Does Enamelin have pleiotropic effects on organs other than the teeth? Lessons from a phenotyping screen of two Enamelin-mutant mouse lines

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Abstract

We analyzed two mutant mouse lines, ATE1 and ATE2, that carry point mutations in the enamelin gene, causing premature stop codons in exons 8 and 7, respectively. Both mutant lines show amelogenesis imperfecta. In order to find out whether mutations within the enamelin gene have effects on other organ functions, we did a systematic, standardized phenotypic analysis of both mutant lines in the German Mouse Clinic. In addition to the initially characterized tooth phenotype that is present in both mutant lines, we detected effects of the enamelin mutations on bone and energy metabolism, as well as alterations in clinical chemical and hematological parameters. These data raise the hypothesis that enamelin defects have pleiotropic effects on organs other than the teeth.

Introduction

Enamelin (ENAM) is one of the three secretory calcium-binding phosphoproteins (enamelin, amelogenin and andameloblastin) and is the largest and least abundant of these, representing about 5% of the total proteins in developing enamel (Uchida et al. 1991). Up to now *Enam* has commonly been considered a tooth-specific gene expressed by the enamel organ and, at a low level, in odontoblasts (Nagano et al. 2003). The mouse gene for enamelin consists of 10 exons, while the human enamelin gene contains 9 exons; the sequence corresponding to mouse exon 2 is absent in humans, although the intron between the first and second exons in man shows a sequence homologous to mouse exon 2.

Enam-knockout mice have no real enamel, developing just a thin, highly irregular, mineralized crust covering the dentin. While the enamel layer is absent in $Enam^{-/-}$ mice, no alterations could be detected in bone, dentin or any other hard tissue. The enamel of the $Enam^{+/-}$ mice was nearly normal in the maxillary incisors, but the mandibular incisors were discolored and showed more signs of tooth wear (Hu et al. 2008). Analysis of an ENU-induced mouse mutant having a C > T transition in exon 8 of the *Enam* gene, which changes the glutamine (Gln) codon at position 176 into a premature stop codon, revealed a similar phenotype: homozygous mice showed a complete loss of enamel in the front and molar teeth, while heterozygous mutants exhibited cracked enamel of reduced thickness in incisor and molar teeth (Seedorf et al. 2004). Masuya et al. (2005) introduced three more ENU-induced mouse mutants with point mutations in the *Enam* gene: two mutants carried substitutions in exon 5, causing putative missense mutations of the translated protein at positions 55 and 57, respectively, and the third mutant carried a mutation at the splice donor site in intron 4, resulting in a frame-shift that caused a premature stop codon. Homozygotes of the latter mutant showed complete loss of enamel on the incisors and the molars. Heterozygotes of all three mutants presented a similar phenotype, with rougher surfaces and abnormal tooth wear (Masuya et al. 2005).

According to the cited studies, effects of point mutations in *Enam* are limited to enamel development; nothing is known about effects of the mutations on organs other than the teeth. No effects of enamelin mutations on body composition or bone metabolism have been described in humans or mice. As many genes have pleiotropic functions, we analyzed two independent ENU mutants, ATE1 and ATE2 (ATE = abnormal teeth), with point mutations in the *Enam* gene in the systematic, standardized phenotyping screen of the German Mouse Clinic (Gailus-Durner et al. 2005, Fuchs et al. 2009) for effects of the enamelin mutations on other organ systems.

Material and Methods

Generation of ATE1 and ATE2 mouse mutant lines

Both mutant lines (ATE1 and ATE2) originate from the Munich ENU mutagenesis screen (Hrabe de Angelis et al. 2000). The mutant lines were established by screening F1 animals from ENU-injected C3HeB/FeJ male mice for dysmorphological abnormalities (Fuchs et al. 2000) and were detected based on the abnormal color of the upper incisors. The mutation and the phenotype of ATE1 mice have been well characterized and published (Seedorf et al. 2004, Seedorf et al. 2007). Briefly, ATE1 mice have a point mutation in exon 8 of the *Enam* gene, which changes the glutamine (Gln) codon at position 176 into a premature stop codon (Gln176X). Homozygous ATE1 mice show total enamel aplasia with exposed dentinal tubules, while heterozygous mutants have a significant reduction in enamel width.

The ATE2-mutant line has recently been discovered. After confirmation of the phenotype by inheritance testing, mapping of the causative mutation was done by applying an outcross-backcross strategy with C57BL/6J mice and subsequent analysis of single nucleotide polymorphism (SNP) markers using MALDI-TOF technology (Sequenom) as described (Klaften et al. 2005).

Phenotypic analysis in the German Mouse Clinic

In the German Mouse Clinic (Gailus-Durner et al. 2005), mutant mouse lines are analyzed for phenotypic abnormalities in the areas Behavior, Dysmorphology, Neurology, Eye, Clinical Chemistry, Immunology, Allergy, Nociception, Lung Function, Energy Metabolism, Molecular Phenotyping and Pathology. For detailed information see Fuchs et al. (2009), Gailus Durner et al. (2009) and Fuchs et al. (2011).

As the ATE1 mutation causes a semi-dominant phenotype and the ATE2 mutation results in a dominant tooth phenotype, we decided to focus on heterozygous animals of both mutant lines in the phenotypic analysis. The tooth phenotypes of heterozygous animals from the two mutant lines are similar, and we expected that the results of the phenotypic analysis might thus serve as an independent confirmation.

The analyzed cohorts resulted from heterozygous x wild-type crosses (of animals that were backcrossed to C3HeB/FeJ for more than 10 generations), and the genotype was assigned based on appearance of the tooth phenotype. From both mutant lines (ATE1 and ATE2), cohorts of 15 heterozygous male mutants, 15 heterozygous female mutants as well as 15 male and 15 (ATE1) or 12 (ATE2) female wild-type littermate controls were analyzed in the German Mouse Clinic primary screen. The data from two animals were excluded from subsequent analysis, as clinical chemistry measurements obtained pathological values.

Mice were housed and handled according to the German Animal Welfare Act (by authority of the government of upper Bavaria/Oberbayern).

Results

Two mouse mutant lines, ATE1 and ATE2, with tooth abnormalities were analyzed in the present study. Both mutant lines originated from an ENU mutagenesis project for dominant mutations on a C3HeB/FeJ genetic background. While the ATE1 mouse mutant line was already well characterized (Seedorf et al. 2004, Seedorf et al. 2007), the ATE2-mutant line was only recently discovered in the Munich ENU project. The tooth phenotype of ATE2 mutants is visible to the naked eye and is similar to that observed in ATE1 mutants: while the incisors of wild-type mice are brown, those of heterozygous ATE2 mice have a whitish appearance. In contrast to homozygous ATE1 mutants, which differ from heterozygotes in their complete loss of enamel on the front teeth, homozygous ATE2 mutants do not show abnormalities any different from the heterozygotes.

For ATE2 the causative mutation was mapped to mouse chromosome 5 between the markers rs29635956 and rs13478429 (67.85 Mb and 103.15 Mb, Build 37.1). Sequencing of the *Enam* coding sequence as a candidate revealed a single nucleotide point mutation within exon 7, which altered the adenosine at position 382 to a thymidine residue (A382T), introducing a premature translational STOP at codon position 128.

As a next step, cohorts of 15 heterozygous mutants of each sex as well as littermate wild-type control animals were generated from both mutant lines and analyzed in the primary phenotyping screen of the German Mouse Clinic. Data is shown only from screens that detected significant differences between the mutant animals and the respective controls.

Clinical Chemistry

Twenty different clinical-chemical parameters were measured. Mean plasma cholesterol values as well as plasma α-amylase activity were decreased in mutant mice of both lines compared to the respective controls. In contrast, alkaline phosphatase (ALP) activity was increased in mutant animals of both lines. Plasma triglyceride concentrations were significantly decreased in ATE2 mutants of both sexes compared to controls, while ATE1 mutants showed mild changes towards the same direction. Transferrin values were below the level of controls in female mutants of both lines, while male ATE1 mutants showed a mild decrease, and no significant difference was detected in male ATE2 mice compared to the respective controls. Plasma lipase activity was slightly decreased compared to controls in mutants of both lines except male ATE2 mice. Additionally, plasma glucose and total protein levels showed a tendency to be reduced in mutant animals irrespective of line and sex (table 1). Taken together, the data reveal several hints that energy, bone and iron metabolism is influenced by the *Enam* mutations in ATE1- as well as ATE2-mutant animals.

Hematology

The red blood cell count (RBC) and hemoglobin concentration (HGB) in male and female ATE1-mutant animals were lower than the values for the control littermates (table 1). The MCV (mean corpuscular volume) was slightly increased only in male mice (data not shown). Therefore, hematocrit (HCT) values were also significantly decreased compared to controls in this line. No significant differences between ATE2-mutant and control animals in hematological parameters were detected.

Bone

In both ATE1- and ATE2-mutant lines, we detected significantly reduced bone mineral density (BMD) in mutants compared to controls. We also found bone mineral content (BMC) and body weight to be significantly decreased in mutants. Body length was significantly decreased in ATE1 mutants and in female ATE2 mutants (with the same tendency in male ATE2 mutants). In order to characterize the findings of the altered bone mineral density in more detail, we analyzed a separate cohort of heterozygous ATE1 mutants by peripheral quantitative computed tomography (pQCT). In the distal femoral metaphysis, total bone density and content were significantly reduced in both sexes of ATE1 mice. This was mainly due to a significant reduction in the trabecular density and cortical content. Concurrently, total bone area was decreased in ATE1 mutants, which was due to significantly reduced cortical bone area. In addition, we observed significantly increased trabecular bone area in female ATE1 mutants compared to controls (table 1).

Energy metabolism

ATE1- and ATE2-mutant mice differed in body mass, with lower values in mutants compared to control animals (-5.5 g and -5.1 g in male and female ATE1 mutants, respectively, at the age of 18 weeks; also see figure 1). The difference was more pronounced in ATE2 mice (-7.5 g and -8.4 g in males and females, respectively). In body composition parameters, fat mass was significantly decreased whereas lean mass was significantly increased in both mutant lines compared to control animals (table 1). Notably, body temperature, food consumption and the efficiency of energy extraction from food were not different between mutant and control mice, even though ATE1 mutants produced less feces per gram consumed food. No differences could be detected in response to a 2-day fasting challenge. Lowered fat mass and elevated lean body mass as well as changes in clinical chemical parameters in both the ATE1 and the ATE2-mutant lines point to changes in the regulation of energy homeostasis.

Discussion

We have analyzed two mutant lines that carry slightly divergent nonsense point mutations in the *Enam* gene, one within exon 7 (ATE2) and the other in exon 8 (ATE1). While the tooth phenotype and the *Enam* mutation in the ATE1-mutant line were described previously (Seedorf et al. 2004, Seedorf et al. 2007), the sequence variation in ATE2 mice is described for the first time in this study.

In order to find out whether the *Enam* mutations have an influence on organ systems other than the teeth, we did a systematic, standardized phenotype assessment of both mutant lines in the German Mouse Clinic. By clinical chemical analysis, we found that parameters associated with energy metabolism differ significantly between mutant and control animals. The significant differences in body weight and reduced body fat, in combination with the lower triglyceride levels in mutant mice, strongly indicate differences in energy metabolism pathways. Lowered fat mass and elevated lean body mass as well as changes in clinical chemical parameters point to changes in energy homeostasis in both ATE1 and ATE2 mutants. The fact that we detected additional alterations in amylase, lipase and alkaline phosphatase activities might indicate subtle changes in liver and/or pancreas function.

It could be argued that the reduced body weight in both *Enam* mutants is due to reduced food consumption because of tooth problems. However, our data show that there is no significant difference in food consumption between the mutant lines and their respective controls. There is a tendency toward reduced absolute food consumption *per se*, but energy uptake was even slightly increased when adjusted to body mass, particularly for ATE1 mutants.

White enamel in rodents can be due to reduced incorporation of iron into the enamel layer during amelogenesis (Yanagawa *et al.* 2004). Taking this into account, the small differences detected in transferrin levels might reflect subtle changes in iron metabolism, which could also be associated with the small differences in red blood cell counts found in ATE1 mutants.

Increased alkaline phosphatase activity can be related to bone metabolism and may be due to increased osteoblast or osteoclast activity. Changes in bone metabolism are also supported by our findings in the analysis of bone mineral density.

There are some human diseases in which patients have skeletal abnormalities in combination with tooth disorders (e.g. osteogenesis imperfecta, tricho-dento-osseous syndrome OMIM #190320, rickets); most of them are known to be hereditary and/or are caused by malnutrition. Enamel defects are often seen in malformation syndromes affecting other body parts such as limbs, bones and ecto-dermal appendages. There are strong similarities between AI hypomaturation-hypoplasia type with taurodontism (AIHHT, OMIM #104510) and the tricho-dento-osseous (TDO) syndrome (OMIM#190320) (Crawford et al. 2007, Dong et al. 2005).

Enamelin is expressed primarily by secretory ameloblasts and is presumed to function only during dental enamel formation (Hu and Yamakoshi, 2003, Hu et al. 2001). No expression was observed in pulp, adjacent bone or along the developing root. Hu et al. (2008) described that *Enam*-null mice show no true enamel covering the dentin, whereas no differences were observed in adjacent bone, dentin or any other tissue besides the enamel layer.

In order to find supporting material for our findings that *Enam* is associated with other organ

functions, we did a co-citation analysis with String (8.3) (Jensen 2009) and the Bibliosphere Pathway Edition of the Genomatix Software Suite (Scherf 2005). We used the interaction partners described by Wang et al. (2005) to analyze their correlations to disease-related co-citation with Genomatix Bibliosphere-Software. Our analysis revealed that the enamelin interaction partners were matched in various combinations to over 300 disease-related MeSH terms, 77 of which also included *Enam*. Table 2 shows results for those 11 disease terms that were most closely related to our analysis: *Enam* is a major component of a few interaction partners involved in amyloid and dental development, but most of the other disease terms describe more complex diseases that are more prevalent in the mature organism. The co-citation analysis indicates that there is an association with other factors but does not allow discrimination of whether this is due to direct interaction(s) or is the result of disease-related interactions (secondary effects). The association of *Enam* with metal metabolism, vitamin D deficiency, calcium disorders, malnutrition, diabetes mellitus and kidney diseases as well as bone and musculoskeletal diseases suggests that *Enam* might also play a role in the adult organism in different areas of the body, supporting the observed pleiotropic effects in the mouse mutants in our study.

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References

Crawford PJ, Aldred M, Bloch-Zupan A (2007). Amelogenesis imperfecta. *Orphanet J Rare Dis* 4;2:17.

Dong J, Amor D, Aldred MJ, Gu T, Escamilla M, MacDougall M (2005). DLX3 mutation associated with autosomal dominant amelogenesis imperfecta with taurodontism. *Am J Med Genet A* Mar 1;133A(2):138-41.

Fuchs H, Schughart K, Wolf E, Balling R, Hrabé de Angelis M (2000). Screening for dysmorphological abnormalities--a powerful tool to isolate new mouse mutants. *Mamm Genome* 11(7):528-30.

Fuchs H, Gailus-Durner V, Adler T, Pimentel JA, Becker L, Bolle I, Brielmeier M, Calzada-Wack J, Dalke C, Ehrhardt N, Fasnacht N, Ferwagner B, Frischmann U, Hans W, Hölter SM, Hölzlwimmer G, Horsch M, Javaheri A, Kallnik M, Kling E, Lengger C, Maier H, Mossbrugger I, Mörth C, Naton B, Nöth U, Pasche B, Prehn C, Przemeck G, Puk O, Racz I, Rathkolb B, Rozman J, Schäble K, Schreiner R, Schrewe A, Sina C, Steinkamp R, Thiele F, Willershäuser M, Zeh R, Adamski J, Busch DH, Beckers J, Behrendt H, Daniel H, Esposito I, Favor J, Graw J, Heldmaier G, Höfler H, Ivandic B, Katus H, Klingenspor M, Klopstock T, Lengeling A, Mempel M, Müller W, Neschen S, Ollert M, Quintanilla-Martinez L, Rosenstiel P, Schmidt J, Schreiber S, Schughart K, Schulz H, Wolf E, Wurst W, Zimmer A, Hrabé de Angelis M (2009). The German Mouse Clinic: a platform for systemic phenotype analysis of mouse models. *Curr Pharm Biotechnol* 10(2):236-43.

Fuchs H, Gailus-Durner V, Adler T, Aguilar-Pimentel JA, Becker L, Calzada-Wack J, Da Silva-Buttkus P, Neff F, Götz A, Hans W, Hölter SM, Horsch M, Kastenmüller G, Kemter E, Lengger C, Maier H, Matloka M, Möller G, Naton B, Prehn C, Puk O, Rácz I, Rathkolb B, Römisch-Margl W, Rozman J, Wang-Sattler R, Schrewe A, Stöger C, Tost M, Adamski J, Aigner B, Beckers J, Behrendt H, Busch DH, Esposito I, Graw J, Illig T, Ivandic B, Klingenspor M, Klopstock T, Kremmer E, Mempel M, Neschen S, Ollert M, Schulz H, Suhre K, Wolf E, Wurst W, Zimmer A, Hrabě de Angelis M (2011). *Mouse phenotyping. Methods* 53(2):120-35.

Gailus-Durner V, Fuchs H, Becker L, Bolle I, Brielmeier M, Calzada-Wack J, Elvert R, Ehrhardt N, Dalke C, Franz TJ, Grundner-Culemann E, Hammelbacher S, Hölter SM, Hölzlwimmer G, Horsch M, Javaheri A, Kalaydjiev SV, Klempt M, Kling E, Kunder S, Lengger C, Lisse T, Mijalski T, Naton B, Pedersen V, Prehn C, Przemeck G, Racz I, Reinhard C, Reitmeir P, Schneider I, Schrewe A, Steinkamp R, Zybill C, Adamski J, Beckers J, Behrendt H, Favor J, Graw J, Heldmaier G, Höfler H, Ivandic B, Katus H, Kirchhof P, Klingenspor M, Klopstock T, Lengeling A, Müller W, Ohl F, Ollert M, Quintanilla-Martinez L, Schmidt J, Schulz H, Wolf E, Wurst W, Zimmer A, Busch DH, Hrabe de Angelis M (2005). Introducing the German Mouse Clinic: open access platform for standardized phenotyping. *Nat Methods* 2(6):403-4.

Gailus-Durner V, Fuchs H, Adler T, Aguilar Pimentel A, Becker L, Bolle I, Calzada-Wack J, Dalke C, Ehrhardt N, Ferwagner B, Hans W, Hölter SM, Hölzlwimmer G, Horsch M, Javaheri A, Kallnik M, Kling E, Lengger C, Mörth C, Mossbrugger I, Naton B, Prehn C, Puk O, Rathkolb B, Rozman J, Schrewe A, Thiele F, Adamski J, Aigner B, Behrendt H, Busch

DH, Favor J, Graw J, Heldmaier G, Ivandic B, Katus H, Klingenspor M, Klopstock T, Kremmer E, Ollert M, Quintanilla-Martinez L, Schulz H, Wolf E, Wurst W, Hrabe de Angelis M (2009). Systemic first-line phenotyping. *Methods Mol Biol* 530:463-509.

Hrabé de Angelis MH, Flaswinkel H, Fuchs H, Rathkolb B, Soewarto D, Marschall S, Heffner S, Pargent W, Wuensch K, Jung M, Reis A, Richter T, Alessandrini F, Jakob T, Fuchs E, Kolb H, Kremmer E, Schaeble K, Rollinski B, Roscher A, Peters C, Meitinger T, Strom T, Steckler T, Holsboer F, Klopstock T, Gekeler F, Schindewolf C, Jung T, Avraham K, Behrendt H, Ring J, Zimmer A, Schughart K, Pfeffer K, Wolf E, Balling R (2000). Genome-wide, large-scale production of mutant mice by ENU mutagenesis. *Nat Genet* 25(4):444-7.

Hu JC, Zhang CH, Yang Y, Karrman-Mardh C, Forsman-Semb K, Simmer JP (2001). Cloning and characterization of the mouse and human enamelin genes. *J Dent Res* 80(898-902.

Hu JC, Sun X, Zhang C, Simmer JP (2001). A comparison of enamelin and amelogenin expression in developing mouse molars. *Eur J Oral Sci* 109(2):125-32.

Hu JC, Hu Y, Smith CE, McKee MD, Wright JT, Yamakoshi Y, et al. (2008). Enamel defects and ameloblast-specific expression in Enam knock-out/lacz knock-in mice. *J Biol Chem* 283(16):10858-71.

Hu JC, Yamakoshi Y (2003). Enamelin and autosomal-dominant amelogenesis imperfecta. *Crit Rev Oral Biol Med* 14(6):387-98.

Jensen LJ, Kuhn M, Stark M, Chaffron S, Creevey C, Muller J, Doerks T, Julien P, Roth A, Simonovic M, Bork P, von Mering C (2009). STRING 8--a global view on proteins and their functional interactions in 630 organisms. *Nucleic Acids Res* 37:D412-6.

Klaften M, Hrabé de Angelis M (2005). ARTS: a web-based tool for the set-up of high-throughput genome-wide mapping panels for the SNP genotyping of mouse mutants. *Nucleic Acids Res* 33(Web Server issue):W496-500.

Masuya H, Shimizu K, Sezutsu H, Sakuraba Y, Nagano J, Shimizu A, et al. (2005). Enamelin (Enam) is essential for amelogenesis: ENU-induced mouse mutants as models for different clinical subtypes of human amelogenesis imperfecta (AI). Hum Mol Genet 14(575-83.

Nagano T, Oida S, Ando H, Gomi K, Arai T, Fukae M (2003). Relative levels of mRNA encoding enamel proteins in enamel organ epithelia and odontoblasts. *J Dent Res* 82(982-6.

Scherf M, Epple A, Werner T (2005). The next generation of literature analysis: integration of genomic analysis into text mining. *Brief Bioinform* 6:287-97.

Seedorf H, Springer IN, Grundner-Culemann E, Albers HK, Reis A, Fuchs H, et al. (2004). Amelogenesis imperfecta in a new animal model--a mutation in chromosome 5 (human 4q21). *J Dent Res* 83(608-612.

Seedorf H, Klaften M, Eke F, Fuchs H, Seedorf U, Hrabe de Angelis M (2007). A mutation in the enamelin gene in a mouse model. *J Dent Res* 86(8):764-8.

Uchida T, Tanabe T, Fukae M, Shimizu M (1991). Immunocytochemical and immunochemical detection of a 32 kDa nonamelogenin and related proteins in porcine tooth germs. *Arch Histol Cytol* 54(5):527-38.

Wang H, Tannukit S, Zhu D, Snead ML, Paine ML (2005). Enamel matrix protein interactions. *J Bone Miner Res* 20:1032-40.

Yanagawa T, Itoh K, Uwayama J, Shibata Y, Yamaguchi A, Sano T, Ishii T, Yoshida H, Yamamoto M (2004). Nrf2 deficiency causes tooth decolourization due to iron transport disorder in enamel organ. Genes to Cells 9: 641-651

Table 1 Significantly changed parameters in clinical chemistry, hematology, bone and energy metabolism (data presented as mean ± SD)

Parameter	ATE1				ATE2			
	males		females		males		females	
	control	mutant	control	mutant	control	mutant	control	mutant
Clinical Chemistry								
Cholesterol [mg/dl]	169,6 ± 12,83	153,6 ± 9,75***	136,6 ± 12,83	126,6 ± 13,23*	165,8 ± 12,28	145,6 ± 16,99***	138,0 ± 10,00	118,8 ± 14,49**
Triglycerides [mg/dl]	377 ± 61,7	337 ± 72,6	346 ± 111,9	277 ± 71,9*	430 ± 79,3	332 ± 101,5 ***	402 ± 94,7	261 ± 110,0**
Glucose [mg/dl]	166,3 ± 24,41	155,7 ± 16,05	146,0 ± 23,69	133,9 ± 18,99	158,0 ± 20,99	157,3 ± 21,28	171,4 ± 30,00	146,8 ± 25,13
α-Amylase [U/l]	2395 ± 97	2150 ± 137***	2054 ± 139	1906 ± 101**	2417 ± 113	2299 ± 237*	2130 ± 91	1901 ± 100***
Lipase [U/l]	52,2 ± 4,6	48,5 ± 3,9*	56,0 ± 5,6	52,2 ± 5,4*	60,5 ± 3,9	75,2 ± 47,6	57,5 ± 5,3	52,8 ± 4,8 *
ALP [U/l]	98,1 ± 6,82	107,2 ± 7,92**	110,7 ± 11,73	120,3 ± 5,34**	95,2 ± 5,54	110,3 ± 8,94***	108,8 ± 10,89	130,9 ± 13,14***
Total Protein [g/dl]	5,40 ± 0,321	5,20 ± 0,185	5,14 ± 0,298	4,99 ± 0,316	5,36 ± 0,188	5,24 ± 0,210	4,87 ± 0,231	4,86 ± 0,318
Transferrin [mg/dl]	188,1 ± 5,83	184,3 ± 3,45*	192,2 ± 5,15	186,6 ± 5,85**	186,6 ± 7,57	185,3 ± 3,49	201,0 ± 6,26	193,3 ± 6,65**
Hematology								
RBC [Mio/µl]	9,39 ± 0,373	9,05 ± 0,318*	9,12 ± 0,463	8,75 ± 0,408*	9,07 ± 0,323	9,24 ± 0,384	8,97 ± 0,337	8,91 ± 0,301
HGB [g/dl]	15,38 ± 0,614	14,85 ± 0,585*	15,81 ± 0,650	15,30 ± 0,441*	15,46 ± 0,619	15,59 ± 0,493	15,20 ± 0,707	15,33 ± 0,570
HCT [%]	44,82 ± 1,69	43,31 ± 1,63*	44,41 ± 2,06	42,46 ± 1,97**	44,92 ± 1,48	45,51 ± 1,92	44,9 ± 1,55	44,8 ± 2,00
Bone (DEXA analysis)								
BMD (mg/cm^2)	73 ± 4	67 ± 4**	75 ± 1	69 ± 5**	74 ± 3	66 ± 5***	76 ± 2	68 ± 6**
BMC (mg)	1295 ± 203	904 ± 150***	1295 ± 192	909 ± 137***	1381 ± 233	969 ± 187***	1390 ± 125	960 ± 257***
Body length (cm)	10.15 ± 0.32	9.90 ± 0.20*	10.39 ± 0.21	9.95 ± 0.27**	10.39 ± 0.21	10.15 ± 0.23	10.36 ± 0.23	9.95 ± 0.35**
Body weight (g)	38.24 ± 3.26	32.43 ± 2.10***	35.80 ± 4.20	29.23 ± 2.33***	40.00 ± 2.83	33.79 ± 3.51**	39.14 ± 3.40	30.18 ± 5.07***
Fat mass (g)	17.87 ± 5.02	8.95 ± 3.66***	19.24 ± 4.83	8.94 ± 3.81***	20.22 ± 4.86	10.33 ± 4.93***	22.77 ± 3.84	9.96 ± 7.27***
Lean mass (g)	15.80 ± 3.23	19.55 ± 2.74**	11.86 ± 1.76	16.32 ± 2.05**	15.07 ± 3.72	19.55 ± 2.62**	11.48 ± 2.07	16.36 ± 3.12**
Energy metabolism								
Body mass (g)	39.3 ± 3.0	33.8 ± 2.3*	36.2 ± 3.8	31.1 ± 3.0*	40.3 ± 2.8	32.8 ± 3.7*	40.1 ± 2.9	31.7 ± 6.7*
Body mass loss (g) after fasting	-6.1 ± 0.7	-5.9 ± 0.6	-4.8 ± 0.6	-4.7 ± 0.4	-6.1 ± 0.7	-6.3 ± 0.6	-5.8 ± 0.4	-5.4 ± 0.5
Body temperature (°C)	36.2 ± 0.2	36.0 ± 0.3	36.7 ± 0.3	36.3 ± 0.4*	36.6 ± 0.2	36.1 ± 0.2*	37.2 ± 0.4	37.0 ± 0.3
Body temperature decrease (°C) after fasting	-1.1 ± 0.4	-1.1 ± 0.4	-0.9 ± 0.7	-0.6 ± 0.7	-1.7 ± 0.6	-0.9 ± 1.2	-2.7 ± 0.6	-3.2 ± 1.1
Food intake (g d^{-1})	3.8 ± 0.3	3.5 ± 0.5	4.1 ± 0.8	3.5 ± 0.6	3.9 ± 0.3	3.6 ± 0.5	3.9 ± 0.3	3.6 ± 0.3
Feces production (g d^{-1})	0.57 ± 0.03	0.50 ± 0.04*	0.56 ± 0.03	0.48 ± 0.06*	0.58 ± 0.05	0.56 ± 0.09	0.48 ± 0.05	0.48 ± 0.05

Energy content feces (kJ g^{-1})	15.60 ± 0.07	15.76 ± 0.37	15.57 ± 0.13	15.26 ± 0.08(*)	15.23 ± 0.15	15.71 ± 0.28*	15.35 ± 0.11	15.45 ± 0.10
Metabolizable energy (kJ d^{-1})	59.14 ± 6.16	55.71 ± 8.78	65.23 ± 13.19	55.31 ± 9.78	62.15 ± 4.38	54.34 ± 14.17	62.68 ± 5.85	58.10 ± 5.67
Assimilation efficiency (%)	85.3 ± 1.5	86.0 ± 1.0	86.3 ± 1.7	86.5 ± 1.7	86.1 ± 0.4	84.0 ± 4.0	87.9 ± 0.8	87.2 ± 1.8
Bone (pQCT, distal femoral metaphysis)								
Total density (mg/cm^3)	673 ± 12	625 ± 21**	787 ± 37	712 ± 15***				
Trabecular density [mg/cm^3]	308 ± 10	259 ± 11***	316 ± 8	248 ± 12***				
Total content (mg)	2.30 ± 0.07	1.95 ± 0.07***	2.76 ± 0.02	2.09 ± 0.11***				
Cortical content (mg]	1.96 ± 0.07	1.63 ± 0.05***	2.58 ± 0.07	1.88 ± 0.09***				
Total area (mm^2)	3.42 ± 0.08	3.12 ± 0.18*	3.51 ± 0.16	2.94 ± 0.17***				
Trabecular area (mm^2)	1.10 ± 0.08	1.20 ± 0.11	0.56 ± 0.20	0.85 ± 0.08***				
Cortical area (mm^2)	2.32 ± 0.09	1.93 ± 0.10***	2.95 ± 0.07	2.09 ± 0.12***				

Statistical analysis: * p < 0.05, ** p < 0.01, *** p < 0.001 (Tukey Test, Two Way ANOVA).

Clinical Chemistry: ATE1 males: 15 control, 15 mutant; females: 14 control, 15 mutant; ATE2 males: 15 control, 14 mutant; females 12 control, 14 mutant

DEXA: ATE1 males: 10 control, 10 mutant; females: 9 control, 10 mutant; ATE2 males: 9 control, 10 mutant; females 7 control, 10 mutant.

Energy metabolism: ATE1 males: 7 control, 7 mutant; females: 7 control, 7 mutant; ATE2 males: 6 control, 6 mutant; females 7 control, 7 mutant.

pQCT: ATE1 males: 4 control, 8 mutant; females: 5 control, 10 mutant.

Table 2: Enamelin disease association table: Co-citation partners are in alphabetical order (top down), and disease associations are sorted according to the number of co-citations (reflecting the complexity of the disease).

	Enam + Co-Citation Partners	Disease Association										
		Amelogenesis Imperfecta	Dentinogenesis Imperfecta	Metal Metabolism, Inborn Errors	Vitamin D Deficiency	Malnutrition	Calcium Metabolism Disorders	Nutrition Disorders	Diabetes Mellitus	Bone Diseases	Kidney Diseases	Musculoskeletal Diseases
1	Ahsg				Ahsg	Ahsg	Ahsg	Ahsg	Ahsg	Ahsg	Ahsg	Ahsg
2	Ambn	Ambn	Ambn		Ambn	Ambn	Ambn	Ambn		Ambn		Ambn
3	Amelx	Amelx		Amelx	Amelx	Amelx	Amelx	Amelx	Amelx	Amelx	Amelx	Amelx
4	Anxa2						Anxa2	Anxa2	Anxa2	Anxa2	Anxa2	Anxa2
5	Bgn		Bgn	Bgn	Bgn	Bgn	Bgn	Bgn	Bgn	Bgn	Bgn	Bgn
6	Bst2									Bst2	Bst2	Bst2
7	Canx					Canx		Canx		Canx	Canx	Canx
8	Car3			Car3	Car3	Car3	Car3	Car3	Car3	Car3	Car3	Car3
9	Cd63								Cd63	Cd63	Cd63	Cd63
10	Col15a1									Col15a1	Col15a1	Col15a1
11	Col2a1		Col2a1	Col2a1	Col2a1	Col2a1	Col2a1	Col2a1	Col2a1	Col2a1	Col2a1	Col2a1
12	Ctsd			Ctsd	Ctsd	Ctsd	Ctsd	Ctsd	Ctsd	Ctsd	Ctsd	Ctsd
13	Enam	Enam	Enam	Enam	Enam	Enam	Enam	Enam	Enam	Enam	Enam	Enam
14	F3							F3	F3		F3	F3
15	Kng1							Kng1	Kng1	Kng1	Kng1	Kng1
16	Xylt2								Xylt2		Xylt2	Xylt2
	Total	3	4	6	8	9	9	12	12	14	15	16

Figure legends:

Figure 1: Body weight curve of ATE1 (A) and ATE2 (B) male (light blue) and female (orange) mutants and control males (dark blue) and females (red).