Bone Mineralization and Calcium Homeostasis are Dependant on Gastric Acid Secretion

INTRODUCTION
Bone is the body’s reservoir for calcium. If the serum level of calcium drops, induction of PTH leads to release of calcium from the bone by osteoclastic action, thereby assuring calcium homeostasis. Consequently, osteoclastic dysfunction in osteoporotic patients should result in major disturbances in serum calcium regulation. Indeed, there are reports on osteoporotic patients displaying hypocalcaemia, hyperparathyroidism and rickets as a complicating medical condition. This combination of osteoporosis (OP) and rickets has been termed osteopetrorickets (OPR). However approximately 50% of the osteoporotic patients do not display a bone mineralization defect, inspite of osteoclastic dysfunction, suggesting an additional mechanism of regulation of bone mineralization.

METHODS
Mice. Oc/+ and Src+/- mice were obtained from the Jackson Laboratory. Cckbr−/− mice on a 129Sv genetic background were kindly provided by A.S. Kopin. Cckbr−/−, oc−/− and Src−/− mice were fed a standard rodent diet. For the determination of life span Src−/−, Src−/− /Cckbr−/− and oc−/oc mice were fed a liquid diet containing 0.5 % calcium carbonate (Altromin #CO199) starting at 2 weeks of age. The same diet containing 2 % calcium carbonate was given to oc−/oc-mice in order to normalize their serum calcium. The skeletons were analyzed by contact Xray, μCT scanning, bone histology and TRAP activity staining.

Primary cell culture. Cultures of primary osteoclasts, chondrocytes or osteoblasts were generated. For the assessment of ex vivo mineralization, primary osteoblasts were isolated from single calvariae of 3 days old wt and oc−/oc littermates and stained after von Kossa at two stages of differentiation induced by ascorbic acid and β-glycerophosphate.

Human bone biopsies. A collection of transiliac bone biopsies from non-genotyped patients with various disorders was established at the Institute of Pathology at the Hamburg University over the last 30 years. All biopsies were embedded non-decalcified into methylmethacrylate, and sections were subjected to Kossa/Gieson or Goldner staining. In addition, non-genotyped patients with various disorders was established at the Institute of Pathology at the Hamburg University over the last 30 years. All biopsies were embedded non-decalcified into methylmethacrylate, and sections were subjected to Kossa/Gieson or Goldner staining.

Histomorphometric analysis was carried out using the OsteoMeasure system (Osteometrics) following the guidelines of the American Society for Bone and Mineral Research. A case of TCIRG1-dependent ARO in a 1.5 years old child was confirmed by automated sequencing of all TCIRG1-encoding exons. A bone biopsy was taken before BMT and a 1.5 years old child was confirmed by automated sequencing of all TCIRG1-encoding exons. A bone biopsy was taken before BMT and subjected to non-decalcified histology as described above. Age- and sex-matched control biopsies for all patients were taken from skeletal stages of differentiation induced by ascorbic acid and β-glycerophosphate.

RESULTS
Osteoclastic dysfunction in osteopetrotic patients should result in major disturbances in serum calcium regulation. Indeed, there are reports on osteoporotic patients displaying hypocalcaemia, hyperparathyroidism and rickets as a complicating medical condition. This combination of osteoporosis (OP) and rickets has been termed osteopetrorickets (OPR). However approximately 50% of the osteoporotic patients do not display a bone mineralization defect, inspite of osteoclastic dysfunction, suggesting an additional mechanism of regulation of bone mineralization.

DISCUSSION
These data prove that calcium homeostasis is dependant on both osteoclastic resorption and gastric acid secretion in the stomach. Our results reveal a novel physiological function of TCIRG1 with potential relevance for the treatment of OPR, patients with decreased gastric acid secretion is of general importance for calcium homeostasis, we observed phenotype is specific to loss of TCIRG1 function or if gastric acid secretion is provided by A.S. Kopin. Cckbr−/− mice on a 129Sv genetic background were kindly provided by A.S. Kopin. Cckbr−/−, oc−/− and Src−/− mice were fed a standard rodent diet. For the determination of life span Src−/−, Src−/− /Cckbr−/− and oc−/oc mice were fed a liquid diet containing 0.5 % calcium carbonate (Altromin #CO199) starting at 2 weeks of age. The same diet containing 2 % calcium carbonate was given to oc−/oc-mice in order to normalize their serum calcium. The skeletons were analyzed by contact Xray, μCT scanning, bone histology and TRAP activity staining.

Primary cell culture. Cultures of primary osteoclasts, chondrocytes or osteoblasts were generated. For the assessment of ex vivo mineralization, primary osteoblasts were isolated from single calvariae of 3 days old wt and oc−/oc littermates and stained after von Kossa at two stages of differentiation induced by ascorbic acid and β-glycerophosphate.

Human bone biopsies. A collection of transiliac bone biopsies from non-genotyped patients with various disorders was established at the Institute of Pathology at the Hamburg University over the last 30 years. All biopsies were embedded non-decalcified into methylmethacrylate, and sections were subjected to Kossa/Gieson or Goldner staining. In addition, non-genotyped patients with various disorders was established at the Institute of Pathology at the Hamburg University over the last 30 years. All biopsies were embedded non-decalcified into methylmethacrylate, and sections were subjected to Kossa/Gieson or Goldner staining.

Histomorphometric analysis was carried out using the OsteoMeasure system (Osteometrics) following the guidelines of the American Society for Bone and Mineral Research. A case of TCIRG1-dependent ARO in a 1.5 years old child was confirmed by automated sequencing of all TCIRG1-encoding exons. A bone biopsy was taken before BMT and a 1.5 years old child was confirmed by automated sequencing of all TCIRG1-encoding exons. A bone biopsy was taken before BMT and subjected to non-decalcified histology as described above. Age- and sex-matched control biopsies for all patients were taken from skeletal stages of differentiation induced by ascorbic acid and β-glycerophosphate.

Expression analysis. RNA was isolated using the Trizol reagent (Invitrogen). A full-length cDNA encoding Tcrg1 was obtained from RZPD (#2416867) and used for Northern Blotting following standard protocols. For in situ-hybridization the tcrigl-cDNA was subcloned into pBluescript in order to generate [35S]-UTP-labeled sense and antisense probes using the riboprobe combination system (Promega). For RT-PCR, RNA was reverse transcribed using the Cloneamc First-Strand cDNA synthesis Kit (Invitrogen). Immunohistochemistry was performed using the standard protocols using a monoclonal antibody against human TCIRG1 (Abnova, #H00010312-M01).

Analysis of calcium homeostasis. Calcium concentrations were measured using a colorimetric assay (Sigma, #587-A). Serum PTH was quantified by ELISA (Immutopics, #60-2300). Urinary deoxypyridinoline (Odp) crosslinks were measured using Pyrilinks-D ELISA (Metra Biosystems, #8007) and normalized to creatinine determined by an alkaline picate assay (Metra Biosystems, #8009). For human patients we applied the routine measurements that were performed in the Department of Clinical Chemistry, Hamburg University.

Determination of gastric pH. Mice were fasted for 2 hours before their luminal gastric pH was determined by insertion of pH sticks through an incision in the greater curvature of the excised stomach. The gastric pH of the human ARO patient was determined over 24 hours using a pH probe positioned into the lumen of the stomach.

All human and animal studies were approved by the respective review boards and informed consent was obtained from the patients, conforming to the laws and regulations of the respective countries.

ACKNOWLEDGEMENTS
We thank O.Winter, M. Dietzmann, C. Erdmann, T.O. Klatte and S. Kessler for technical assistance, and A. Kopin for providing the Cckbr−/− mice. This work was supported by grants from the Deutsche Forschungsgemeinschaft given to M.A. (AM103/14-1) and M.B. (BL423/4-3), from Telethon to A.T. (#GGP06119) and by the NOBEL program from Fondazione Cariplo to A.V.