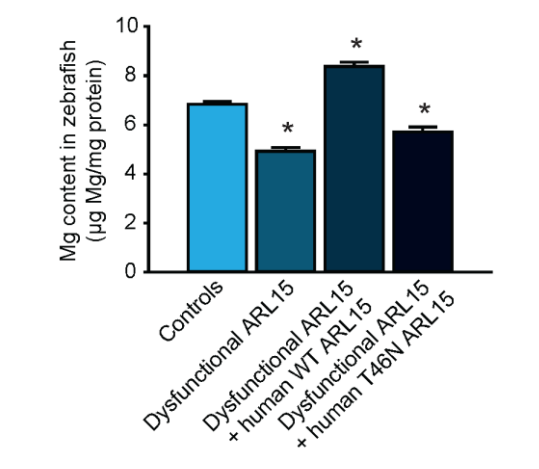


SNP-by-environment interaction

CONCLUSION ARL15 is a new player for renal Mg²⁺ handling that provides the first genetic link between Mg²⁺ balance and metabolism



Renal cells



Zebrafish

Bauguy Corre*, Francisco J. Arjona*, Caroline Hayward*, Sonia Youhanna, Jeroen H.F. de Baaij, Hendrica Belge, Nadine Nägele, Huguette Debaix, Maxime G. Bonchard, Michela Traglia, Sarah E. Harris, Sheila Ulivi, Rico Rueedi, David Lamparter, Aurélien Macé, Cinza Sala, Stefania Lenarduzzi, Belen Ponte, Menno Ruijijm, Daniel Ackermann, Georg Ehret, Daniela Baptista, Ozren Polasek, Igor Rudan, Toby Hurd, Nicholas D. Hastie, Veronique Vitart, Gerard Waeber, Zoltán Kutalik, Sven Bergmann, Rosa Vargas-Poussou, Martin Konrad, Paolo Gasparini, Ian J. Deary, John M. Starr, Daniela Toniolo, Peter Vollenweider, Jobst G.J. Hoenderop, René J.M. Bindels#, Murielle Bochud#, and Olivier Devuyst#: **Genome-wide Meta-analysis Unravels Novel Interactions Between Magnesium Homeostasis and Metabolic Phenotypes.** J Am Soc Nephrol. doi: 10.1681/ASN. *equally contributed to the study; #co-directed the study