# Identification of novel regulators of fibroblast growth factor 23 (FGF23) production: the role of high-fat diet and AMP-activated protein kinase (AMPK)

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| | |
|---|---|
| 2-APB | 2-aminoethoxydiphenyl borate |
| ADHR | Autosomal dominant hypophosphatemic rickets |
| ADP | Adenosine diphosphate |
| ADPKD | Autosomal dominant polycystic kidney disease |
| AGEs | Advanced glycation end products |
| AICAR | 5-aminoimidazole-4-carboxamide ribonucleotide |
| AKI | Acute kidney injury |
| AMP | Adenosine monophosphate |
| AMPK | AMP-activated protein kinase |
| $ampk^{-/-}$ mice | AMPKα1-deficient mice |
| $ampk^{+/+}$ mice | Wild-type mice |
| ANKH | Progressive ankylosis protein homologue |
| ANP | Atrial natriuretic peptide |
| ARHR | Autosomal recessive hypophosphatemic rickets |
| ASARM | Acidic serine aspartate-rich matrix extracellular phosphoglycoprotein (MEPE)-associated motif |
| AT | Adipose tissue |
| ATP | Adenosine triphosphate |
| BNP | Brain natriuretic peptide |
| $Ca^{2+}$ | Calcium |
| CaM | Calmodulin |
| CaMKKβ | $Ca^{2+}$/calmodulin-dependent protein kinase kinase β |
| CKD | Chronic kidney disease |
| $Cl^-$ | Chloride |
| CRAC channel | $Ca^{2+}$ release-activated $Ca^{2+}$ channel |
| CRP | C-reactive protein |
| CRT | Creatine transporter |
| CYP24A1 | Cytochrome P450 Family 24 Subfamily A Member 1 (also known as: 1,25-dihydroxyvitamin $D_3$ 24-hydroxylase) |
| CYP27B1 | Cytochrome P450 family 27 subfamily B member 1 (also known as: 25-hydroxyvitamin D-1α-hydroxylase) |
| DAG | Diacylglycerol |
| DMP1 | Dentin matrix acidic phosphoprotein-1 |
| E.g. | Exempli gratia (for example) |
| ENaC | Epithelial $Na^+$ channel |

| | |
|---|---|
| ENPP1 | Ectonucleotide pyrophosphatase/phosphodiesterase family member 1 |
| EPO | Erythropoietin |
| ER/SR | Endoplasmic/sarcoplasmic reticulum |
| ERK1/2 | Extracellular signal-regulated kinases 1/2 |
| ESRD | End-stage renal disease |
| FAM20C | Family with sequence similarity-20 member C |
| FGF | Fibroblast growth factor |
| FGFR | Fibroblast growth factor receptor |
| FOXO1 | Forkhead box protein O1 |
| FRS2α | FGFR substrate 2α |
| GALNT3 | UDP-*N*-acetyl-*α*-D-galactosamine-polypeptide *N*-acetylgalactosaminyltransferase 3 |
| GFR | Glomerular filtration rate |
| HBS | Heparan sulphate glycosaminoglycan binding site |
| HFD | High-fat diet |
| HFTC | Hyperphosphatemic familial tumoral calcinosis |
| HIF-1α | Hypoxia-inducible factor-1α |
| HPT | Hyperparathyroidism |
| IGF1 | Insulin-like growth factor 1 |
| IL | Interleukin |
| $IP_3$ | Inositol-1,4,5-trisphosphate |
| $IP_3R$ | $IP_3$-receptor |
| $K^+$ | Potassium |
| KCNQ1 | $K^+$ voltage-gated channel subfamily Q member 1 |
| kDa | Kilodalton |
| LKB1 | Liver kinase B1 |
| LPS | Lipopolysaccharide |
| LVH | Left ventricular hypertrophy |
| MAPK | Mitogen-activated protein kinase |
| MCP-1 | Monocyte chemoattractant protein-1 |
| mRNA | Messenger ribonucleic acid |
| MRS 1845 | *N*-propylargylnitrendipine |
| mTOR | Mechanistic target of rapamycin |
| $Na^+$ | Sodium |
| NaPi-IIa/b/c | $Na^+$-coupled phosphate transporter IIa/b/c |
| NASH | Nonalcoholic steatohepatitis |
| NCC | $Na^+$-$Cl^-$ cotransporter |

| | |
|---|---|
| NCX | $Na^+/Ca^{2+}$ exchanger |
| Nedd4-2 | Neural precursor cell expressed developmentally down-regulated protein 4-2 |
| NFAT | Nuclear factor of activated T cells |
| NF-κB | Nuclear factor-kappa B |
| NHERF-1 | $Na^+/H^+$ exchange regulatory cofactor-1 |
| NKCC2 | $Na^+$-$K^+$-$2Cl^-$ cotransporter |
| Nurr1 | Nuclear receptor-associated protein1 |
| PHEX | Phosphate regulating gene with homologies to endopeptidases on the X chromosome |
| PI3K | Phosphoinositide 3-kinase |
| PKB | Protein kinase B |
| PLCγ | Phospholipase Cγ |
| PPARα | Peroxisome proliferator-activated receptor α |
| PTH | Parathyroid hormone |
| RXR | Retinoid X receptor |
| SERCA | Sarco/endoplasmic reticulum $Ca^{2+}$-ATPase |
| SFAs | Saturated fatty acids |
| SGK1 | Serum/glucocorticoid-regulated kinase-1 |
| siRNA | Small interfering RNA |
| SOCE | Store-operated $Ca^{2+}$ entry |
| SPCs | Subtilisin-like proprotein convertases |
| STIM1 | Stromal interaction molecule 1 |
| TGF | Transforming growth factor |
| TIO | Tumor-induced osteomalacia |
| TNAP | Tissue non-specific alkaline phosphatase |
| $tnf^{-/-}$ mice | TNFα-deficient mice |
| $tnf^{+/+}$ mice | Wild-type mice |
| TNFα | Tumor necrosis factor alpha |
| TRPC1 | Transient receptor potential canonical 1 |
| TRPV5 | Transient receptor potential vanilloid 5 |
| VDR | Vitamin D receptor |
| WNK4 | With-no-lysine kinase-4 |
| XLH | X-linked hypophosphatemia |

# 1 Introduction

## 1.1 Fibroblast growth factor 23 (FGF23) – a bone-derived phosphaturic hormone

### 1.1.1 General aspects of the FGF family

Fibroblast growth factors (FGFs) are considered as a superfamily of polypeptides exerting versatile biological functions in developmental growth and homeostasis of cells, tissues, and organs including mitogenesis, cellular differentiation, and angiogenesis, and play a crucial role in the regulation of energy and mineral metabolism [1–5]. In humans and mice, 22 members of the *FGF/Fgf* gene have been identified, with *FGF19* being the human ortholog of mouse *Fgf15* [4, 6, 7]. All FGFs are structurally related by sharing a highly conserved β-trefoil core domain of 12 antiparallel β-strands consisting of approximately 120 amino acids in length flanked by distinctive N- and C-terminal sequences [1, 2, 4, 6, 8, 9]. Based on phylogenetic sequence homology, human and mouse FGFs can be classified into seven subfamilies comprising three groups according to their biochemical characteristics and developmental properties [2, 4, 6, 10]. In detail, it can be distinguished between intracellular, paracrine, and endocrine FGF subgroups as depicted in Figure 1 [6, 11].

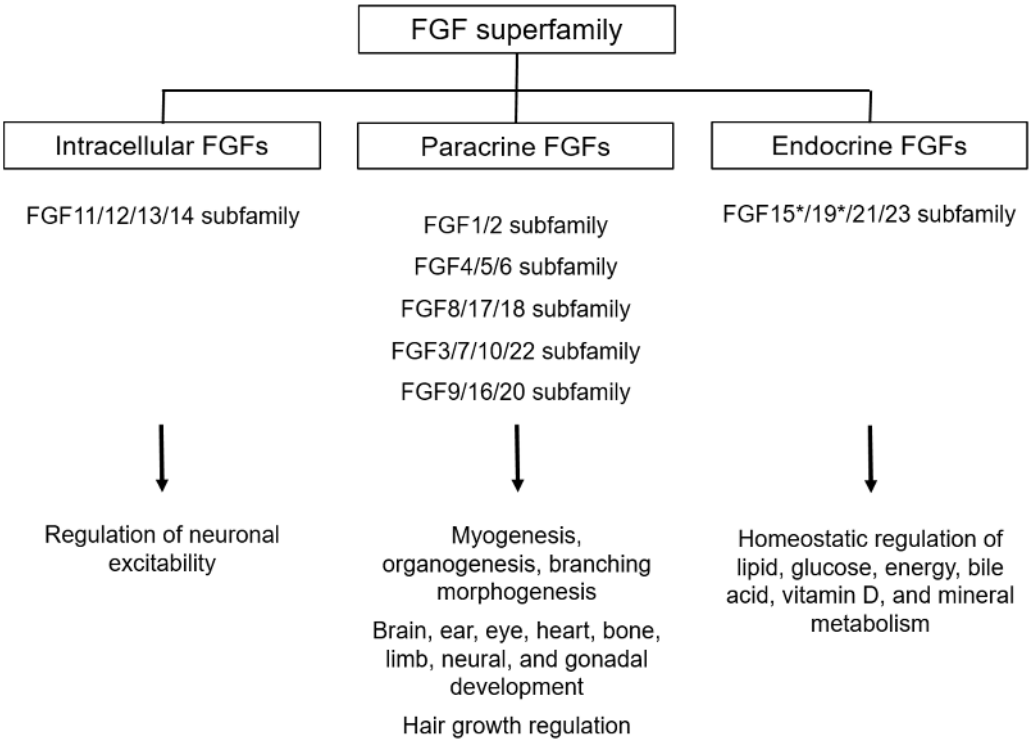


**Figure 1. Schematic overview of the FGF superfamily and physiological functions of group-specific FGFs.** FGFs are classified into seven phylogenetic subfamilies composing intracellular, paracrine, and endocrine FGF groups. *indicates that FGF19 is the human ortholog of mouse FGF15. Adapted and modified from [6, 7, 9, 11].

In contrast to para- and endocrine FGFs, intracellular (or intracrine) FGFs including FGF11/12/13/14 are not secreted extracellularly, thus mediating their function as intracellular signaling molecules in an FGF receptor (FGFR)-independent manner [4, 6, 11]. Intracellular FGFs are implicated in the regulation of synaptic plasticity and neuronal excitability through modulation of voltage-gated sodium ($Na^+$) channels [12–14]. Specifically, FGF14 is considered to play a critical role in neuronal motor function and coordination as an autosomal dominant missense mutation in the human *FGF14* gene leads to a slowly progressive spinocerebellar ataxia [13, 15].

Characterized by a heparan sulphate glycosaminoglycan binding site (HBS) located within the FGF core domain, paracrine-acting FGFs use heparin/heparan sulphate as a cofactor to mediate their physiological reponses as extracellular proteins *via* interaction with cell surface tyrosine kinase FGFRs [2, 6, 9, 16, 17]. The FGFR family comprises four genes (*FGFR1-FGFR4*) which encode seven FGFR isoforms (FGFRs 1b, 1c, 2b, 2c, 3b, 3c, and 4) generated by alternative splicing within the extracellular immunoglobin-like domain D3 of *FGFR1-FGFR3*, thus ensuring distinct ligand binding specificities [5, 11, 18]. The structural basis of FGFRs further contains a transmembrane domain and a split cytoplasmic domain with protein tyrosine kinase activity [9, 11, 19]. Heparin/heparan sulphate-facilitated FGF binding to FGFRs initiates FGFR dimerization, stabilization, and subsequent activation of tyrosine kinase-mediated signaling pathways involved in the regulation of developmental growth and differentiation of cells, tissues, and organs [9, 11, 20–26].

Unlike paracrine FGFs, the HBS of endocrine FGFs, namely FGF15/19, FGF21 and FGF23, shows low affinity to heparin/heparan sulphate which enables endocrine FGFs to be secreted and to circulate in the bloodstream and not being captured in the extracellular matrix [2, 6, 7, 9, 11]. In order to execute their hormone-like activity on their target organs, endocrine FGFs thus require alternate cofactors to interact with FGFRs [1, 6]. Therefore, endocrine actions of FGF15/19 and FGF21 are dependent on the protein β-Klotho [1, 6, 7, 27, 28]. Specifically, FGF15/19 is implicated in the regulation of bile acid metabolism [29–31], whereas FGF21 is reported to stimulate lipolysis, hepatic ketogenesis, and glucose uptake in adipocytes [28, 32]. On the other hand, α-Klotho serves as the obligate co-receptor for FGF23 and its functions in phosphate homeostasis and vitamin D metabolism targeting the kidneys and parathyroid glands as addressed in section 1.1.2 and 1.1.3 [33–35].

### 1.1.2 Structural characteristics and renal effects of FGF23

FGF23 is a bone-derived hormone mainly produced by osteoblasts and osteocytes [5, 36], but is also expressed to a lesser extent in extraosseous tissues such as thymus, brain, heart, spleen, and skeletal muscle [37–39]. Yamashita *et al.* (2000) initially identified FGF23

in the mouse brain being predominantly expressed in the ventrolateral thalamic nucleus [40]. Positional cloning approaches further revealed that missense mutations in the human *FGF23* gene located on chromosome 12p13 are responsible for the clinical phenotype of autosomal dominant hypophosphatemic rickets (ADHR) characterized by disturbed phosphate homeostasis [41]. Moreover, Shimada and coworkers (2001) were the first who confirmed that the tumoral overproduction of FGF23 is a causative factor for renal phosphate wasting and hypophosphatemia as seen in tumor-induced osteomalacia (TIO) [42].

The biologically active *FGF23* gene product is a 32-kilodalton (kDa) glycoprotein comprised of 251 amino acid residues containing a hydrophobic secretory signal sequence of 24 amino acids [6, 40]. The FGF23 protein further contains a conserved $NH_2$ terminal FGF core homology region and a unique 73-amino acid COOH-terminus [6, 43]. The mature FGF23 protein contains a specific consensus sequence (arginine$^{176}$-histidine$^{177}$-threonine$^{178}$-arginine$^{179}$) which functions as a recognition site for proteolytic cleavage between arginine$^{179}$ and serine$^{180}$ by subtilisin-like proprotein convertases (SPCs) like furin [3, 6, 44]. Consequently, three molecular species of FGF23 circulate in the bloodstream: the biologically active full-length FGF23, a shorter fragment lacking the cleaved C-terminal portion, and the cleaved C-terminal fragment itself [3, 6, 45, 46].

FGF23 is nowadays considered as a major regulator of phosphate homeostasis and vitamin D metabolism [33, 47]. Targeting the kidney, evidence from several *in vivo* studies using genetically modified mouse models indicates that FGF23 acts as a phosphaturic hormone and suppresses the synthesis of 1,25-dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$ or calcitriol, the active form of vitamin D] (Figure 2) [48–51]. The renal actions of FGF23 are dependent on membrane-bound α-Klotho as an obligate co-receptor [5, 52]. Kuro-o *et al.* (1997) initially identified α-Klotho as a membrane protein involved in the regulation of ageing and morbidity in age-related diseases [53]. α-Klotho can be detected in various tissues [54], but is primarily expressed in the kidney [55], choroid plexus [55], and parathyroid glands [35]. Membrane-bound full length α-Klotho is an approximately 140-kDa type I single-pass transmembrane protein comprised of a short intracellular tail and a large two-parted ectodomain that can be proteolytically cleaved off by α-, β-, and γ-secretases, yielding a 130-kDa soluble α-Klotho fragment [47, 56, 57]. Soluble α-Klotho is a humoral factor circulating in the blood, but can also be detected in cerebrospinal fluid and urine mediating pleiotropic functions including the regulation of renal ion transport [56, 58, 59]. In addition, a truncated isoform of soluble α-Klotho is reported to be produced by alternative splicing [33, 60]. There are three known paralogous members of the Klotho family, namely α-, β-, and γ-Klotho, of which α- and β-Klotho are required for endocrine FGF signaling, whereas the physiological function of γ-Klotho is largely ill-defined to date [5, 56].

Targeting the proximal tubule, circulating FGF23 preferably binds to an FGFR1c-α-Klotho complex leading to receptor heterodimerization and activation of the intracellular FGFR-tyrosine kinase domain [6, 56]. This event, in turn, primes the phosphorylation of FGFR substrate 2α (FRS2α) and downstream activation of extracellular signal-regulated kinases 1/2 (ERK1/2) and serum/glucocorticoid-regulated kinase-1 (SGK1) [52, 56, 61]. Subsequently, SGK1-mediated phosphorylation of $Na^+/H^+$ exchange regulatory cofactor-1 (NHERF-1) leads to internalization and degradation of brush border membrane residing $Na^+$-coupled phosphate transporters NaPi-IIa/c [51, 61]. Reduced apical membrane abundance of especially NaPi-IIa, the key transporter mediating renal phosphate reabsorption, results in increased renal phosphate excretion [33, 49]. Thus, FGF23 consequently lowers serum phosphate levels by fostering phosphaturia as confirmed in various transgenic mouse models (Figure 2) [48–51].

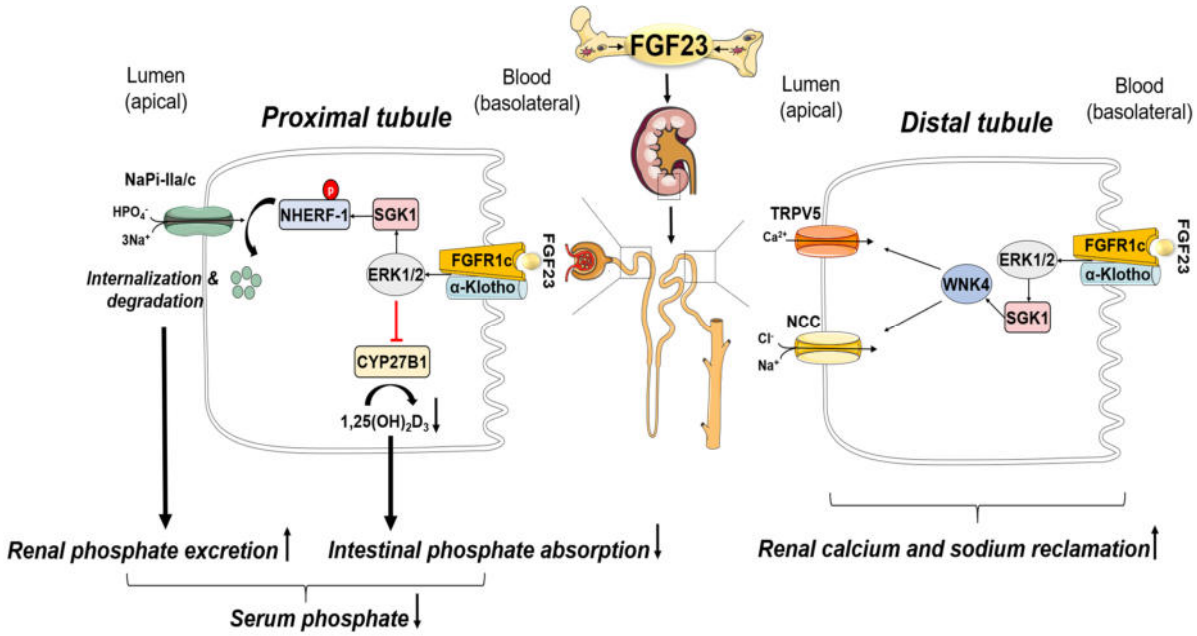


**Figure 2. Renal effects of FGF23.** See text for details of renal actions of FGF23 and its impact on phosphate, $Ca^{2+}$-, and $Na^+$ homeostasis. Figure is adapted and modified according to [33, 62, 63] and was created with images adapted and modified from Servier Medical Art by Servier licensed under a Creative Commons Attribution 3.0 Unported License (https://smart.servier.com/; https://creativecommons.org/licenses/by/3.0/legalcode).

In addition to its phosphaturic properties, it is well documented that FGF23 acts as a counter-regulator of vitamin D [6, 64]. In fact, FGF23 suppresses the rate-limiting step in calcitriol formation by ERK1/2-dependent inhibition of proximal tubular 25-hydroxyvitamin D-1α-hydroxylase (*Cyp27b1*) expression (Figure 2) [33, 47, 48, 65, 66]. Concomitantly, FGF23 upregulates the expression of proximal tubular 1,25-dihydroxyvitamin $D_3$ 24-hydroxylase (*Cyp24a1*), whose gene product facilitates calcitriol degradation, subsequently leading to reduced circulating calcitriol levels [34]. Calcitriol is reported to stimulate intestinal phosphate absorption by increasing the brush-border membrane abundance of NaPi-IIb [67]. Since

FGF23 antagonizes renal calcitriol formation, it is thus assumed that FGF23 indirectly decreases NaPi-IIb-mediated phosphate absorption in the small intestine, thereby underlining the significance of FGF23 as a serum phosphate lowering hormone [68, 69]. Given that calcitriol stimulates intestinal absorption and renal conservation of calcium ($Ca^{2+}$) and phosphate to promote bone mineralization [64, 70, 71], FGF23-elicited phosphaturia and inhibition of calcitriol formation counteracts ectopic tissue calcifications that would occur due to imbalances of serum $Ca^{2+}$ and phosphate concentrations [48, 64]. Accordingly, FGF23-deficient mice exhibit hyperphosphatemia, hypercalcemia, exaggerated elevations of serum calcitriol along with renal vascular calcifications, severe defects in bone mineralization, and a drastically shortened life span [48]. Interestingly, the phenotypic features of FGF23-null mice are similar to the pathologic derangements resembling human age-related disorders as observed in the Klotho-deficient mouse strain [48, 53]. Consequently, dietary calcitriol or phosphate restrictions are reported to prolong survival and alleviate the aforementioned ageing-like phenotypes in both mouse strains [72–74].

In addition, there is solid evidence from *in vitro* and *in vivo* studies that bone-derived FGF23 expression is strikingly upregulated by calcitriol, indicating a negative feedback loop controlling circulating calcitriol levels by inducing FGF23 [75, 76]. In osteoblasts, calcitriol thus interacts with the vitamin D receptor (VDR) which heterodimerizes with its co-receptor protein retinoid X receptor (RXR) leading to nuclear translocation and subsequent binding to vitamin D-responsive consensus sites in the *Fgf23* promoter, thereby inducing transcriptional activation of *Fgf23* [56, 77].

Besides its actions on the proximal tubule, FGF23 also targets the distal tubular epithelium by serving as a regulator of renal $Ca^{2+}$ and $Na^{+}$ reclamation as shown in Figure 2 [66]. Albeit its indirect calciuretic actions *via* suppression of calcitriol synthesis [34], FGF23 is reported to stimulate renal $Ca^{2+}$ conservation in the distal tubule *via* upregulation of apical membrane abundance of the epithelial $Ca^{2+}$ channel transient receptor potential vanilloid 5 (TRPV5) involving ERK1/2-SGK1-with-no-lysine kinase-4 (WNK4) signaling [78]. Furthermore, Andrukhova *et al.* (2014) revealed that injection of recombinant FGF23 to wild-type mice promotes distal tubular $Na^{+}$ reabsorption and apical membrane expression of the $Na^{+}$-chloride ($Cl^{-}$) cotransporter NCC *via* induction of the ERK1/2-SGK1-WNK4 signaling cascade [79]. Conversely, FGF23- and Klotho-deficient mice display renal $Na^{+}$ wasting due to diminished apical membrane abundance of NCC in the distal tubule [79]. Targeting the kidney, these data support the notion that FGF23 acts not only as a phosphaturic endocrine factor, but also serves as a $Ca^{2+}$- and $Na^{+}$-conserving hormone, thereby underlining its physiological significance in maintaining serum phosphate, $Ca^{2+}$, and $Na^{+}$ concentrations in narrow ranges [33, 78, 79].

### 1.1.3 Extrarenal actions of FGF23

Besides its key functions on the kidney, FGF23 has emerged as a humoral factor exerting pleiotropic effects on various extrarenal tissues and cell types including the parathyroid glands [35], heart [80], liver [81], immune cells [82, 83] or the bone itself as the primary source of circulating FGF23 (Figure 3) [84].

Considering the regulation of phosphate homeostasis, FGF23 functions as a key player in the endocrine network between bone, kidney, and parathyroid glands [6, 47]. Similar to FGF23, the parathyroid gland-derived parathyroid hormone (PTH) targets the renal proximal tubule and inhibits phosphate reabsorption by decreasing the apical membrane abundance of NaPi-IIa/c [85, 86]. PTH has further been shown to act as a powerful stimulator of *Fgf23* gene expression in osteoblasts and osteocytes preferably *via* activation of the nuclear orphan receptor nuclear receptor-associated protein1 (Nurr1) [87, 88]. Reciprocally, FGF23 targets the parythyroid glands and inhibits PTH synthesis and secretion in a FGFR1/α-Klotho-dependent manner involving mitogen-activated protein kinase (MAPK)-ERK1/2 signaling, thereby ensuring a negative feedback loop regulating circulating PTH levels by FGF23 induction [6, 35, 47, 89, 90]. Interestingly, FGF23 has also been shown to inhibit PTH secretion in the absence of α-Klotho by alternative induction of the calcineurin/nuclear factor of activated T cells (NFAT) signaling pathway (Figure 3) [91]. It is well established that PTH is secreted in response to hypocalcemia associated with calcitriol deficiency [92]. As a consequence, PTH stimulates calcitriol synthesis *via CYP27B1* induction to counteract hypocalcemia [93]. In addition to the direct inhibition of calcitriol synthesis [33, 65] as described above, FGF23 indirectly ensures its inhibitory effect on calcitriol formation by downregulation of PTH synthesis [35], thereby underlining the importance of FGF23 as a key player in the regulation of $Ca^{2+}$ and phosphate homeostasis. However, contrary to the postulated inhibitory actions of FGF23 on PTH formation and secretion there are conflicting data suggesting a rather stimulatory effect of FGF23 on circulating PTH levels especially in clinical settings [94–101]. In this regard, patients with hyperparathyroidism (HPT) display elevations in serum FGF23 along with exaggerated PTH levels in the blood [94–97]. Similarly, serum concentrations of both FGF23 and PTH are elevated in chronic kidney disease (CKD) [98–101]. Several reasons may account for these discrepancies: First, evidence from both animal models and human studies indicates a downregulation of the α-Klotho-FGFR1 receptor complex in the parathyroid glands of advanced CKD, thus potentially explaining the reduced ability of FGF23 to suppress PTH overproduction in CKD-associated HPT [69, 102, 103]. Secondly, a large portion of elevated PTH undergoes inactivation due to increased oxidative stress-related processes in pathologies such as CKD [69, 104, 105]. However, commonly used test kits do not distinguish between intact and oxidized PTH fragments with reduced biologic action, thus limiting the assumption

of proposed stimulatory actions of FGF23 on circulating active PTH levels [69, 104]. In addition, elevated FGF23 levels are paralleled by low calcitriol levels and hypocalcemia in CKD, which, in turn, favor secondary HPT in CKD [106, 107]. The underlying role of FGF23 in the regulation of PTH is thus difficult to discern from rather complex interactions causing pathologic derangements of phosphate homeostasis in CKD [56, 69, 106].

Targeting the heart, cardiac actions of FGF23 are implicated in pathological conditions such as left ventricular hypertrophy (LVH) and myocardial fibrosis representing major cardiovascular complications in patients with CKD (see section 1.2.2) [47, 108]. Thus, FGF23 is reported to induce hypertrophic remodeling of cardiomyocytes in an α-Klotho-independent manner [80]. According to crucial findings by Faul *et al.* (2011) and Grabner *et al.* (2015) using isolated neonatal rat ventricular cardiomyocytes, FGF23 mediates its pro-hypertrophic effects *via* FGFR4 and downstream activation of the phospholipase Cγ (PLCγ)-calcineurin-NFAT signaling pathway (Figure 3), thereby upregulating the gene expression of pro-hypertrophic markers of pathological LVH including atrial and brain natriuretic peptide (*ANP, BNP*) [80, 109]. Notably, there is evidence that FGF23 is expressed by cardiomyocytes [38, 110]. Moreover, Leifheit-Nestler and colleagues (2016) observed excessively high myocardial *FGF23* expression in CKD patients with diagnosed LVH [38]. These findings support the notion that not only circulating but also locally produced FGF23 may contribute to cardiac hypertrophic remodeling and myocardial fibrosis in an autocrine and/or paracrine manner [66, 108].

Current data suggest that FGF23 also acts on the liver (Figure 3) [81, 111]. According to Mattinzoli *et al.* (2018), FGF23 stimulates the gene expression of pro-inflammatory cytokines including *tumor necrosis factor alpha (TNFα)* and *interleukin (IL)-6* in cultured hepatocytes [111]. Moreover, Singh and coworkers (2016) demonstrated that FGF23 promotes hepatic expression and secretion of pro-inflammatory mediators such as C-reactive protein (CRP) and IL-6 in primary mouse hepatocytes and in wild-type mice in the absence of α-Klotho [81]. In detail, both *in vitro* and *in vivo* approaches confirmed that the activation of FGFR4-mediated PLCγ-calcineurin-NFAT signaling is crucial for the pro-inflammatory and α-Klotho-independent actions of FGF23 on the liver [81]. It is important to note that also immune cells, specifically leukocytes, respond to FGF23 [47]. In detail, FGF23 induces TNFα formation in different macrophage populations (Figure 3) [83, 112]. Since hepatic tissue and also cultures of primary hepatocytes contain specialized resident phagocytic macrophages, designated as Kupffer cells capable to secrete TNFα and IL-6 [47, 113], it can be speculated that FGF23 may affect TNFα and/or IL-6 synthesis in Kupffer cells, thereby, at least to some extent, potentially explaining the pro-inflammatory effects of FGF23 in primary hepatocytes as observed in the study of Singh *et al.* (2016) [81].

As outlined above, FGF23 serves as a central regulator of $Ca^{2+}$ and phosphate homeostasis and thus indirectly affects skeletal mineralization by controlling calcitriol and PTH

synthesis [6, 35, 65]. Interestingly, recent data indicate that the bone is not only the predominant source of circulating FGF23, but may also respond to local FGF23 production *via* autocrine and/or paracrine mechanisms indicating that FGF23 is directly involved in the regulation of bone mineralization (Figure 3) [62, 66]. A crucial step during skeletal mineralization is the hydrolysis of the mineralization-inhibiting molecule pyrophosphate, yielding inorganic phosphate mediated by the ecto-enzyme tissue non-specific alkaline phosphatase (TNAP) [62, 84]. According to Murali *et al.* (2016), FGF23 may exert an inhibitory role on skeletal mineralization as it is reported to suppress *Tnap* gene expression in murine osteoblasts independently of α-Klotho and concomitantly decreases the inorganic phosphate concentration in the cell culture supernatant [84]. In line with this finding, FGF23-deficient mice show increased femur *Tnap* mRNA expression levels, indicating a regulatory function of FGF23 on bone mineralization also *in vivo* [84].

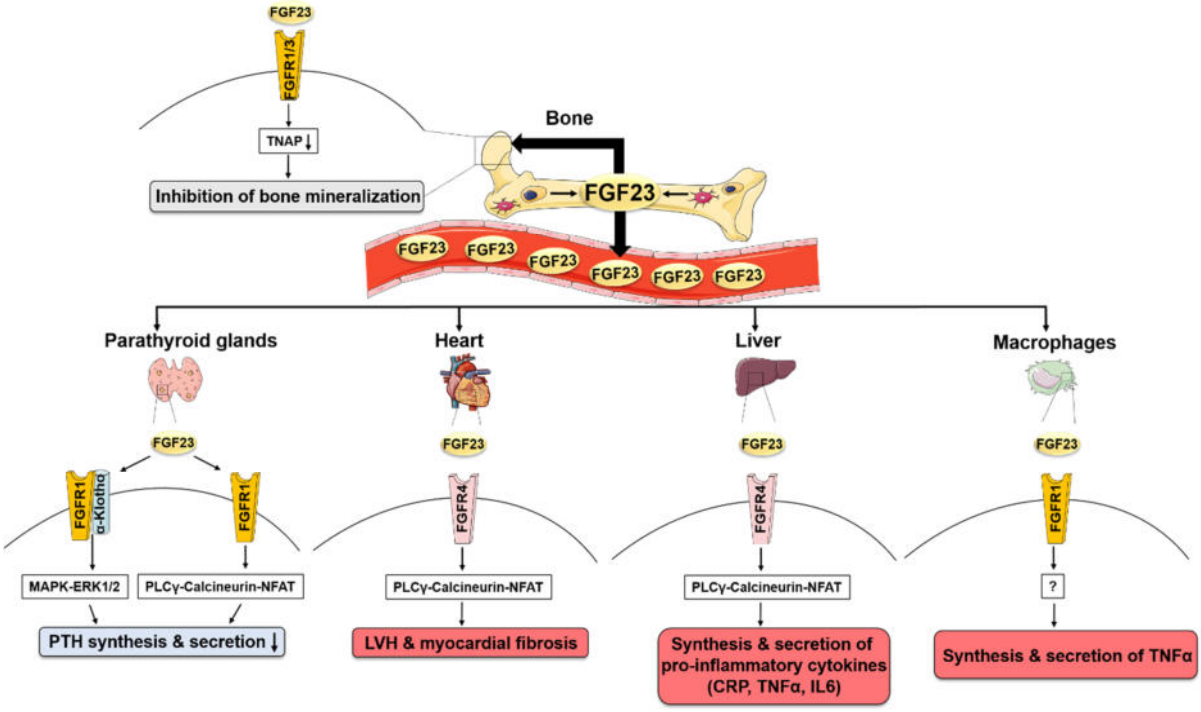


**Figure 3. Extrarenal effects of FGF23.** See text for details of FGF23 actions on various extrarenal tissues and cell types. Figure is adapted and modified according to [47, 62, 91, 114] and was created with images adapted and modified from Servier Medical Art by Servier licensed under a Creative Commons Attribution 3.0 Unported License (https://smart.servier.com/; https://creativecommons.org/licenses/by/3.0/legalcode).

## 1.2 Pathophysiological implications of FGF23

### 1.2.1 FGF23 and genetic disorders of phosphate metabolism

Upon its discovery, FGF23 has been implicated in various diseases and pathological conditions primarily defined by perturbations in phosphate homeostasis and bone metabolism

[5, 56]. Primary FGF23-related hereditary diseases are either characterized by FGF23 hyperfunction due to excess production and/or increased biological activity or deficiency of serum FGF23, respectively [56, 63, 115]. Furthermore, elevated serum FGF23 levels and concomitant renal phosphate wasting as observed in primary non-hereditary disorders are either acquired due to mesenchymal tumoral overproduction (as seen in TIO) [42] or exaggerated bone remodeling (as seen in hypophosphatemic linear nevus sebaceous syndrome) [6, 116]. Moreover, secondary excess in circulating FGF23 levels is observed in various renal diseases including CKD [100, 101, 117–119] (as addressed in section 1.2.2), autosomal dominant polycystic kidney disease (ADPKD) [120, 121], and acute kidney injury (AKI) [39, 122, 123], but is also implicated in hepatic [124, 125] and pulmonary diseases [69, 126, 127]. Furthermore, elevated serum FGF23 levels are nowadays considered as a clinical biomarker for the severity of various scenarios of acute and chronic illness including inflammatory and cardiovascular diseases [115].

Gain-of-function missense mutations in the human *FGF23* gene leading to resistance to proteolytic inactivation of FGF23 are the causative factor for excess serum FGF23 concentrations in ADHR [41, 56, 63, 69]. Concomitantly, patients with ADHR display hypophosphatemia, low serum levels of calcitriol, and osteomalacia [46, 128].

Elevations in serum FGF23 found in X-linked hypophosphatemia (XLH) are caused by inactivating mutations in the gene encoding PHEX *(phosphate regulating gene with homologies to endopeptidases on the X chromosome)* [56, 63, 129]. PHEX is a cell-surface-bound zinc metalloproteinase primarily expressed in osteoblasts and osteocytes in bone, but also found in teeth, muscles, lungs, and ovaries [63, 130, 131]. PHEX was initially suggested to cleave FGF23, thus assuming that PHEX is a systemic regulator of circulating levels of active FGF23 [6, 132]. However, instead of cleaving FGF23, PHEX rather appears to control *Fgf23* gene expression as it has been shown that *Hyp* mice – a *Phex*-deficient mouse homologue of XLH – display upregulated *Fgf23* mRNA transcript levels in bone [37, 133]. Nonetheless, it is still incompletely understood how loss-of-function mutations of *PHEX* are translated into increased serum FGF23 levels and the consequential clinical features of XLH including hypophosphatemic rickets, renal phosphate wasting, and calcitriol deficiency [56, 63, 133, 134].

Autosomal recessive hypophosphatemic rickets (ARHR) 1 is another hereditary disorder characterized by primary excess of serum FGF23 levels due to homozygous loss-of-function mutations in the *dentin matrix acidic phosphoprotein-1* (*DMP1*) gene [6, 135, 136]. DMP1 is primarily expressed in bone cells (osteoblasts/osteocytes) and implicated to interact with PHEX *via* an acidic serine aspartate-rich matrix extracellular phosphoglycoprotein (MEPE)-associated motif (ASARM), thereby reducing osseous *Fgf23* expression [6, 136, 137]. Whilst the exact molecular pathomechanisms underlying the clinical picture of ARHR1 remain

to be elucidated [56, 62], the ablation of *Dmp1* in *Dmp1*-null mice has been shown to result in increased bone expression of *Fgf23* accompanied by phenotypic aberrations resembling those in XLH [135, 138].

In addition, the monogenic hypophosphatemic disorder ARHR2 is caused by loss-of-function mutations in the gene encoding ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (ENPP1) [43, 139, 140]. ENPP1 is a transmembrane glycoprotein implicated in the generation of extracellular pyrophosphate, a known inhibitor of hydroxyapatite crystal deposition and bone calcification, respectively [69, 140, 141]. Hence, inactivating mutations in *ENPP1* result in low pyrophosphate levels and aberrant arterial calcifications [142], whereas elevated FGF23 levels in ARHR2 are accompanied with ossification of posterior longitudinal ligament [143]. Of note, a pathophysiological interrelation between extracellular matrix pyrophosphate and increased *Fgf23* bone expression has also been suggested in the context of a mouse model for craniometaphysial dysplasia carrying a mutation in the gene encoding the pyrophosphate transporter progressive ankylosis protein homologue (ANKH) [143, 144].

In contrast to the aforementioned hypophosphatemic diseases caused by elevated serum FGF23 levels, patients with hyperphosphatemic familial tumoral calcinosis (HFTC) display a primary deficiency of intact FGF23 in the serum [43, 56, 145]. It is reported that both loss-of-function mutations in the gene encoding UDP-*N*-acetyl-*α*-D-galactosamine-polypeptide *N*-acetylgalactosaminyltransferase 3 (GALNT3) or in the *FGF23* gene are causative factors for HFTC [145–148]. GALNT3 mediates post-translational *O*-glycosylation to FGF23 at threonine$^{178}$ located in the SPC recognition sequence motif, thereby stabilizing and protecting intact FGF23 from proteolytic degradation by furin-like proteases [47, 56, 149]. Inactivating *GALNT3* mutations consequently result in increased intracellular proteolysis of full-length FGF23 and subsequent secretion of inactive C-terminal FGF23 fragments. This, in turn, fosters hyperphosphatemia, elevations of serum calcitriol levels, and concomitant vascular and ectopic calcifications [145, 146, 149].

### 1.2.2 FGF23 and chronic kidney disease (CKD)

CKD is considered as a global epidemic health burden affecting millions of patients worldwide [150, 151]. The disease is characterized by progressive decline of kidney function as defined by proteinuria and decreased glomerular filtration rate (GFR) (as classified into five stages) due to chronical loss of functional nephron number caused by multiple factors including hypertension, diabetes, obesity, and other primary renal disorders [5, 56, 92, 150–154]. CKD is associated with poor clinical outcome as patients exhibit a markedly increased risk for end-stage renal disease (ESRD) with the necessity of renal replacement therapy [150]. Moreover, CKD is a well-known risk factor for cardiovascular morbidities such as LVH and

arteriosclerosis, which contribute to the substantially elevated mortality in CKD patients [118, 155–158].

Accumulating evidence from clinical trials suggests that a surge in the serum FGF23 concentration is the earliest detectable biomarker of deteriorating kidney function in CKD [5, 92, 99–101, 119, 159]. In order to maintain phosphate homeostasis, FGF23 secretion is increased prior to the onset of hyperphosphatemia and secondary HPT as an adaptive response to decreased nephron number to ensure renal phosphate elimination [5, 64, 92, 100, 160]. As FGF23 levels dramatically rise up to 1000-fold with disease progression, active vitamin D levels decrease whereas PTH levels concomitantly rise, eventually leading to hyperphosphatemia and secondary HPT [56, 100, 115, 152, 161]. PTH overproduction further stimulates excessive FGF23 formation and accentuates calcitriol deficiency in a vicious feed-forward loop [56, 162–164]. Low calcitriol levels and the effort to maintain phosphate balance by increasing PTH- and FGF23-mediated phosphaturia aggravates kidney damage and involves suppression of renal α-Klotho expression, which, in turn, accounts for renal resistance to FGF23 and its downstream phosphaturic signaling [5, 56, 152, 165, 166]. Additionally, CKD patients are further reported to exhibit reduced parathyroid α-Klotho and FGFR1 expression [167], indicating parathyroid resistance to FGF23 which may explain why PTH levels remain elevated in secondary HPT and CKD despite severely high serum FGF23 concentrations [102, 103, 162, 168]. Ultimately, impaired kidney function in CKD results in progressive renal failure as the increased demand for urinary phosphate elimination exceeds the excretory capacity of remaining functional nephron mass [56, 106].

According to a multitude of clinical observations, an incremented circulating FGF23 level may not only serve as a sensitive biomarker for CKD progression [100, 101, 119, 163], but is also implicated as a prognostic marker of increased mortality in dialysis-requiring CKD patients [152, 156, 159, 169]. Moreover, FGF23 is suggested as a disease indicator for CKD-related comorbidities including cardiovascular, inflammatory, and metabolic disorders [115, 152, 170]. In fact, compelling evidence from *in vitro* approaches, animal studies, and clinical observations indicates that FGF23 induces pathologic cardiac remodeling, including LVH and myocardial fibrosis (see section 1.1.3 for details), which are important mechanisms in the pathogenesis of cardiovascular disease, a characteristic sequela of CKD [38, 47, 80, 108, 109, 118, 155]. Furthermore, current studies found positive associations between elevated systemic FGF23 levels, body fat mass, adverse lipid metabolism, and obesity [115, 171–173], a hallmark of the metabolic syndrome contributing to increased cardiovascular risk and CKD development [153, 172, 174]. Given its emerging role as a putative biomarker for declining renal function in CKD and cardiovascular risk [108, 152], it is of current scientific need to provide more detailed insights into the regulatory mechanisms of FGF23 production and its implications in health and pathophysiological processes.

## 1.3 Regulation of FGF23 production

### 1.3.1 Systemic and local regulators of FGF23 synthesis

Within the scope of its central function as a phosphaturic hormone and its relevance in several pathologic conditions, the regulation of FGF23 production has been a subject of ongoing research since its discovery in the early 21st century [175, 176]. FGF23 formation is controlled by a coordinated network of systemic and local bone-derived factors involving regulatory mechanisms of *FGF23/Fgf23* gene transcription as well as post-translational modifications and processing [47, 63, 177].

With regard to its crucial role in $Ca^{2+}$ and phosphate homeostasis, systemic regulators of FGF23 have been extensively examined in *in vitro* settings, animal studies, and clinical trials [48, 49, 75–77, 100, 161–163]. As delineated in more detail in the previous sections, FGF23 release from bone is classically stimulated by high serum phosphate levels [177, 178], calcitriol [75, 76], extracellular $Ca^{2+}$ [179–181], and PTH [64, 87, 88] involving complex endocrine feedback loops as depicted in Figure 4.

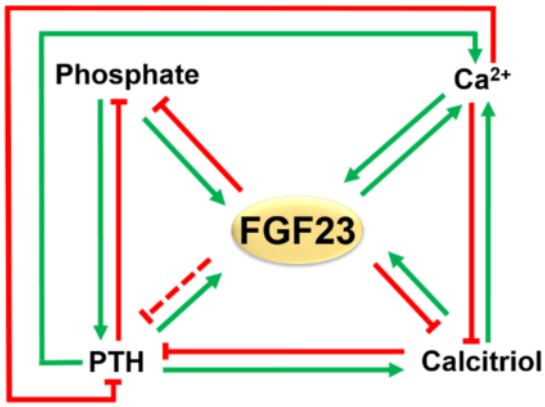


**Figure 4. Systemic regulators of FGF23.** See text for details of FGF23 as a pivotal regulator of $Ca^{2+}$ and phosphate homeostasis and the involvement of multifaceted endocrine feedback loops. Green lines indicate stimulatory effects, red lines correspond to inhibitory effects. The dashed line refers to rather conflicting data regarding the effect of FGF23 on PTH secretion especially in clinical settings of HPT and CKD. Figure is adapted and modified according to [35, 63, 69, 75].

Although extracellular phosphate is reported to upregulate *FGF23* mRNA expression in human osteoblast-like bone cells [182], available data regarding the physiological regulation of FGF23 secretion in response to dietary phosphate changes remain inconsistent [6]. In particular, dietary phosphate loading has been shown to increase FGF23 synthesis, whereas phosphate depletion appears to lower circulating FGF23 levels in healthy subjects [183–185]. In contrast, others demonstrated only modest changes or even no rapid postprandial regulation of FGF23 after oral phosphate loading or deprivation [99, 186]. Those differing findings may result from distinct study designs concerning the magnitude or duration of oral phosphate

intervention and whether other nutrients (such as $Ca^{2+}$) were modified in the study setting [183, 185]. It is thus difficult to discriminate the effects of oral phosphate intake from putatively more determinant factors for FGF23 formation such as calcitriol and PTH [6, 177]. Of note, soluble α-Klotho, the cleavage product of membrane-bound α-Klotho, has also been implicated as a systemic stimulus for FGF23 production as it increases circulating FGF23 levels and elicits hypophosphatemia in mice [187]. Conversely, Bär *et al.* (2018), recently identified insulin and insulin-like growth factor 1 (IGF1) as systemic suppressors of FGF23 production, presumably *via* activation of the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB)/Akt signaling pathway and subsequent inhibition of the transcription factor forkhead box protein O1 (FOXO1) [188].

Referring to section 1.2.1, the study of FGF23-related genetic diseases provides molecular insight into the transcriptional and post-translational regulation of FGF23 [6, 63, 69]. In particular, *FGF23/Fgf23* gene transcription is inhibited by the proteins PHEX [37, 133], DMP1 [135–138], ENPP1 [139, 140], and ANKH [144]. Consequently, loss-of-function mutations in the genes encoding these proteins lead to hypophosphatemic rickets, incremented FGF23 levels, renal phosphate wasting, and calcitriol deficiency [136, 189].

Besides the transcriptional control of *FGF23/Fgf23* gene expression, FGF23 homeostasis is regulated by post-translational processes [47]. Briefly, GALNT3-mediated *O*-glycosylation at threonine[178] facilitates FGF23 stabilization and ensures proteolytic protection of intact FGF23 [47, 56, 149]. Vice versa, phosphorylation at serine[180] by the serine/threonine protein kinase family with sequence similarity-20 member C (FAM20C) impedes *O*-glycosylation and thus promotes proteolytic cleavage and inactivation of FGF23 by furin-like proteases [189–191]. A regulated balance between *FGF23/Fgf23* transcription, post-translational modifications, and cleavage is crucial as deleterious mutations in *GALNT3* result in hyperphosphatemia owing to low intact FGF23 levels as seen in HFTC [145, 146], whereas *FAM20C* mutations are causative for aberrant elevations of full-length biologically active FGF23 as apparent in the hypophosphatemic disease ARHR3 [192]. Interestingly, Takashi *et al.* (2019) recently showed that the increase of serum intact FGF23 in high phosphate diet-fed mice is not attributed to inductions of bone *Fgf23* expression but of reduced proteolytic degradation of biologically active FGF23 according to enhanced femoral *Galnt3* expression [193]. Moreover, *in vitro* approaches using IDG-SW3 osteocyte-like cells revealed that calcitriol administered in the presence of physiological phosphate concentrations induces *Fgf23* and *Galnt3* gene expression, but also increases the mRNA expression of negative regulators of *Fgf23* transcription, namely *Dmp1*, *Phex,* and *Enpp1* [194]. These findings demonstrate the complex interdependent interactions between local and systemic regulators involved in the transcriptional and post-translational control of FGF23 homeostasis and associated negative feedback loops [69, 194]. In respect of distinct pathologic conditions causing either alterations

in *FGF23* transcription and/or FGF23 cleavage, it is thus of higher significance to better assess the ratio of intact:C-terminal FGF23 fragments than detecting only one FGF23 form by using either a C-terminal or intact FGF23 assay [189].

During the last years, a growing number of other determinants of FGF23 regulation have been identified. In particular, recent publications provide evidence that components of the renin-angiotensin-aldosterone system, namely angiotensin II and aldosterone, lead to the induction of *Fgf23* mRNA expression in cardiac myocytes [195], whereas aldosterone also induces *Fgf23* transcription in osteoblasts [196]. Moreover, iron deficiency [197, 198], hypoxia *via* activation of the transcription factor hypoxia-inducible factor-1α (HIF-1α) [199], erythropoietin (EPO) [200, 201], circadian profile-associated sympathetic activation [202], leptin [203, 204], cadmium [205, 206], and lithium [207, 208] have further been linked to increased FGF23 formation in bone.

### 1.3.2 Inflammation as a stimulator of FGF23 formation

Evidence from numerous clinical and epidemiological studies indicates strong positive associations between elevated serum FGF23 concentrations and increased levels of systemic markers of inflammation such as CRP, IL-6, and TNFα in several inflammatory diseases including CKD [209, 210], inflammatory bowel diseases [211], and rheumatoid arthritis [212]. In this regard, a wide range of inflammation-related mediators including the pro-inflammatory cytokines TNFα, IL-6, IL-1β, bacterial lipopolysaccharide (LPS), and advanced glycation end products (AGEs) have been shown to be major triggers of osseous FGF23 synthesis and secretion *in vitro* and in mice models of acute inflammation and CKD [36, 197, 213, 214]. Vice versa, FGF23 itself induces pro-inflammatory cytokine production from hepatocytes, cardiomyocytes, and immune cells, thereby promoting a vicious cycle of amplifying systemic inflammation and exaggerated FGF23 formation as seen in the aforementioned pathologies [47, 81, 83, 175, 195]. Of note, FGF23-associated chronic inflammation has a broad clinical impact as it is a well-established determinant for the increased risk of cardiovascular morbidity and mortality in patients with CKD [47, 155, 215].

The underlying mechanisms by which inflammation regulates FGF23 synthesis are not fully understood, yet multiple reports suggest the involvement of complex interactions between systemic and local factors triggering FGF23 production [36, 69, 112, 216]. According to numerous studies utilizing different cell types, the induction of *Fgf23* gene expression in response to inflammatory stimuli, including TNFα and IL-1β, essentially requires the activation of nuclear factor-kappa B (NF-κB) [36, 112, 214], a pivotal transcription factor regulating the expression of a wide range of pro-inflammatory genes such as TNFα, IL-6, and IL-1β [215, 217, 218]. Interestingly, p38MAPK, a known inducer of NF-κB transcriptional activity [219], has

recently been demonstrated to participate in the stimulatory effect of NF-κB on FGF23 synthesis [220]. Ito *et al.* (2015) further showed that the increase in *Fgf23* mRNA expression mediated by TNFα and IL-1β is paralleled by concomitant suppression of negative transcriptional regulators of *Fgf23*, namely *Phex*, *Dmp1,* and *Enpp1* [36]. Moreover, calcineurin-NFAT signaling, which is also implicated in FGF23-driven LVH [108], has been demonstrated as another pathway linking inflammation to enhanced FGF23 formation [216, 221–223].

Inflammation is known to induce iron sequestration in the reticulo-endothelial system (e.g., in terms of infections), thereby eliciting functional iron deficiency [189]. Inflammation and related functional iron deficiency, both commonly occuring in CKD [189, 224], are suggested to increase *Fgf23* mRNA expression and concomitant secretion of C-terminal FGF23 *via* HIF-1α activation and subsequent upregulation of EPO [189, 197, 225].

### 1.3.3 The role of store-operated $Ca^{2+}$ entry (SOCE) in the regulation of FGF23

Emerging evidence from multiple investigations indicates a putative sensitivity of FGF23 formation not only to dietary/extracellular $Ca^{2+}$ [179–181] but also to intracellular $Ca^{2+}$ signaling [196, 207, 226, 227]. $Ca^{2+}$ acts as an ubiquitous intracellular messenger involved in the regulation of versatile cellular functions and signaling pathways including muscle contraction, exo- and endocytos, cell survival/death, and gene expression [228]. Thus, the intracellular $Ca^{2+}$ concentration has to be tightly controlled and is kept approximately $10^4$ times lower than in the extracellular milieu [228, 229]. Maintenance of low resting cytosolic $Ca^{2+}$ concentrations is ensured by extracellular directed $Ca^{2+}$ efflux accomplished by the plasma membrane located $Ca^{2+}$-adenosine triphosphate (ATP)ase and the $Na^+/Ca^{2+}$ exchanger (NCX) [228–230]. Additionally, $Ca^{2+}$ removal from the cytosol into intracellular stores, particularly the endoplasmic/sarcoplasmic reticulum (ER/SR) or lysosomes, is mediated by the sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) [228, 229, 231]. Numerous physiological agonists (e.g., histamine or thrombin in endothelial cells [232]) are capable to elicit receptor-mediated activation of PLCγ and subsequent generation of the second messenger molecules inositol-1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG) [231]. Binding of $IP_3$ to $IP_3$-receptor ($IP_3R$) channels located in the ER-membrane causes $Ca^{2+}$ release from the ER into the cytosol and leads to translocation of stromal interaction molecule 1 (STIM1), a single pass transmembrane protein sensing ER $Ca^{2+}$ depletion, to the plasma membrane where it activates Orai1, a key pore subunit of the $Ca^{2+}$ release-activated $Ca^{2+}$ (CRAC) channel [228, 231, 233, 234]. The resultant cytosolic $Ca^{2+}$ influx triggered upon intracellular $Ca^{2+}$ store depletion is referred to as store-operated $Ca^{2+}$ entry (SOCE) [228, 231]. In addition to CRAC channels

formed by Orai1 subunits, the cation permeable SOC channels consisting of Orai1 and transient receptor potential canonical 1 (TRPC1) proteins are also implicated in SOCE [229].

Zhang *et al.* (2016) were the first who identified SOCE as an essential mechanism in the regulation of FGF23 formation [226], as depicted in Figure 5. In particular, stimulation of SOCE mimicked by the $Ca^{2+}$ ionophore ionomycin was shown to enhance *Fgf23* transcript levels in rat UMR106 osteoblastic cells, an effect disrupted by pharmacological inhibition of SOCE and small interfering RNA (siRNA)-mediated gene silencing of *Orai1* [64, 226]. Zhang and colleagues (2016) further demonstrated that inhibition of the pro-inflammatory transcription factor NF-κB attenuates SOCE and concomitantly suppresses *Fgf23* gene expression [226], underlining a decisive stimulatory role of NF-κB in the regulation of FGF23 synthesis. Notably, the stimulatory effects of lithium [207], aldosterone [196], and transforming growth factor (TGF)-β2 [227] on FGF23 formation have also been shown to be mediated *via* Orai1-sensitive SOCE (Figure 5).

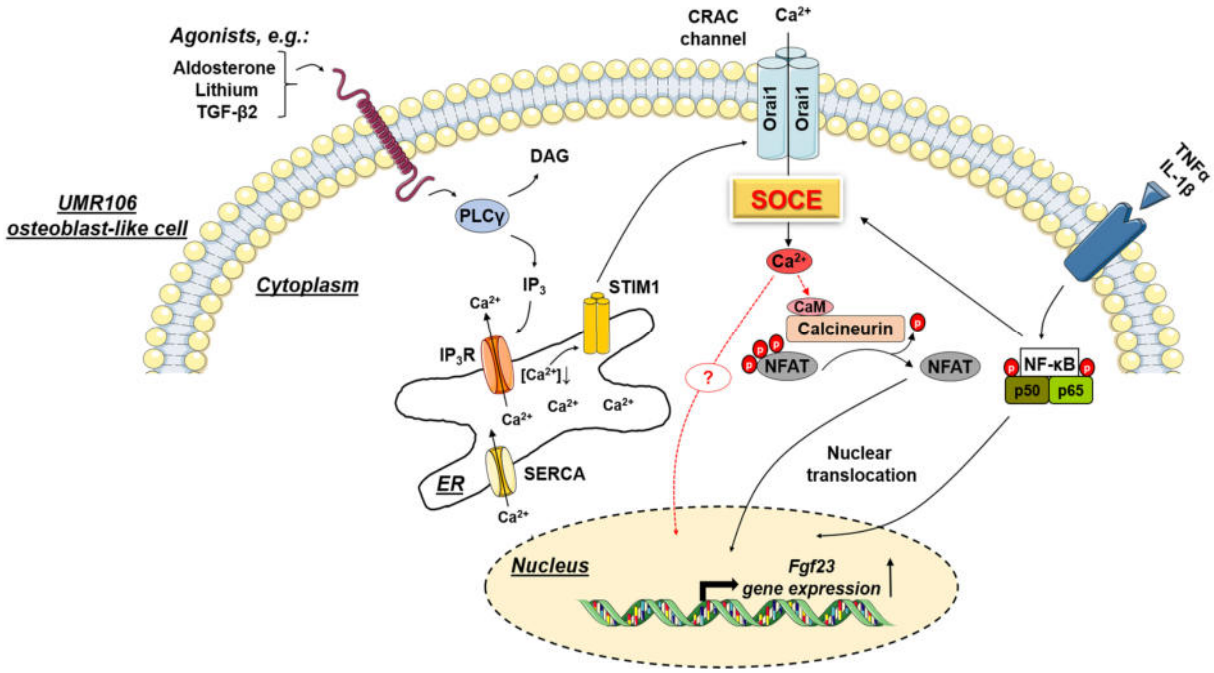


**Figure 5. Tentative scheme illustrating SOCE as a pivotal mechanism regulating *Fgf23* gene expression.** See text for details of the proposed role of SOCE in the transcriptional control of *Fgf23* and the putative involvement of the transcription factors NF-κB and NFAT. Figure is in accordance with [36, 196, 207, 221, 222, 226, 227, 229, 231, 235] and was created with images adapted and modified from Servier Medical Art by Servier licensed under a Creative Commons Attribution 3.0 Unported License (https://smart.servier.com/; https://creativecommons.org/licenses/by/3.0/legalcode).

Orai1-driven $Ca^{2+}$ entry in T lymphocytes triggers calmodulin (CaM)-mediated activation of calcineurin, which, in turn, dephosphorylates cytoplasmic NFAT, exposing its translocation into the nucleus where it functions as a transcription factor for genes involved in cytokine production or cell proliferation [235]. Interestingly, inhibition of the calcineurin-dependent activation of NFAT decreased *Fgf23* transcript levels and protein production in UMR106 cells, pointing to a stimulating effect of the calcineurin-NFAT pathway on FGF23

synthesis [221]. However, a comprehensive understanding of the detailed molecular mechanisms underlying the regulation of SOCE-mediated FGF23 formation remains to be defined. Regarding the significance of FGF23 not only as a major regulator of phosphate homeostasis but also being involved in various aforementioned pathologies, it is thus of scientific need to elucidate novel regulators of Orai1-mediated SOCE and its impact on the regulation of FGF23 production.

### 1.3.4 SOCE and AMP-activated protein kinase (AMPK)

The 5´-adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a heterotrimeric serine/threonine protein kinase expressed in all eukaryotes and consists of catalytic α (α1 or α2) and regulatory β (β1 or β2) and γ (γ1, γ2, or γ3) subunits [236, 237]. AMPK is considered as a key regulator of cellular energy status as it is activated in states of energy deprivation during metabolic stress by sensing increments in intracellular AMP/ATP and adenosine diphosphate (ADP)/ATP ratios [237, 238]. Induction of AMPK activity thereby serves to conserve cellular energy by stimulating ATP-providing catabolic pathways such as cellular glucose uptake, glycolysis, and fatty acid oxidation [236, 238–241], whereas biosynthetic anabolic processes including gluconeogenesis, protein-, fatty acid-, and glycogen synthesis are inhibited in response to AMPK activation [238, 242, 243].

As delineated in more detail in **Paper 3** (see section 3.3), canonical (nucleotide-dependent) activation of AMPK involves AMP or ADP binding to the γ subunit and subsequent liver kinase B1 (LKB1)-mediated phosphorylation of the α subunit's kinase domain at threonine$^{172}$ [237, 238, 244–246]. In addition, non-canonical (nucleotide-independent) regulation of AMPK activity comprises phosphorylation of threonine$^{172}$ by $Ca^{2+}$/calmodulin-dependent protein kinase kinase β (CaMKKβ) which is activated in response to increases in intracellular $Ca^{2+}$ levels caused by $Ca^{2+}$ release from intracellular stores [237, 238, 247]. Hence, this alternate $Ca^{2+}$-dependent pathway mediates transient activation of AMPK by hormonal stimuli [236–238] including thrombin in endothelial cells [248, 249] or ghrelin in neuronal cells [250]. Along those lines, AMPK appears to be a key player in a negative feedback mechanism regulating cytosolic $Ca^{2+}$ activity in endothelial cells [249]. In detail, Sundivakkam *et al.* (2013) showed that SOCE triggers CaMKKβ-mediated AMPK activation, which, in turn, results in phosphorylation of ER-localized STIM1 and subsequent inhibition of SOCE [249]. In view of the fact that Orai1-sensitive SOCE has been verified as an important mechanism in the regulation of FGF23 formation [196, 207, 226, 227], it has, however, not been explored so far whether AMPK potentially affects SOCE-dependent FGF23 production.

# 2 Objective of this study

Initially discovered as a causative factor for renal phosphate wasting in rare forms of hypophosphatemic rickets, FGF23 has emerged as a major regulator of phosphate homeostasis and vitamin D metabolism [47, 63, 175]. Over the last two decades, a myriad of experimental studies and clinical investigations yielded decisive insights into the versatile actions of FGF23 in health and disease settings, particularly in the pathogenesis of CKD and its related comorbidities including cardiovascular, inflammatory, and metabolic disorders [47, 115, 152, 175]. Despite extensive ongoing research, the current understanding of the complex network of endocrine feedback loops as well as the interaction of systemic and local factors involved in the regulation of FGF23 synthesis is, however, still incomplete. Therefore, this thesis aimed to elucidate novel regulators of FGF23 production and to identify the underlying cellular and molecular mechanisms involved. Characterizing hitherto unknown determinants of FGF23 synthesis will thus provide a more comprehensive understanding of FGF23 regulation, which may be important for the development of new therapeutic strategies targeting aberrated FGF23 production in pathologies such as CKD and associated diseases.

***Paper 1***

As outlined in the previous sections, inflammation, particularly the pro-inflammatory cytokine TNFα, is a major trigger of FGF23 formation and markedly important in the pathology of CKD [36, 209, 210]. Moreover, it is well established that the accumulation of excess body fat in obesity, owing to a sedentary life style and an immoderate intake of energy-dense diets rich in fats, is accompanied by chronic low-grade systemic inflammation [251, 252]. In this regard, the first study presented in this thesis sought to address the following question:

**I) What is the relevance of high-fat diet (HFD)-induced inflammation on FGF23 production?**

Among other pro-inflammatory cytokines, TNFα is a critical factor for the development of low-grade inflammation associated with obesity, a hallmark of the metabolic syndrome [251–254]. Accordingly, the role of TNFα in the context of HFD-mediated FGF23 formation was examined in an animal study. To this end, HFD feeding was performed in TNFα-deficient ($tnf^{-/-}$) and in age- and sex-matched wild-type ($tnf^{+/+}$) mice (C57BL/6 mouse strain). Serum and tissue specimen were subjected to subsequent analysis. Furthermore, rat UMR106 osteoblast-like cells, an osteosarcoma cell line endogenously expressing FGF23 [75], were used to assess the impact of TNFα on *Fgf23* gene expression *in vitro*.

***Paper 2***

Whilst **Paper 1** was concerned with the putative role of HFD-associated inflammation in FGF23 synthesis in conditions of nutritional energy excess, the purpose of **Paper 2** was to elaborate on the underlying mechanisms of FGF23 regulation in terms of energy deficiency as determined by studying the role of the intracellular energy sensor AMPK [236] in the regulation of FGF23 formation. To this end, the following question was addressed:

**II) What is the impact of AMPK in the regulation of FGF23 synthesis and what are the underlying mechanisms involved?**

AMPK is ubiquitously expressed in eukaryotic cells [236, 237], whereas FGF23 is mainly produced by osteoblasts and osteocytes [36, 75]. Hence, cell culture experiments were carried out in rat UMR106 osteoblast-like cells, whereas AMPK activation and/or inhibtion was attained by pharmacological manipulation or siRNA-mediated gene silencing. Given that Orai1-mediated SOCE is critical for regulating *Fgf23* gene expression [226], it was further explored whether AMPK participates in this signaling pathway. A next series of experiments aimed to explore the role of AMPK in the regulation of FGF23 in an animal model using AMPKα1-deficient ($ampk^{-/-}$) and sex- and age-matched wild-type ($ampk^{+/+}$) mice. Serum and tissue specimen as well as renal function parameters were characterized utilizing versatile analytical methods.

***Paper 3***

AMPK functions not only as a pivotal regulator of energy homeostasis being activated in low energy states [236], but is also implicated in kidney function and renal pathophysiology, particularly in CKD [255, 256]. Conceived as a systematic review, **Paper 3** comprehensively explored recent data regarding the mechanistic insights of AMPK-dependent regulation of renal tubular transport as emphasized on physiological and pathophysiological implications. Given that the kidney represents the major target organ of FGF23 and in light of a putative role of AMPK on FGF23 synthesis, the following question was subject for subsequent discussion:

**III) Is there a possible interrelation between AMPK and FGF23 concerning the homeostatic regulation of renal mineral handling?**

In this view, renal function parameters of $ampk^{-/-}$ and $ampk^{+/+}$ mice obtained from **Paper 2** were reconsidered in the discussion of this thesis.

# 3 Publications

## 3.1 Paper 1: A high-fat diet stimulates fibroblast growth factor 23 formation in mice through TNFα upregulation

**Glosse, P.**; Fajol, A.; Hirche, F.; Feger, M.; Voelkl, J.; Lang, F.; Stangl, G.I.; Föller, M. A high-fat diet stimulates fibroblast growth factor 23 formation in mice through TNFα upregulation. *Nutr Diabetes* **2018**, *8*, 36, DOI: 10.1038/s41387-018-0037-x.

Glosse et al. *Nutrition and Diabetes* (2018)8:36
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Nutrition & Diabetes

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# A high-fat diet stimulates fibroblast growth factor 23 formation in mice through TNFα upregulation

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## Abstract

**Background/objectives:** Bone-derived fibroblast growth factor 23 (FGF23) is a hormone that suppresses renal phosphate reabsorption and calcitriol (i.e., $1,25(OH)_2D_3$) formation together with its co-receptor Klotho. FGF23- or Klotho-deficient mice suffer from rapid aging with multiple age-associated diseases, at least in part due to massive calcification. FGF23 is considered as a disease biomarker since elevated plasma levels are observed early in patients with acute and chronic disorders including renal, cardiovascular, inflammatory, and metabolic diseases. An energy-dense diet, which induces sequelae of the metabolic syndrome in humans and mice at least in part by enhancing pro-inflammatory TNFα formation, has recently been demonstrated to stimulate FGF23 production.

**Methods:** We investigated the relevance of TNFα for high-fat diet (HFD)-induced FGF23 formation in wild-type ($tnf^{+/+}$) and TNFα-deficient ($tnf^{-/-}$) mice.

**Results:** Within 3 weeks, HFD feeding resulted in a strong increase in the serum FGF23 level in $tnf^{+/+}$ mice. Moreover, it caused low-grade inflammation as evident from a surge in hepatic *Tnfa* transcript levels. TNFα stimulated *Fgf23* transcription in UMR106 osteoblast-like cells. Serum FGF23 was significantly lower in $tnf^{-/-}$ mice compared to $tnf^{+/+}$ mice following HFD. Serum phosphate and calcitriol were not significantly affected by genotype or diet.

**Conclusions:** We show that HFD feeding is a powerful stimulator of murine FGF23 production through TNFα formation.

## Introduction

The hormone fibroblast growth factor 23 (FGF23) is mainly produced by osteoblasts and osteocytes in the bone[1]. Its renal effects include inhibition of phosphate reabsorption and calcitriol formation[1, 2]. Calcitriol is the biologically active form of vitamin D. The renal effects of FGF23 are mediated by a receptor which requires the protein α-Klotho (referred to as Klotho in the following) as an obligatory co-receptor[1].

Klotho was originally discovered in 1997 as an anti-aging protein[3–5]. Klotho-deficient mice have an extremely short life span of a few weeks only and exhibit many disorders associated with aging in humans[3]. FGF23-deficient mice have a similar phenotype[6]. Both mouse strains suffer from drastically elevated plasma levels of phosphate and calcitriol due to the primary renal effect of FGF23 and Klotho. Importantly, the premature aging of Klotho- or FGF23-deficient mice is also a direct or indirect consequence of the hyperphosphatemia of the

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mice since maintaining them on a low phosphate or low vitamin D diet normalizes their life span[7].

A high plasma FGF23 level has been found in patients with various acute and chronic disorders including renal (acute kidney injury, chronic kidney disease), cardiovascular (coronary heart disease, myocardial infarction, atrial fibrillation), inflammatory, and metabolic diseases[8]. The role of FGF23 in chronic kidney disease is established best: Plasma FGF23 is elevated before a marked decrease of glomerular filtration rate (GFR), and it exhibits a strong positive correlation with mortality, hypertrophy of the left ventricle, and disease progression[9]. Therefore, it is presently being considered as a valuable disease biomarker. However, it is yet incompletely understood whether and to which extent FGF23 contributes to pathophysiological processes rather than merely indicating them. At least, FGF23 has been shown to induce hypertrophy of the left ventricle independently of Klotho[10].

Recently, inflammation has been shown to be a major trigger of FGF23 formation[11–14]. In line with this, pro-inflammatory cytokines including TNFα induce FGF23 production[15].

Metabolic syndrome is characterized by hypertension, glucose intolerance, dyslipidemia, as well as obesity, and affects millions of patients world-wide and represents a significant health burden particularly in industrialized countries[16]. Although the complex pathophysiological processes have not yet been uncovered completely, it is clear that an imbalance between caloric needs and intake is the predominant factor. In mice, a diet rich in fats (high-fat diet (HFD)) induces metabolic syndrome[17–19]. Low-grade inflammation associated with metabolic syndrome is relevant especially for the development of glucose intolerance[20]. In this respect, pro-inflammatory cytokines derived from adipose tissue or the liver are a major source of inflammation in metabolic syndrome. Among those cytokines, TNFα has been found to play a predominant role[21]. Interestingly, an energy-dense diet has recently been demonstrated to upregulate the production of FGF23 in rats[22].

Here, we sought to define the role of metabolic syndrome-associated TNFα production in HFD-induced FGF23 formation.

## Materials and methods

### Animals and treatments

All animal experiments were conducted according to the German law for the welfare of animals and were approved by the authorities of the state of Saxony-Anhalt. Experiments were performed in TNFα-deficient ($tnf^{-/-}$) mice (from The Jackson Laboratory, Sulzfeld, Germany; Stock No: 005540; the generation and genotyping is available on the website of The Jackson laboratory) and in age- and sex-matched wild-type mice ($tnf^{+/+}$) fed a control diet (Ssniff, Soest, Germany; standard diet for maintenance V1534).

At the age of 8–10 weeks, the mice were fed a HFD containing 70% kcal from fat (Altromin, Lage, Germany; C1090-70) for 3 weeks, and the body weight was recorded weekly. The animals had free access to food and tap water. Serum was taken before and on the last day of the treatment. The exact number of mice and the number of replications is provided in the figure legends. For all animal experiments, no randomization was used, no blinding was done, and no statistical test was applied to estimate the sample size.

### Serum parameters

To obtain blood specimens, the animals were lightly anesthetized with ether, and blood was drawn into heparinized capillaries by puncturing the retro-orbital plexus. Since the entire procedure takes less than a minute, it is unlikely to have a significant impact on our study. Serum concentrations of intact FGF23 and calcitriol were determined by ELISA kits (Immutopics, San Clemente, CA, USA; IDS, Frankfurt am Main, Germany). Inorganic phosphate was measured by a photometric method (Biocon® Diagnostik, Vöhl/Marienhagen, Germany).

### Tissue collection and quantification of liver and adipose tissue Tnfα mRNA expression

For the determination of *Tnfα* mRNA abundance, total RNA was extracted from the liver and gonadal adipose tissue using the peqGold Trifast™ reagent (Peqlab, Erlangen, Germany) according to the manufacturer's protocol. The RNA integrity was assessed by agarose gel electrophoresis and the RNA purity by measurement of the optical density at 260 and 280 nm. Single-strand cDNA was synthesized from 1.2 µg of total RNA at 42 °C for 60 min by use of the RevertAid™ M-MuLV Reverse Transcriptase (MBI Fermentas, St. Leon-Rot, Germany) and oligo dT18 primers (Eurofins MWG Operon, Ebersberg, Germany). The mRNA expression level was determined by real-time polymerase chain reaction (RT-PCR) with the Rotor-Gene 6000 system (Corbett Research, Mortlake, Australia) using 2 µl cDNA templates, SYBR® Green I (Sigma-Aldrich, München, Germany), 1.25 U Taq DNA polymerase (Promega, Mannheim, Germany), 500 µM dNTP (Ares Bioscience, Köln, Germany), and 13.3 pmol of a primer pair specific for *Tnfα* (NM_013693.2; forward 5′-AGT CCG GGC AGG TCT ACT TT-3′, reverse 5′-GGT CAC TGT CCC AGC ATC TT-3′). The *Tnfα* expression was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*, forward 5′-AAC GAC CCC TTC ATT GAC-3′, reverse 5′-TCC ACG ACA TAC TCA GCA C-3′) (in liver) or *18S* (forward 5′-GGG AGC CTG AGA AAC GGC-3′, reverse

5′-GGG TCG GGA GTG GGT AAT TT-3′) (in adipose tissue) using the ΔΔCt method.

### Cell culture

Cell culture was performed as previously described[23]. Briefly, UMR106 rat osteosarcoma cells (ATCC, Manassas, VA, USA) were cultured in DMEM high glucose medium (Gibco, Grand Island, NY, USA) supplemented with 10% FCS (Gibco) and 100 U/ml penicillin/100 µg/ml streptomycin (Gibco) under standard culture conditions. After 24 h, the cells were treated with or without TNFα (Sigma-Aldrich) for different periods.

### Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from the cells using Trifast reagent (Peqlab) according to the manufacturer's instructions. Messenger RNA was transcribed with GoScript™ Reverse Transcription System (Promega) using 1.2 µg of total RNA and random primers. For qRT-PCR analysis, the final volume of the qRT-PCR reaction mixture was 20 µl and contained: 2 µl cDNA, 0.5–1 µM of a primer pair specific for rat *Fgf23* (forward 5′-TAGAGCCTATTCAGACACTTC-3′, reverse 5′-CATCA GGGCACTGTAGATAG-3′) or the housekeeping gene TATA box-binding protein (*Tbp*, forward 5′-ACTCCT GCCACACCAGCC-3′, reverse 5′-GGTCAAGTTTACA GCCAAGATTCA-3′), 10 µl GoTaq® qPCR Master Mix (Promega), and sterile water up to 20 µl. PCR conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 57 °C for 30 s, and 72 °C for 30 s. Quantitative RT-PCR was performed on a Rotor-Gene Q (QIAGEN, Hilden, Germany).

### Statistics

Data are provided as means ± SEM, *n* represents the number of independent experiments or number of mice per group, respectively. All data were tested for significance using the tests indicated in the figure legends. For serum FGF23, normal distribution was assumed. The data meet the assumptions of the respective tests. Variance was similar between the groups apart from the data in Fig. 2B and Fig. 4E. Therefore, Welch's correction was applied in these cases. Only results with $p < 0.05$ were considered statistically significant.

## Results

At the age of 8–10 weeks, we started to feed wild-type mice ($tnf^{+/+}$) a HFD ad libitum for 3 weeks. Similar to what has recently been demonstrated in rats[22], the HFD caused a strong increase (by almost four times) in the serum intact FGF23 level (Fig. 1).

HFD feeding and subsequent adipose tissue accumulation are associated with subclinical inflammation and the generation of the key pro-inflammatory cytokine TNFα.

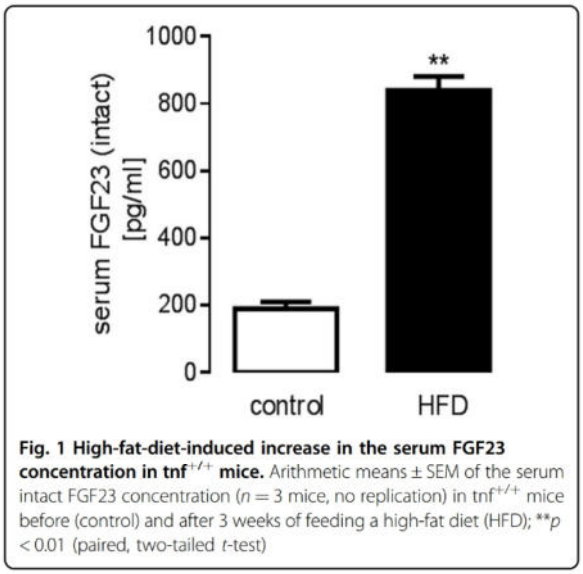


**Fig. 1 High-fat-diet-induced increase in the serum FGF23 concentration in $tnf^{+/+}$ mice.** Arithmetic means ± SEM of the serum intact FGF23 concentration ($n = 3$ mice, no replication) in $tnf^{+/+}$ mice before (control) and after 3 weeks of feeding a high-fat diet (HFD); $^{**}p < 0.01$ (paired, two-tailed *t*-test)

Hence, we found that feeding HFD indeed resulted in a significant increase in liver *Tnfα* mRNA expression levels (Fig. 2A) in $tnf^{+/+}$ mice pointing to HFD-associated low-grade inflammation. Moreover, also adipose tissue *Tnfα* mRNA expression levels (Fig. 2B) tended to be higher in $tnf^{+/+}$ mice on HFD, a difference, almost reaching statistical significance ($p = 0.106$).

Next, we carried out cell culture experiments with UMR106 osteoblast-like cells to test whether TNFα is capable of stimulating FGF23 production as has been shown for IDG-SW3 cells[15]. According to Fig. 3a, a 24 h incubation with TNFα resulted in a dose-dependent upregulation of *Fgf23* mRNA transcript levels in UMR106 cells with significance at 5 and 10 ng/ml TNFα. The time dependence for the effect of 5 ng/ml TNFα is illustrated in Fig. 3B.

Our last series of experiments explored whether the HFD-induced FGF23 production is dependent on TNFα formation. To this end, we compared $tnf^{+/+}$ mice with $tnf^{-/-}$ mice. On control diet, the serum intact FGF23 concentration was not significantly different between $tnf^{+/+}$ mice and $tnf^{-/-}$ mice (Fig. 4A). However, after 3 weeks of feeding the HFD, the serum intact FGF23 level was significantly different between the genotypes being nearly 50% lower in $tnf^{-/-}$ mice compared to $tnf^{+/+}$ mice (Fig. 4A). Serum calcitriol was not significantly different between $tnf^{-/-}$ and $tnf^{+/+}$ mice on either control or HFD, but was significantly lower in a group of HFD-fed mice compared to mice on control diet (Fig. 4B). Similarly, the serum phosphate concentration was not significantly affected by neither genotype nor diet (Fig. 4C). On control diet, no significant difference between the body weight of $tnf^{+/+}$ mice and $tnf^{-/-}$ mice could be observed (Fig. 4D).

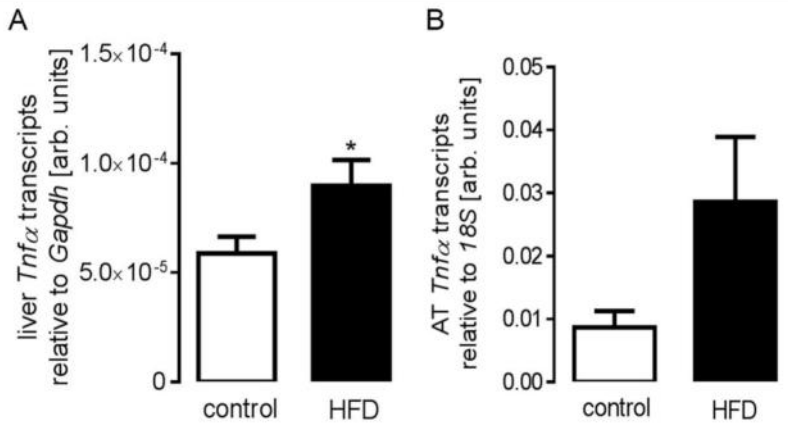


**Fig. 2 High-fat-diet-induced increase in liver *Tnfα* transcript levels in tnf$^{+/+}$ mice.** Arithmetic means ± SEM ($n = 7$ mice per group, one replication) of relative hepatic (**A**; unpaired, two-tailed *t*-test) and adipose tissue (AT). (**B**; unpaired, two-tailed *t*-test with Welch's correction) *Tnfa* mRNA abundance (relative to *Gapdh* or *18S* mRNA) in tnf$^{+/+}$ mice on control diet and on high-fat diet (HFD) for 3 weeks. *$p < 0.05$

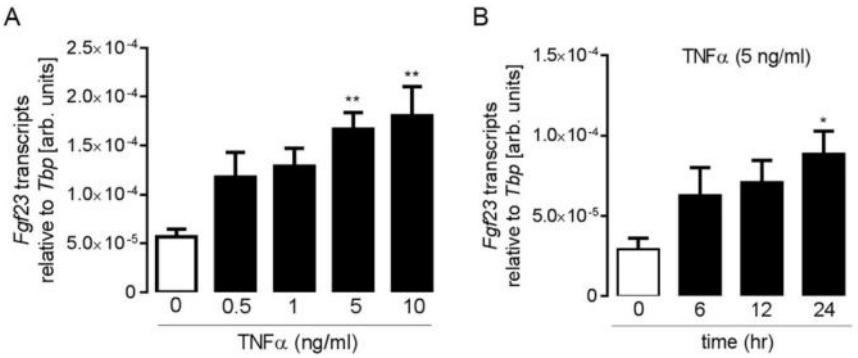


**Fig. 3 TNFα upregulated *Fgf23* transcripts in UMR106 cells.** Arithmetic means ± SEM of relative *Fgf23* mRNA abundance (relative to *Tbp* mRNA) in UMR106 cells incubated for 24 h without (white bar) or with (black bars) TNFα at the indicated concentration (**A**; $n = 5$) or incubated with TNFα (5 ng/ml) for the indicated periods (**B**; $n = 6$); *$p < 0.05$ and **$p < 0.01$ (one-way ANOVA followed by Dunnett's multiple comparisons test)

However, the HFD resulted in significantly stronger weight gain in tnf$^{+/+}$ mice than in tnf$^{-/-}$ mice (Fig. 4D, E).

## Discussion

According to our study, the stimulatory effect of a HFD on FGF23 formation was significantly blunted in gene-targeted mice devoid of pro-inflammatory TNFα (tnf$^{-/-}$). This result suggests that a HFD stimulates FGF23 production in large part by inducing low-grade inflammation.

It is well established that energy-dense diets including a HFD favor the development of metabolic syndrome characterized by insulin resistance, dyslipidemia, obesity, and hypertension[24, 25]. This pathophysiological condition is associated with systemic low-grade inflammation[26]. In particular, a pivotal role for the pro-inflammatory cytokine TNFα in the development of obesity-induced insulin resistance has been demonstrated[21].

Inflammation has emerged as a powerful factor driving FGF23 production[11]. Our study demonstrates that TNFα upregulated *Fgf23* transcript levels in UMR106 osteosarcoma-like cells. Importantly, TNFα is effective through transcription factor NF-κB[15] and in line with this, NF-κB has also been demonstrated to enhance FGF23 synthesis[14].

Elevated serum FGF23 concentrations are observed in acute and chronic renal, metabolic, and cardiovascular diseases[8]. Most of these clinical conditions are associated with inflammation. Therefore, similar to HFD feeding, these disorders may at least in part be effective in stimulating FGF23 production by enhancing the production of pro-inflammatory cytokines.

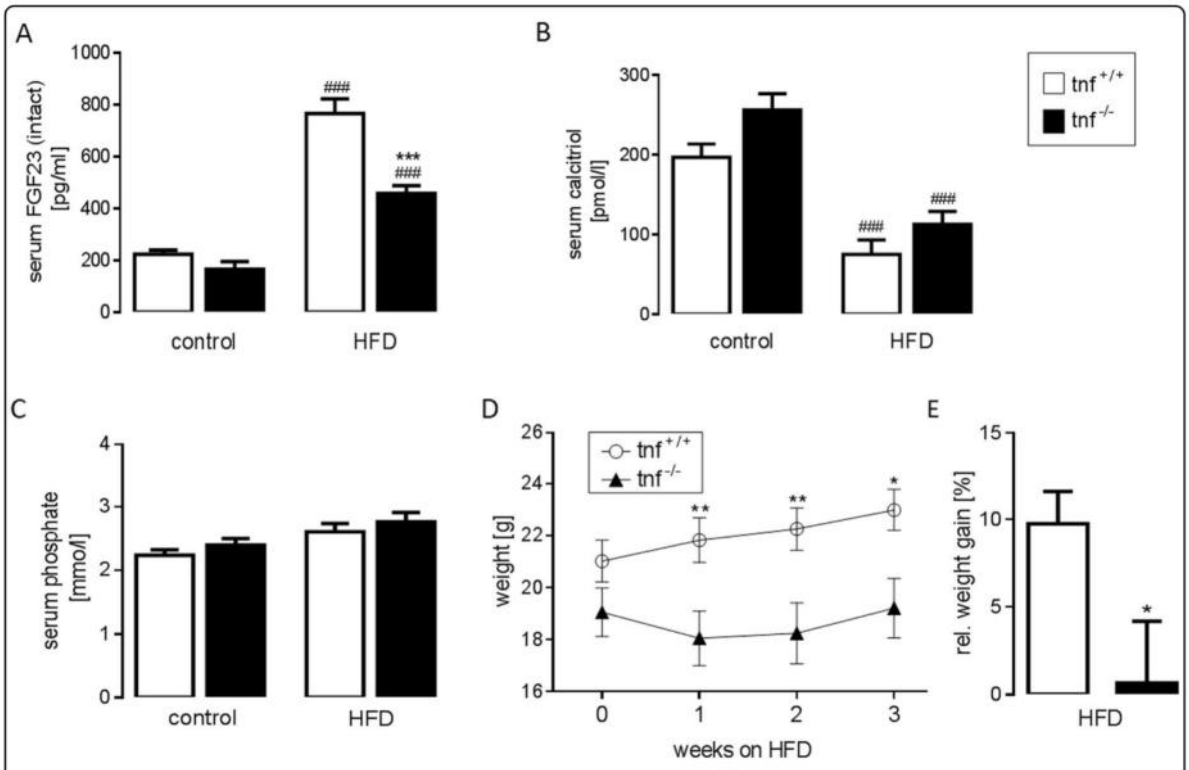


**Fig. 4 High-fat-diet-induced FGF23 production was blunted in TNFα-deficient ($tnf^{-/-}$) mice.** Arithmetic means ± SEM of the serum intact FGF23 concentration (**A**; $n = 3$ $tnf^{+/+}$ mice and 7 $tnf^{-/-}$ mice, one replication) and serum phosphate concentration (**C**; $n = 11$ $tnf^{+/+}$ mice, and 8 $tnf^{-/-}$ mice, two replications) in $tnf^{+/+}$ mice (white bars) and $tnf^{-/-}$ mice (black bars) before (control) and after 3 weeks of feeding a high-fat diet (HFD). Arithmetic means ± SEM of the serum calcitriol concentration (**B**; $n = 8$ mice per group) in a group of $tnf^{+/+}$ mice (white bars) and $tnf^{-/-}$ mice (black bars) on control diet or HFD fed for 3 weeks. Arithmetic means ± SEM ($n = 13$ $tnf^{+/+}$ mice and 12 $tnf^{-/-}$ mice, two replications) of the total body weight (**D**) in dependence of the duration of HFD and relative weight gain after 3 weeks of HFD (**E**); $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate significant difference between the genotypes; $^{\#\#\#}p < 0.001$ indicates significant difference between control and HFD. (**A**–**C**: one-way ANOVA followed by Tukey's multiple comparisons test; **D**: Holm-Sidak method; **E**: unpaired, two-tailed *t*-test with Welch's correction)

On control diet, the serum concentration of intact FGF23 was not significantly different between $tnf^{+/+}$ mice and $tnf^{-/-}$ mice although a tendency toward lower FGF23 in $tnf^{-/-}$ mice was apparent. A pro-inflammatory milieu in HFD-treated animals, however, resulted in strong TNFα-dependent FGF23 generation.

Since an increase in serum FGF23 has been observed very early in some chronic disorders including chronic kidney disease, FGF23 has been suggested as a biomarker[27]. According to our results, an increase in serum FGF23 by almost four times was observed after 3 weeks of HFD feeding, a relatively short period as evident from a moderate increase in total body weight by only some 10% in $tnf^{+/+}$ mice. Therefore, lower FGF23 may also indicate a better metabolic profile of an individual.

A major effect of FGF23 is the inhibition of renal calcitriol formation thereby lowering the serum calcitriol concentration[1]. Elevated FGF23 formation in HFD-fed mice could therefore be expected to decrease the serum calcitriol concentration. We did not, however, observe a significant difference in the serum calcitriol between the genotypes, although a tendency toward higher values in $tnf^{-/-}$ mice was obvious.

Taken together, our study demonstrates that a HFD stimulates FGF23 production at least in part by inducing TNFα formation.

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**Authors' contribution**
P.G., A.F., F.H., and M.F. performed the experiments. G.J.S. and J.V. provided the essential tools. P.G. and M.F. analyzed the data and wrote the paper. F.L. and M. F. designed the study.

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**Conflict of interest**
The authors declare that they have no conflict of interest.

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## 3.2 Paper 2: AMP-activated kinase is a regulator of fibroblast growth factor 23 production

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# AMP-activated kinase is a regulator of fibroblast growth factor 23 production

see commentary on page 453

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**Fibroblast growth factor 23 (FGF23) is a proteohormone regulating renal phosphate transport and vitamin D metabolism as well as inducing left heart hypertrophy. FGF23-deficient mice suffer from severe tissue calcification, accelerated aging and a myriad of aging-associated diseases. Bone cells produce FGF23 upon store-operated calcium ion entry (SOCE) through the calcium selective ion channel Orai1. AMP-activated kinase (AMPK) is a powerful energy sensor helping cells survive states of energy deficiency, and AMPK down-regulates Orai1. Here we investigated the role of AMPK in FGF23 production. *Fgf23* gene transcription was analyzed by qRT-PCR and SOCE by fluorescence optics in UMR106 osteoblast-like cells while the serum FGF23 concentration and phosphate metabolism were assessed in AMPKα1-knockout and wild-type mice. The AMPK activator, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) down-regulated, whereas the AMPK inhibitor, dorsomorphin dihydrochloride (compound C) and AMPK gene silencing induced *Fgf23* transcription. AICAR decreased membrane abundance of Orai1 and SOCE. SOCE inhibitors lowered *Fgf23* gene expression induced by AMPK inhibition. AMPKα1-knockout mice had a higher serum FGF23 concentration compared to wild-type mice. Thus, AMPK participates in the regulation of FGF23 production *in vitro* and *in vivo*. The inhibitory effect of AMPK on FGF23 production is at least in part mediated by Orai1-involving SOCE.**

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KEYWORDS: calcium; FGF23; Klotho; Orai1; phosphate; parathyroid hormone



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Fibroblast growth factor 23 (FGF23) is a protein mainly produced by osteocytes in bone. Being a classical hormone, FGF23 acts on target organs to which it travels via the bloodstream.[1,2] An important target is the kidney where FGF23 inhibits phosphate transporter NaPi-IIa and *Cyp27b1* expression, the gene coding the key enzyme for the synthesis of calcitriol or $1,25(OH)_2D_3$, the active form of vitamin D.[2–5] Thus, FGF23 lowers the plasma phosphate and calcitriol concentration. FGF23 also acts on the heart, inducing hypertrophy of the left ventricle.[6] The renal receptor for FGF23 depends on the protein Klotho as a coreceptor,[2,7] whereas the cardiac receptor does not.[6] Membrane-bound Klotho has an extracellular domain that can be cleaved off, yielding soluble Klotho. Soluble Klotho can be found in the blood, in urine, and in cerebrospinal fluid and exerts hormone-like effects.[8]

The significance of FGF23 and Klotho goes far beyond the regulation of phosphate metabolism. FGF23- or Klotho-deficient mice have a very short life span and exhibit a wide range of aging-associated features that are also typical of aging humans including muscle, skin, neuronal, metabolic, and cardiovascular abnormalities.[9,10] Massive calcification can be found in most tissues and organs of FGF23- or Klotho-deficient mice and is the result of deranged phosphate metabolism. Consequently, a low vitamin D or low phosphate diet attenuates or even prevents calcification, premature aging, and early death of these mice.[8]

The production of FGF23 by bone cells is regulated by parathyroid hormone (PTH),[11] $1,25(OH)_2D_3$,[12] the iron status,[13] dietary phosphate,[14] and inflammation.[13,15] Proinflammatory cytokines such as tumor necrosis factor α or interleukin-1 up-regulate FGF23.[16] Moreover, the inflammatory transcription factor complex nuclear factor κB is also involved in the formation of FGF23: nuclear factor κB up-regulates $Ca^{2-}$ release-activated $Ca^{2+}$ channel Orai1/stromal interaction molecule 1 (STIM1).[17] Orai1/STIM1–mediated store-operated calcium entry (SOCE) induces the transcription of the *Fgf23* gene.[17] Polycystic kidneys are another source of FGF23 production.[18]

Apart from its role as a hormone, FGF23 has gained broad attention as a disease biomarker.[19] In particular, in chronic kidney disease (CKD),[20] an elevated FGF23 plasma concentration has been observed early before onset of hyperparathyroidism and hyperphosphatemia, typical sequelae of

CKD.[21,22] An elevation of the plasma FGF23 concentration is also observed in cardiovascular, metabolic, and inflammatory diseases,[19] and for several of those diseases, a positive correlation between severity of the disorder and plasma FGF23 has been established. Therefore, FGF23 is under investigation to uncover its suitability as a sensitive biomarker for several acute and chronic clinical conditions.[19]

The 5′–adenosine monophosphate (AMP)–activated protein kinase (AMPK; Enzyme Commission 2.7.11.31) is a serine and threonine protein kinase expressed in all organs and tissues.[23–25] As its name suggests, it is activated by an increase in the cellular AMP level indicating a lack of adenosine triphosphate, the main cellular energy substrate.[23–25] AMPK is a heterotrimer consisting of an α (α1 or α2), β (β1 or β2), and γ (γ1, γ2, or γ3) subunit.[23] The α subunit is catalytically active, whereas the γ subunit binds AMP.[23–25] In addition to binding AMP, the activation of AMPK requires the phosphorylation of the protein, which is accomplished by the tumor suppressor liver kinase B1, another serine and threonine kinase, or by calcium-calmodulin–dependent protein kinase 2, a $Ca^{2+}$-activated protein kinase. Thus, at least in some cells, $Ca^{2+}$ influx also triggers activation of AMPK.[23–25]

Taken together, AMPK acts as a cellular energy sensor, which protects the cell against energy deficiency.[23–25] It induces energy-providing cellular pathways (e.g., fatty acid oxidation, glycolysis) and inhibits energy-consuming processes (e.g., protein synthesis, lipogenesis).[23–25] AMPK also regulates membrane transport including glucose transporter 4,[26] sodium-dependent glucose transporter 1,[27] or phosphate transporter NaPi-IIa.[28] In addition, AMPK controls ion channels including the epithelial Na channel[29–31] or big potassium $K^+$ channel.[32] Importantly, AMPK has also been demonstrated to down-regulate $Ca^{2-}$ release-activated $Ca^{2}$ channel Orai1, which accomplishes SOCE.[33] SOCE is relevant for a broad range of cellular functions including cell proliferation, migration, and differentiation in many cell types.[34,35] Recently, the production of FGF23 has been shown to be dependent on SOCE through Orai1.[17]

In view of the regulation of Orai1 by AMPK and Orai1-dependent formation of FGF23, we investigated whether AMPK is relevant for the production of FGF23.

## RESULTS

### AMPK down-regulates *Fgf23* expression in UMR106 cells

First, we analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) whether UMR106 osteoblast-like cells express AMPK subunits. As demonstrated in Figure 1a, PCR products specific for *Ampkα1*, *Ampkα2*, *Ampkβ1*, and *Ampkγ1* could readily be detected. Weaker bands for *Ampkβ2* and *Ampkγ2* were observed (Figure 1a). Western blotting confirmed these results at the protein level (Figure 1b). Thus, UMR106 osteoblast-like cells express functional AMPK. Next, we explored in UMR106 cells whether AMPK activity influences FGF23 production. We incubated UMR106 cells with or without AMPK activator 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and inhibitor compound C and determined *Fgf23* transcripts. AICAR decreased whereas compound C increased *Fgf23* gene expression (Figure 1c). Similar to pharmacological inhibition, the joint small, interfering RNA (siRNA)-mediated silencing of *Ampkα1* and *Ampkα2* genes resulted in a significant increase in *Fgf23* gene expression (Figure 1d). Silencing was effective as specific siRNA reduced *Ampkα1* transcript levels by 62% ± 7% ($n = 7$; $P < 0.001$) relative to nonsense siRNA. Hence, AMPK is a regulator of FGF23 down-regulating the production of this hormone.

### The inhibition of Orai1-mediated SOCE participates in the AMPK effect on FGF23 formation

The synthesis of FGF23 is driven by SOCE involving $Ca^{2+}$ release-activated $Ca^{2+}$ channel Orai1 in UMR106 cells.[17] Orai1-mediated SOCE has been demonstrated to be inhibited by AMPK.[33] To test whether AMPK activity influences SOCE in UMR106 cells, we determined intracellular $Ca^{2+}$ by measuring Fura-2-dependent fluorescence. SOCE was estimated from the increase in $Ca^{2+}$-dependent fluorescence following readdition of $Ca^{2}$ to the extracellular fluid of UMR106 cells that had been treated with the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) inhibitor thapsigargin in $Ca^{2+}$-free solution before. Thapsigargin depletes intracellular $Ca^{2+}$ stores, enabling SOCE. According to Figure 2a and c, activation of AMPK with AICAR resulted in reduced SOCE, pointing to an inhibitory effect of AMPK on SOCE in UMR106 cells. Interestingly, also the thapsigargin-induced increase in intracellular $Ca^{2+}$ was moderately, but significantly reduced by AICAR (Figure 2b).

AMPK regulates various ion channels by influencing their membrane abundance. Because Orai1 is relevant for SOCE in UMR106 cells, we investigated whether AMPK activity altered Orai1 expression or membrane insertion or both. As depicted in Figure 2d, AMPK activation with AICAR did not change *Orai1* transcript levels in UMR106 cells. However, AICAR significantly reduced the membrane abundance of Orai1 (Figure 2e). These results suggest that AMPK activity reduced the insertion of Orai1 in the cell membrane of UMR106 cells. Our next series of experiments explored whether the inhibitory effect of AMPK on Orai1-mediated SOCE is required for AMPK to down-regulate *Fgf23* gene expression. Inhibition of AMPK with compound C again elevated *Fgf23* transcript levels (Figure 2f). This effect was significantly and almost completely abolished by SOCE inhibitors 2-aminoethoxydiphenyl borate or MRS 1845 (*N*-Propylargylnitrendipine) or by specific Orai1 inhibitor AnCoA4 (Figure 2f). Thus, the inhibitory effect of AMPK on FGF23 production is dependent on Orai1-mediated SOCE.

### FGF23 production is enhanced in AMPKα1-deficient mice

We sought to test whether the regulation of FGF23 by AMPK is also relevant *in vivo*. To this end, we analyzed AMPKα1-deficient mice ($ampk^{-/-}$) and compared them with wild-type mice ($ampk^{+/+}$). First, we determined the serum

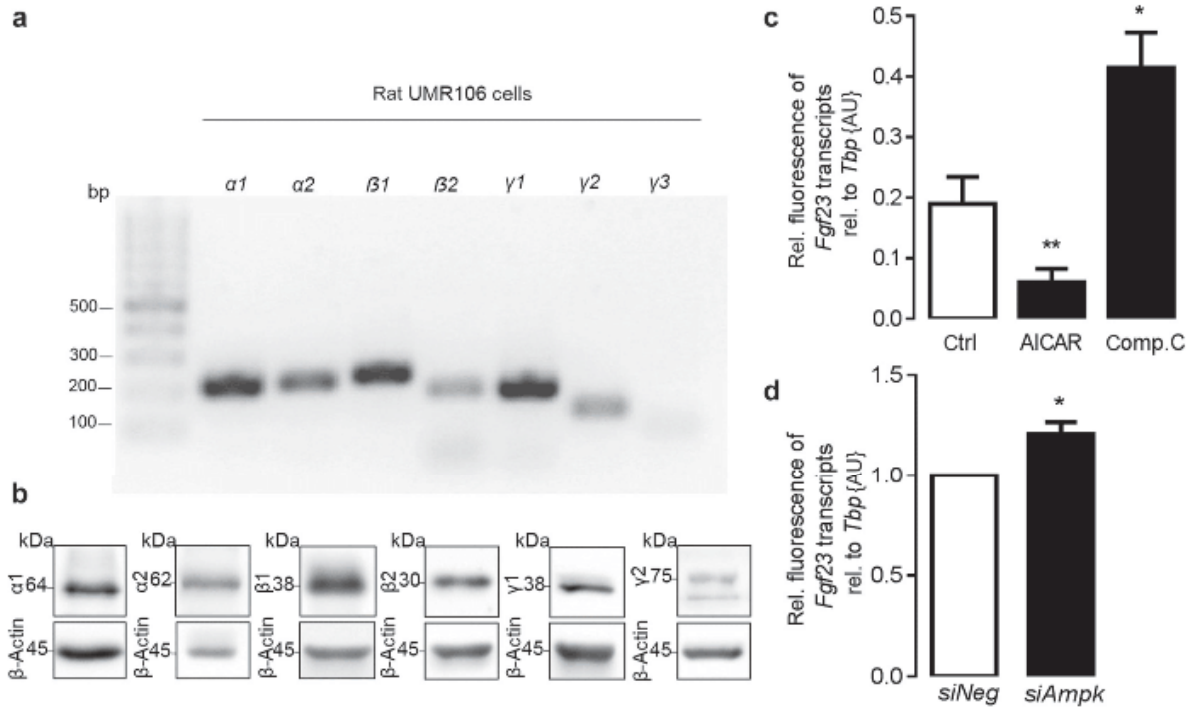


**Figure 1 | Effect of 5′–adenosine monophosphate–activated protein kinase (AMPK) on *Fgf23* gene expression in UMR106 cells.** (**a**) Original agarose gel photo demonstrating amplified *Ampkα1* (224 base pairs [bp])-, *α2* (223 bp)-, *β1* (246 bp)-, *β2* (197 bp)-, *γ1* (201 bp)-, *γ2* (144 bp)-, and *γ3* (90 bp)-specific cDNA in UMR106 rat osteoblast-like cells. (**b**) Original Western blots demonstrating protein abundance of AMPKα1, α2, β1, β2, γ1, and γ2 subunits as well as β-actin (loading control) in UMR106 cells. (**c**) Arithmetic means ± SEMs ($n = 7$) of relative (rel.) *Fgf23* mRNA abundance (relative to *Tbp* mRNA) in UMR106 cells incubated without (ctrl, white bar) or with (black bars) AMPK activator 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (250 µmol/l) or inhibitor compound C (1 µmol/l) for 24 hours. $^*P < 0.05$, $^{**}P < 0.01$ indicate significant difference from UMR106 cells treated with 100 nmol/l $1,25(OH)_2D_3$ only (analysis of variance). (**d**) Arithmetic means ± SEMs ($n = 7$) of relative *Fgf23* mRNA abundance (relative to *Tbp* mRNA) in UMR106 cells treated with control small, interfering (si)RNA (*siNeg*, white bar) or with specific *Ampkα1* and *Ampkα2* siRNA (*siAmpk*, black bar) for 72 hours. $^*P < 0.05$ indicates significant difference from 1. AU, arbitrary units; Comp. C, compound C; rel., relative. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

FGF23 concentration. As illustrated in Figure 3a, the serum concentration of C-terminal FGF23 was markedly and significantly higher in ampk$^{-/-}$ mice compared with ampk$^{+/-}$ mice. Similarly, the serum level of intact FGF23 was significantly higher in ampk$^{-/-}$ mice than in ampk$^{+/+}$ mice (Figure 3b).

FGF23 is mainly produced by osteocytes and osteoblasts in bone. Quantitative RT-PCR (qRT-PCR) analysis of bone tissues revealed higher *Fgf23* mRNA abundance in ampk$^{-/-}$ mice compared with ampk$^{-/+}$ mice, suggesting enhanced FGF23 production in the bone of ampk$^{-/-}$ mice (Figure 3c). Thus, the absence of AMPKα1 activity resulted in more FGF23 formation in mice, pointing to a relevant inhibitory effect of AMPK on FGF23 *in vivo* as observed *in vitro*.

### Normal phosphate metabolism but moderate cardiac hypertrophy in AMPKα1-deficient mice

Because FGF23 induces[36] and AMPK activity counteracts[37] left heart hypertrophy, we determined heart weights. As shown in Figure 4a, the heart weight was slightly, but significantly higher in ampk$^{-/-}$ mice than in ampk$^{+/+}$ mice, an observation in line with left heart hypertrophy in AMPKα1 deficiency. Histological analysis confirmed a slightly, but significantly higher myocyte diameter in ampk$^{-/-}$ mice, suggesting moderate heart hypertrophy (Figure 4b and c). Hypertrophy marker brain natriuretic peptide[36] as determined by qRT-PCR was not different between the genotypes ($0.09 \pm 0.01$ [ampk$^{+/+}$]; $0.09 \pm 0.02$ [ampk$^{-/-}$], $n = 10$–11). Western blotting revealed that the activity of phospholipase C γ1 (PLCγ1), which participates in FGF23-induced cardiac hypertrophy,[38] was not different between the genotypes either (Figure 4d).

The renal effects of AMPK include inhibition of phosphate reabsorption. Thus, we studied phosphate metabolism in ampk$^{-/-}$ mice and ampk$^{+/+}$ mice. The serum phosphate concentration was, however, not significantly different between ampk$^{-/-}$ mice and ampk$^{-/+}$ mice (Table 1). Moreover, no significant difference in urinary phosphate excretion was found either (Table 1). Thus, phosphate metabolism was not significantly affected by AMPKα1 deficiency in mice fed

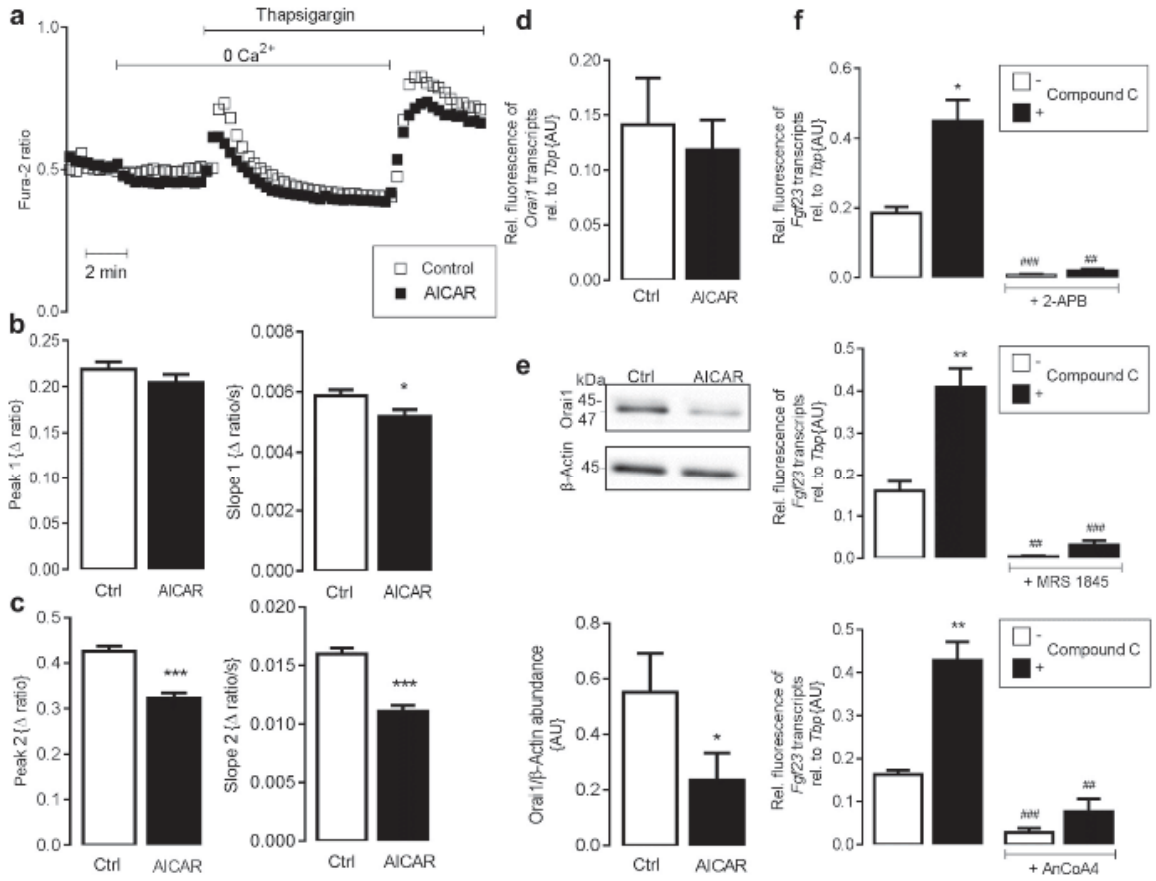


**Figure 2| Store-operated $Ca^{2+}$ entry (SOCE) in UMR106 cells.** (**a**) Representative original tracings showing intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in Fura-2 (Fura-2 acetoxymethyl ester)/AM-loaded UMR106 rat osteoblast-like cells before and after removal of extracellular $Ca^{2+}$, addition of the sarco-endoplasmic $Ca^{2+}$–adenosine triphosphatase inhibitor thapsigargin (1 μmol/l), and readdition of extracellular $Ca^{2+}$ in the absence (open squares) and presence (closed squares) of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (250 μmol/l, 4 hours). (**b**) Arithmetic means ± SEMs ($n$ = 171–200 cells measured on 4 different days) of peak (left) and slope (right) values of $[Ca^{2+}]_i$ increase after the addition of thapsigargin reflecting $Ca^{2+}$ release from intracellular stores. (**c**) Arithmetic means ± SEMs ($n$ = 171–200 cells measured on 4 different days) of peak (left) and slope (right) values of $[Ca^{2+}]_i$ increase following the readdition of extracellular $Ca^{2+}$ reflecting SOCE. White bars: without (Ctrl), black bars: with AICAR (250 μmol/l, 4 hours). $^{*}P < 0.05$, $^{***}P < 0.001$ indicate significant difference ($t$-test). (**d**) Arithmetic means ± SEMs ($n$ = 7) of relative *Orai1* mRNA abundance (relative to *Tbp* mRNA) in UMR106 cells incubated without (Ctrl, white bar) or with (black bar) AICAR (250 μmol/l) for 24 hours ($t$-test). (**e**) Original Western blot (upper panel) and densitometric analysis (lower panel) (arithmetic means ± SEMs; $n$ = 7) demonstrating the protein abundance of Orai1 and β-actin (loading control) in the cell membrane of UMR106 cells incubated without (Ctrl, white bar) or with (black bar) AICAR (250 μmol/l) for 24 hours. $^{*}P < 0.05$ indicates significant difference ($t$-test). (**f**) Arithmetic means ± SEMs ($n$ = 7) of *Fgf23* mRNA abundance (relative to *Tbp* mRNA) in UMR106 cells incubated without (white bars) or with compound C (black bars, 1 μmol/l) in the absence or presence of SOCE inhibitors 2-aminoethoxydiphenyl borate (APB) (25 μmol/l; upper panel) or MRS 1845 (50 μmol/l; middle panel) or specific Orai1 inhibitor AnCoA4 (50 μmol/l; lower panel) for 24 hours. $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant difference from the absence of compound C, $^{\#\#}P < 0.01$, $^{\#\#\}P < 0.001$ indicate significant difference from the absence of 2-APB, MRS 1845 (*N*-Propylargylnitrendipine), or AnCoA4 (analysis of variance). AU, arbitrary units; rel., relative. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

normal chow. Because FGF23 is a potent regulator of calcitriol formation, we also determined the serum calcitriol level, which was again not significantly different between $ampk^{-/-}$ mice and $ampk^{-/+}$ mice (Table 1). Serum PTH was not different, either (Table 1). To study renal function, we also determined the glomerular filtration rate and serum $Na^+$ and $K^+$ as well as the urinary $Na^+$ and $K^+$ excretion. All these parameters were not significantly different between $ampk^{-/-}$ mice and $ampk^{+/+}$ mice (Table 1), suggesting gross normal renal function in AMPKα1 deficiency.

Because enhanced FGF23 production in $ampk^{-/-}$ mice could point to rather subtle phosphate retention, we exposed the animals to a high phosphate diet containing 2% phosphorus. As expected, dietary phosphate loading resulted in an increase in the serum phosphate level and urinary phosphate excretion in both genotypes (Table 2). Under this diet, the

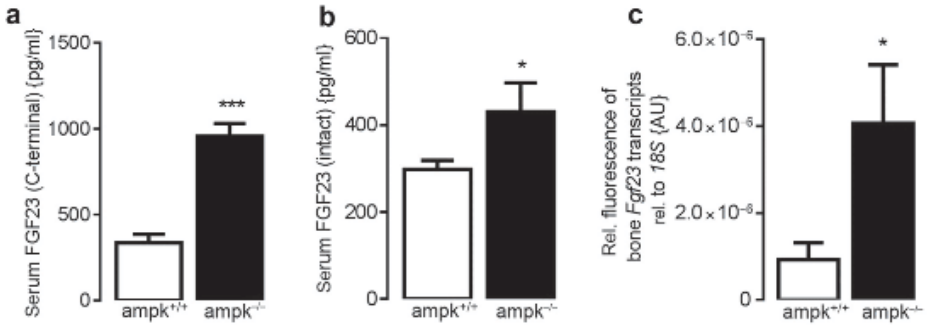


**Figure 3 | Serum fibroblast growth factor 23 (FGF23) levels and bone *Fgf23* mRNA abundance in ampk$^{+/+}$ mice and ampk$^{-/-}$ mice.** Arithmetic means + SEMs of the serum concentration of C-terminal FGF23 (**a**) ($n = 5$), serum intact FGF23 concentration (**b**) ($n = 6$), and relative bone *Fgf23* mRNA abundance (**c**) ($n = 6$) (relative [rel.] to *18S* mRNA) in ampk$^{+/+}$ mice (white bars) and ampk$^{-/-}$ mice (black bars). *$P < 0.05$, ***$P < 0.001$ indicate significant difference between the genotypes (*t*-test). AU, arbitrary units.

serum phosphate concentration tended to be higher in ampk$^{-/-}$ mice than in ampk$^{+/+}$ mice, the difference was almost statistically significant ($P = 0.051$) (Table 2). The FGF23 serum concentration was, again, markedly and significantly higher in ampk$^{-/-}$ mice compared with ampk$^{+/+}$ mice (Table 2).

Finally, the renal effects of FGF23 are mediated by Klotho, the expression of which we determined by qRT-PCR analysis and Western blotting. As demonstrated in Figure 5a and b, we did not observe a significant difference in renal Klotho mRNA and protein expression between ampk$^{-/-}$ mice and ampk$^{+/-}$ mice.

Renal phosphate reabsorption is mainly accomplished by $Na^+$-dependent phosphate transporter NaPi-IIa (*Slc34a1*). We therefore analyzed NaPi-IIa expression in the kidneys from ampk$^{-/-}$ mice and ampk$^{-/+}$ mice by qRT-PCR analysis and Western blotting and found no significant difference (Figure 5c and d). In addition, we employed immunohistochemistry to study the cellular localization of NaPi-IIa in the proximal tubule. As a result, surface expression of NaPi-IIa was similar in ampk$^{-/-}$ mice and ampk$^{+/+}$ mice, a result in line with unaltered renal phosphate excretion (Figure 5e). Downstream FGF23 signaling in renal tubular cells includes phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2), which we determined by Western blotting in kidney tissues from ampk$^{-/-}$ mice and ampk$^{+/+}$ mice. As demonstrated in Figure 5f, the abundance of phosphorylated ERK1/2 was not different between the genotypes despite a markedly elevated FGF23 level in ampk$^{-/-}$ mice, which would be expected to induce ERK1/2 phosphorylation.

## DISCUSSION

Our study suggests that AMPK is a regulator of FGF23 *in vitro* as well as *in vivo*. AMPK activation down-regulated, whereas AMPK inhibition or siRNA-mediated gene silencing enhanced FGF23 production. In line with this, AMPKα1-deficient mice had a markedly higher FGF23 serum concentration, but seemingly normal mineral metabolism.

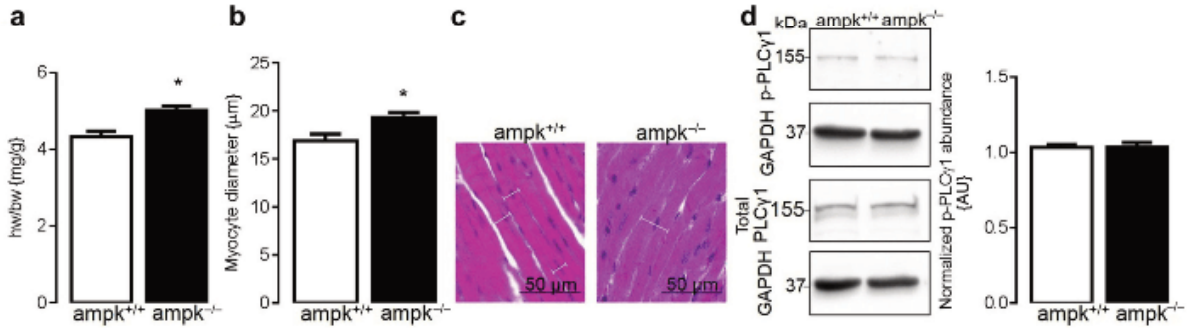


**Figure 4 | Heart hypertrophy markers in ampk$^{+/+}$ mice and ampk$^{-/-}$ mice.** (**a**) Arithmetic means ± SEMs (n = 5) of the heart weight (hw)/body weight (bw) ratio in ampk$^{+/+}$ mice (white bar) and ampk$^{-/-}$ mice (black bar). *$P < 0.05$ indicates significant difference between the genotypes (*t*-test). (**b**) Arithmetic means + SEMs ($n = 9$–10) of the myocyte diameter in hearts from ampk$^{+/+}$ mice (white bar) and ampk$^{-/-}$ mice (black bar). *$P < 0.05$ indicates significant difference between the genotypes (*t*-test). (**c**) Representative images of hematoxylin and eosin–stained heart sections from ampk$^{-/+}$ mice and ampk$^{-/-}$ mice. The white lines indicate the myocyte diameter. Bars = 50 µm. (**d**) Original Western blot (left panel) and densitometric analysis (right panel) (arithmetic means ± SEMs; $n = 6$) demonstrating cardiac protein abundance of phosphorylated and total phospholipase C γ1 (PLCγ1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (loading control) in ampk$^{-/+}$ mice (white bar) and ampk$^{-/-}$ mice (black bar). AU, arbitrary units. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

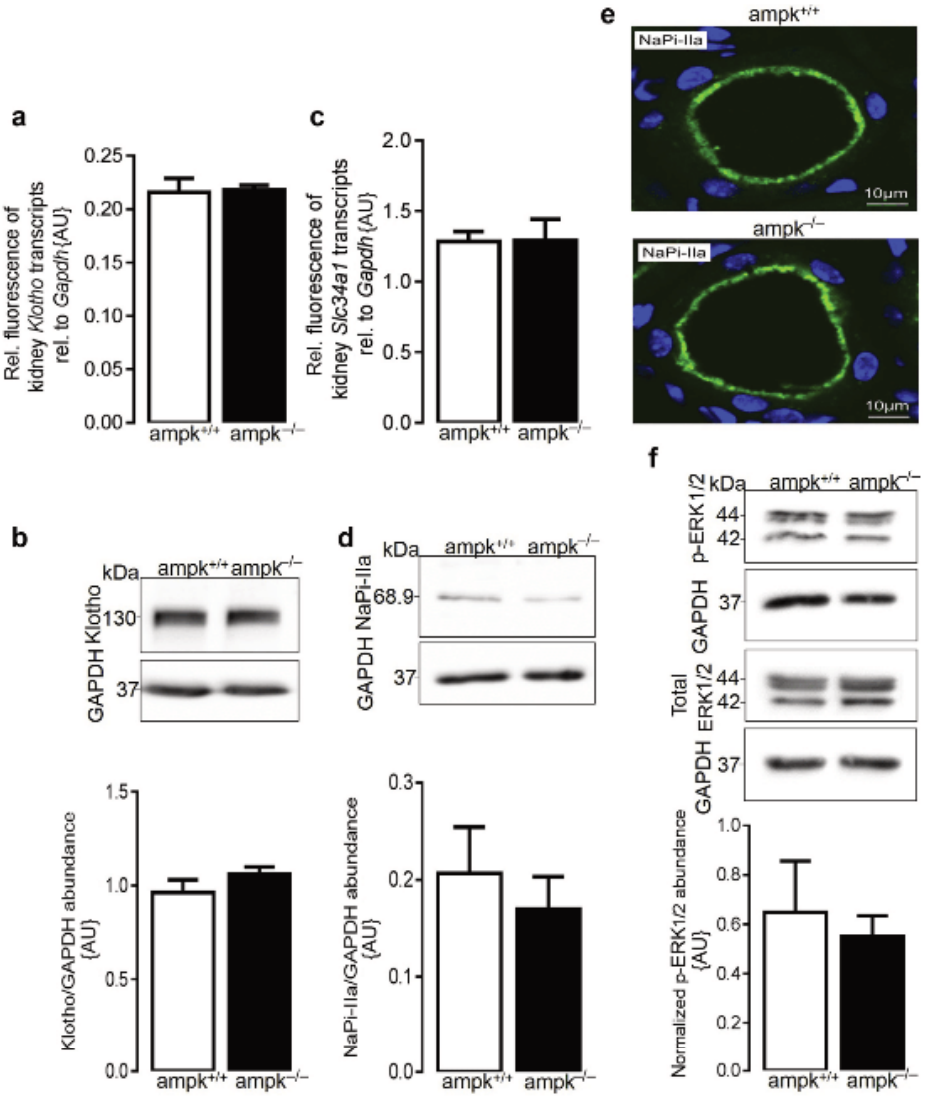


**Figure 5 | Renal Klotho and NaPi-IIa expression and downstream fibroblast growth factor 23 (FGF23) signaling in kidneys from ampk$^{+/+}$ mice and ampk$^{-/-}$ mice.** (**a**) Arithmetic means ± SEMs ($n$ — 3) of relative kidney *Klotho* mRNA abundance (relative [rel.] to *Gapdh* mRNA) in ampk $^{+/+}$ mice (white bar) and ampk $^{-/-}$ mice (black bar). (**b**) Original Western blot (upper panel) and densitometric analysis (lower panel) (arithmetic means + SEMs; $n = 3$) demonstrating the renal protein abundance of full length Klotho and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (loading control) in ampk$^{+/+}$ mice (white bar) and ampk$^{-/-}$ mice (black bar). (**c**) Arithmetic means ± SEMs ($n = 3$) of relative kidney *Slc34a1* mRNA abundance (relative to *Gapdh* mRNA) in ampk$^{+/+}$ mice (white bar) and ampk$^{-/-}$ mice (black bar). (**d**) Original Western blot (upper panel) and densitometric analysis (lower panel) (arithmetic means ± SEMs; $n = 5$–6) demonstrating the renal protein abundance of NaPi-IIa and GAPDH (loading control) in ampk$^{-/+}$ mice (white bar) and ampk$^{-/-}$ mice (black bar). (**e**) Cellular localization of NaPi-IIa in the proximal tubule from ampk$^{-/+}$ and ampk$^{-/-}$ kidneys. Confocal microscopic images showing representative S3 proximal tubule sections labeled for NaPi-IIa (green signal). The nuclei are counterstained with 4′,6-diamidino-2-phenylindole (blue signal). Note the similar NaPi-IIa distribution patterns within the brush border in the 2 genotypes. Bars = 10 µm. (**f**) Original Western blot (upper panels) and densitometric analysis (lower panel) (arithmetic means ± SEMs; $n = 6$) demonstrating the renal protein abundance of phosphorylated (p) and total extracellular signal-regulated kinases 1/2 (ERK1/2) and GAPDH (loading control) in ampk$^{+/+}$ mice (white bar) and ampk$^{-/-}$ mice (black bar). AU, arbitrary units. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

AMPK is activated by cellular energy deficiency.[25] Under this condition, it serves to provide cells with energy and limits energy-consuming pathways.[25] According to our results, the production of FGF23 is among the latter processes, which are limited in energy deficiency. On the one hand, FGF23 induces hypertrophy of the left ventricle, a clearly energy-consuming effect. On the other hand, the inhibitory effect of FGF23 on renal phosphate reabsorption, which is secondary active, and calcitriol formation is expected to save energy.

**Table 1 | Renal function parameters and serum levels of hormones in ampk$^{+/+}$ mice and ampk$^{-/-}$ mice**

| Parameters | ampk$^{+/+}$ ($n$ = 5-7) | ampk$^{-/-}$ ($n$ = 5-7) |
|---|---|---|
| GFR (µl/min per g bw) | 14.6 ± 2.3 | 13.0 ± 1.9 |
| Serum sodium (mmol/l) | 132.4 ± 0.9 | 133.0 ± 0.9 |
| Urinary sodium excretion (µmol/24 h per g bw) | 10.3 ± 0.8 | 9.5 ± 0.9 |
| Serum potassium (mmol/l) | 4.5 ± 0.2 | 4.9 ± 0.4 |
| Urinary potassium excretion (µmol/24 h per g bw) | 15.9 ± 1.4 | 14.0 ± 1.2 |
| Serum phosphate (mg/dl) | 5.8 ± 0.3 | 6.2 ± 0.3 |
| Urinary phosphate excretion (µmol/24 h per g bw) | 5.0 ± 0.4 | 4.7 ± 0.5 |
| Calcitriol (pg/ml) | 110.1 ± 10.2 | 124.7 ± 16.1 |
| PTH (pg/ml) | 63.1 ± 5.2 | 60.9 ± 8.2 |

bw, body weight; GFR, glomerular filtration rate; PTH, parathyroid hormone.
Data are presented as arithmetic means ± SEMs.

**Table 2 | Impact of high phosphate diet on renal phosphate excretion in ampk$^{+/+}$ mice and ampk$^{-/-}$ mice**

| Parameters | ampk$^{+/+}$ ($n$ = 10) | ampk$^{-/-}$ ($n$ = 9) |
|---|---|---|
| GFR (µl/min per g bw) | 7.1 ± 0.5 | 6.8 ± 0.5 |
| Serum phosphate (mg/dl) | 6.0 ± 0.2 | 6.8 ± 0.3 ($P$ = 0.051) |
| Urinary phosphate excretion (µmol/24 h per g bw) | 24.3± 1.5 | 27.8 ± 2.8 |
| C-terminal FGF23 (pg/ml) | 789.3 ± 62.6 | 2170.6 ± 538.8[a] |

bw, body weight; GFR, glomerular filtration rate; FGF23, fibroblast growth factor 23.
[a]$P$ < 0.001 indicates significant difference between the genotypes (Kolmogorov-Smirnov test).
Data are presented as arithmetic means ± SEMs.

AMPK is known to counteract cardiac hypertrophy with AMPKα2 being most relevant for this effect[37,39] and AMPKα1 having a minor role.[40] Patients with CKD suffer from left ventricular hypertrophy,[41] and FGF23 is not only associated with this cardiac condition as a biomarker but actively induces left heart hypertrophy by inducing PLCγ activity.[36,38] We indeed found a small but significant increase in heart weight of ampk$^{-/-}$ mice compared with ampk$^{+/+}$ mice, an effect paralleled by a higher cardiac myocyte diameter in ampk$^{-/-}$ mice pointing to moderate cardiac hypertrophy. However, cardiac PLCγ activity was not different between the genotypes. Hence, it seems possible that FGF23 does not have a dominant role in the moderate heart hypertrophy of ampk$^{-/-}$ mice. Instead, FGF23-independent inhibitory effects of AMPK may be more relevant.[37,39]

In CKD, renal AMPK activity has been found to be lower than in healthy kidneys.[42] Therefore, pharmacological AMPK activation may be beneficial in this disease[42] as it may be in other renal diseases[43] including polycystic kidney disease that may ultimately result in CKD.[44] Our results are in line with this assumption as a high FGF23 level is clearly predictive of declining kidney function and earlier death in CKD.[45] Because FGF23 may not only indicate disease but may actively contribute to progression in CKD, AMPK-dependent suppression of enhanced FGF23 production in this disease may not only be beneficial with regard to renal function, but also with regard to the prevention of left heart hypertrophy, a very severe sequela of CKD.[41] Surely, apart from AMPK, a plethora of other novel factors are relevant for CKD–mineral and bone disorder including a disintegrin and metalloproteinase 17 (ADAM17). This enzyme generates soluble Klotho from transmembrane Klotho and tumor necrosis factor α, molecules influencing CKD–mineral and bone disorder pathophysiology, and has recently been shown to be a novel player in this disorder.[46]

SOCE involving $Ca^{2+}$ release-activated $Ca^{2+}$ channel Orai1 is part of the cellular signaling resulting in the production of FGF23.[17] As AMPK has been shown to down-regulate Orai1-mediated SOCE,[33,47,48] we tested whether this mechanism is responsible for the AMPK effect on FGF23. Our results indeed suggest that AMPK lowers the cell surface expression of Orai1 in osteoblast-like cells. Orai1 is targeted by ubiquitin ligase neural precursor cell expressed developmentally down-regulated protein 4-2 (Nedd4-2), resulting in proteasomal degradation of the channel protein.[49] AMPK has been demonstrated to regulate Nedd4-2,[29,30] and the AMPK effect on ion channels including epithelial $Na^+$ channel [30] and KCNQ1 (potassium voltage-gated channel subfamily Q member 1)[50] is indeed also mediated by Nedd4-2. As a consequence of decreased Orai1 membrane expression upon treatment with AICAR, we observed reduced SOCE. Conversely, the stimulating effect of AMPK inhibition was abolished in the presence of SOCE inhibitors 2-aminoethoxydiphenyl borate and MRS 1845 or by Orai1 inhibitor AnCoA4, suggesting that the AMPK effect on FGF23 is indeed dependent on Orai1-mediated SOCE.

Increased FGF23 production in ampk$^{-/-}$ mice would be expected to impact on renal phosphate handling and calcitriol metabolism. We, however, did not see a significant difference in renal phosphate excretion and the serum phosphate concentration between ampk$^{-/-}$ mice and ampk$^{+/+}$ mice. Hence, renal expression and cellular localization of NaPi-IIa, the main $Na^+$-dependent phosphate transporter, was similar in ampk$^{-/-}$ mice and ampk$^{+/+}$ mice. Neither did we observe a significant difference in the serum calcitriol and PTH level between ampk$^{-/-}$ mice and ampk$^{+/+}$ mice. It should be kept in mind that AMPK is an inhibitor of the renal tubular phosphate transporter NaPi-IIa[28] and that renal AMPK deficiency is expected to enhance renal tubular phosphate transport, thereby counteracting the effect of FGF23. It is tempting to speculate that AMPK contributes to the decline of renal tubular phosphate transport following high phosphate diet and that the effect of high phosphate diet on plasma phosphate concentration is thus augmented in ampk$^{-/-}$ mice.

The lack of hypophosphatemia in ampk$^{-/-}$ mice may further reflect the complexity of mineral metabolism compensating moderate derangements of a regulator. Notably, lack of functional vitamin D receptor similarly does not lead to hypophosphatemia.[51] AMPK deficiency may cause

some FGF23 resistance. Along those lines, FGF23-downstream ERK1/2 signaling[52] was not different between ampk$^{-/-}$ and ampk$^{+/-}$ kidneys despite a markedly higher FGF23 concentration in ampk$^{-/-}$ mice. Therefore, unaltered phosphate metabolism in ampk$^{-/-}$ mice may at least in part be explained by the fact that the higher FGF23 concentration of ampk$^{-/-}$ mice ultimately resulted in the same ERK1/2 activity in ampk$^{-/-}$ kidneys as did the lower FGF23 concentration of ampk$^{-/+}$ mice in ampk$^{+/+}$ kidneys.

Moreover, renal $1,25(OH)_2D_3$ formation is not only regulated by FGF23/Klotho, but by several further negative feedback loops including $1,25(OH)_2D_3$ itself,[53] which are expected to limit alterations of plasma phosphate concentration and renal phosphate excretion at enhanced FGF23 plasma levels.

In line with a previous report,[54] renal function including glomerular filtration rate, $Na^+$ and $K^+$ excretion was not affected by AMPKα1 deficiency under control conditions, whereas AMPK-dependent down-regulation of renal outer medullary $K^+$ channel results in failure of ampk$^{-/-}$ mice to adapt to an acute $K^+$ load.[54] Also, $K^+$ channel KCNQ1 is expressed in the kidney and regulated by AMPK.[50]

Taken together, our study shows that AMPK is an important regulator of FGF23 production both *in vitro* and *in vivo*. The AMPK effect on FGF23 is at least in part mediated by Orai1-involving SOCE (Figure 6). AMPK-dependent FGF23 regulation is expected to participate in the beneficial effects of AMPK activation in CKD as well as in other diseases.

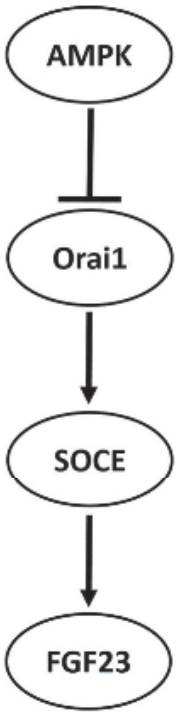


**Figure 6| Scheme illustrating the signaling of the 5′–adenosine monophosphate–activated protein kinase (AMPK) effect on fibroblast growth factor 23 (FGF23).** SOCE, store-operated calcium entry.

## METHODS

### Cell culture

Cell culture experiments were conducted as previously described.[56] UMR106 cells do not have appreciable amounts of *Fgf23* mRNA *per se*. *Fgf23* expression was therefore induced by pretreatment with 100 nmol/l $1,25(OH)_2D_3$ (Tocris, Bristol, UK). After 24 hours, cells were additionally treated with or without 250 μmol/l AICAR, 1 μmol/l compound C with or without 25 μmol/l 2-aminoethoxydiphenyl borate (Sigma-Aldrich, Schnelldorf, Germany), 50 μmol/l MRS 1845 (Tocris) or 50 μmol/l AnCoA4 (Merck, Darmstadt, Germany) for another 24 hours or treated with vehicle only. For $Ca^{2+}$ measurements, cells were treated with or without 250 μmol/l AICAR for 4 hours.

### Silencing

A total of $1.5 \times 10^5$ cells were seeded 24 hours before the experiment in antibiotic-free medium. Cells were transfected with ON-TARGETplus Rat SMARTpool *Ampkα1* and *Ampkα2* siRNA (each 50 nmol/l) (Thermo Fisher Scientific, Waltham, MA) or 100 nmol/l nonspecific siRNA (Thermo Fisher Scientific) using DharmaFECT1 transfection reagent. Forty-eight hours after transfection, cells were treated with $1,25(OH)_2D_3$ (100 nmol/l) for another 24 hours and harvested.

### Expression analysis

Total RNA was extracted from UMR106 cells treated for 48 hours with 100 nmol/l $1,25(OH)_2D_3$ only. PCR was carried out using GoTaq Green Master Mix (Promega, Mannheim, Germany). PCR conditions were 94 °C (5 minutes), 35 cycles of 94 °C (30 seconds), 58 to 60 °C (30 seconds), and 72 °C (1 minute).

### Primers

*Ampkα1*
CTCAACCGGCAGAAGATTCG
TGGAACAGACGTCGACTCTC
*Ampkα2*
GGAGGGTTGAAGAGGTGGAA
TCCGGTGCTGCATAATTTGG
*Ampkβ1*
ACGATCCTTCCGAGCCAATA
TGTTCAAGATGACCTGCAGC
*Ampkβ2*
CTGGCAGCAGGATTTGGATG
ACTTGTACTGGTGCTCTCCC
*Ampkγ1*
TCGCTGCTATGACCTGATCC
AGGGCTGACTTGTAGTATCGG
*Ampkγ2*
CCTCCAGCTTTTTATGTCTG
CTCTCCACAAAGATGTTCAAG
*Ampkγ3*
ATATATTTGGTGCCCTGTTG
CTACAGCTAAATCTCGGAATG

### qRT-PCR

Total RNA was extracted with Tri-Fast (Peqlab, Erlangen, Germany) (in case of bone also with RNeasy Mini Kit [QIAGEN, Hilden, Germany]) and transcribed with GoScript Reverse Transcription System (Promega) using 1.2 µg of total RNA and random primers. The RT-PCR was carried out with GoTaq qPCR Master Mix (Promega) containing a proprietary fluorescent double-stranded DNA-binding dye exhibiting spectral properties similar to those of SYBR Green I. PCR conditions were 95 °C (3 minutes), 29 to 40 cycles of 95 °C (10 seconds), 56 to 58 °C (30 seconds), and 72 °C (30–45 seconds).

### Primers

*Tbp*
ACTCCTGCCACACCAGCC
GGTCAAGTTTACAGCCAAGATTCA
*Fgf23*
TGGCCATGTAGACGGAACAC
GGCCCCTATTATCACTACGGAG
*Ampkα1*
CTCAACCGGCAGAAGATTCG
TGGAACAGACGTCGACTCTC
*Orai1*
CGTCCACAACCTCAACTCC
AACTGTCGGTCCGTCTTAT
*Fgf23*
TCGAAGGTTCCTTTGTATGGA
AGTGATGCTTCTGCGACAAGT
*Klotho*
CCTTAAAAGCAATCAGACTGG
GAAAGCCATTGTCCTCTATC
*Slc34a1*
AATGCAACCATATCTTCGTG
GGAAAGTCTGTGTTGATGAC
*18S*
GGGAGCCTGAGAAACGGC
GGGTCGGGAGTGGGTAATTT
*Gapdh*
GGTGAAGGTCGGTGTGAACG
CTCGCTCCTGGAAGATGGTG
*Nppb*
ATGGATCTCCTGAAGGTGCTGTC
CTACAACAACTTCAGTGCGTTAC

Calculated mRNA expression levels were normalized to the expression levels of *Tbp* (TATA-box binding protein in rat UMR106 cells), *Gapdh* (in murine kidney and heart), or *18S* (in murine bone), respectively, of the same cDNA sample as internal reference. Quantification of gene expression was based on the $2^{-\Delta\Delta Ct}$ method ($\Delta\Delta Ct$ = [$\Delta Ct$ of a target sample]−[$\Delta Ct$ of a reference sample]), whereas normalized data are presented after the $2^{-\Delta Ct}$ ($\Delta Ct$ = Ct [target gene]−Ct[reference gene]) transformation.[55]

### $Ca^{2+}$ measurements

The measurements were performed as described previously using Fura-2.[56] SOCE was determined after extracellular $Ca^{2+}$ removal and subsequent $Ca^{2+}$ readdition in the presence of thapsigargin (1 µmol/l; Tocris). For quantification of $Ca^{2-}$ entry, the slope (delta ratio per second) and peak (delta ratio) were calculated following readdition of $Ca^{2+}$. Experiments were performed in Ringer solution containing (in mmol/l): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.8 $CaCl_2$, 2 $Na_2HPO_4$, 32 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 5 glucose, pH 7.4. To reach nominally $Ca^{2+}$-free conditions, $Ca^{2+}$-free Ringer solution was used (in mmol/l: 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 2 $Na_2HPO_4$, 32 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 0.5 ethylene glycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 5 glucose, pH 7.4).

### Animals

All animal experiments were conducted according to the German law for the welfare of animals and were approved by the authorities of the state of Saxony-Anhalt. Experiments were performed in AMPK-deficient ($ampk^{-/-}$) mice[57] and in sex- and age- (3–6 months old) matched wild-type mice ($ampk^{+/+}$).

### Serum and urine parameters

Mice were placed individually in metabolic cages (Tecniplast, Hohenpeissenberg, Germany) and allowed a 2-day habituation period. Subsequently, 24-hour urine was collected on the following 3 to 4 days in siliconized metabolic cages under water-saturated oil as described previously.[58] For phosphate loading, mice were fed a high phosphate diet (C1049; Altromin, Lage, Germany) containing 2% phosphorus for the duration of urine collection. Blood was drawn from lightly anesthetized mice into heparinized capillaries by puncturing the retro-orbital plexus. Serum concentrations of intact and C-terminal FGF23, PTH (all from Immutopics, San Clemente, CA) and calcitriol (IDS, Frankfurt am Main, Germany) were determined by enzyme-linked immunosorbent assay kits according to the manufacturer's protocol. For evaluation of the glomerular filtration rate, urinary creatinine was measured using the Jaffé method (Labor + Technik, Berlin, Germany) and serum creatinine using an enzymatic reaction (PAP; Labor + Technik), respectively. Urinary and serum inorganic phosphate concentrations were determined by a photometric method (Roche, Mannheim, Germany). Sodium and potassium concentrations in serum and urine were analyzed by flame photometry (Eppendorf, Hamburg, Germany).

### Western blotting

Orai1 protein abundance was detected using the Pierce Cell Surface Protein Isolation Kit (Thermo Fisher Scientific). Proteins from lysed cells or tissues (30–50 µg) or total protein lysate (15 µl) for Orai1 detection were subjected to standard Western blotting using the following antibodies: anti-AMPKα1, anti-AMPKα2, anti-AMPKβ1, anti-SLC34A1 (Novus Biologicals, Littleton, CO), anti-AMPKβ2, anti-AMPKγ2, anti-p44/42-MAPK, anti-phospho-p44/42-MAPK, anti-PLCγ1, anti-phospho-PLCγ1 (Cell Signaling Technology, Danvers, MA), anti-AMPKγ1 (Abcam, Cambridge, UK), anti-Orai1 (Proteintech, Chicago, IL), anti-Klotho (R&D Systems, Minneapolis, MN), horseradish peroxidase–conjugated-β-Actin-antibody or anti-glyceraldehyde-3-phosphate dehydrogenase–antibody (Cell Signaling) as loading controls. Horseradish peroxidase–conjugated anti-rabbit or anti-goat secondary antibody was used, and the bands were visualized using enhanced chemiluminescence reagent (GE Healthcare-Amersham, Amersham, UK) and detected by Syngene G:BOX Chemi XX6 (VWR, Dresden, Germany).

### Analysis of myocyte diameter

Hearts were fixed in Roti-Histofix 4% (Carl Roth, Karlsruhe, Germany), dehydrated in graded ethanol, cleared in Roticlear (Carl Roth), and embedded in paraffin. Sections were stained with hematoxylin and eosin and analyzed as described before.[59]

### Histochemistry

The immunofluorescence protocol was described previously.[60] Primary anti-NaPi-IIa antibody[61] was added for 1 hour at room temperature followed by overnight incubation at 4 °C, detected by a Cy3-coupled fluorescent secondary antibody (Dianova, Hamburg, Germany) and evaluated by confocal microscopy (LSM 5; Zeiss, Oberkochen, Germany).

### Statistics

Data are provided as means ± SEMs, and $n$ represents the number of independent experiments or animals studied. All data were tested for significance using Student's paired or unpaired $t$-test or one-way analysis of variance (followed by Tukey's multiple comparisons test), as appropriate. Nonparametric data were analyzed using Kolmogorov-Smirnov test. Only results with $P < 0.05$ were considered statistically significant.

### DISCLOSURE

All the authors declared no competing interests.

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## 3.3 Paper 3: AMP-Activated Protein Kinase (AMPK)-Dependent Regulation of Renal Transport

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# AMP-Activated Protein Kinase (AMPK)-Dependent Regulation of Renal Transport

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**Abstract:** AMP-activated kinase (AMPK) is a serine/threonine kinase that is expressed in most cells and activated by a high cellular AMP/ATP ratio (indicating energy deficiency) or by $Ca^{2+}$. In general, AMPK turns on energy-generating pathways (e.g., glucose uptake, glycolysis, fatty acid oxidation) and stops energy-consuming processes (e.g., lipogenesis, glycogenesis), thereby helping cells survive low energy states. The functional element of the kidney, the nephron, consists of the glomerulus, where the primary urine is filtered, and the proximal tubule, Henle's loop, the distal tubule, and the collecting duct. In the tubular system of the kidney, the composition of primary urine is modified by the reabsorption and secretion of ions and molecules to yield final excreted urine. The underlying membrane transport processes are mainly energy-consuming (active transport) and in some cases passive. Since active transport accounts for a large part of the cell's ATP demands, it is an important target for AMPK. Here, we review the AMPK-dependent regulation of membrane transport along nephron segments and discuss physiological and pathophysiological implications.

**Keywords:** transporter; carrier; pump; membrane; energy deficiency

## 1. Introduction

The 5′-adenosine monophosphate (AMP)–activated protein kinase (AMPK) is a serine/threonine protein kinase that is evolutionarily conserved and functions as an intracellular energy sensor in mammalian cells [1–5]. It is a central regulator of energy homeostasis and affects many important cellular functions including growth, differentiation, autophagy, and metabolism [1,2,6]. During energy depletion when cellular AMP levels are high relative to the adenosine triphosphate (ATP) concentration, AMPK activates energy-providing pathways including glucose uptake, glycolysis, or fatty acid oxidation [7–10]. Simultaneously, processes consuming ATP (e.g., gluconeogenesis, lipogenesis, or protein synthesis) are inhibited [7–10].

Being expressed in most mammalian cells, AMPK is a heterotrimeric protein consisting of a catalytic α (α1 or α2), scaffolding β (β1 or β2), and a regulatory nucleotide-binding γ (γ1, γ2, or γ3) subunit with the expression pattern differing from cell type to cell type [1,2,11–14]. Induction of AMPK activity involves phosphorylation of the conserved threonine residue Thr172 within the activation loop of the α subunit's kinase domain by various protein kinases including the tumor suppressor liver kinase B1 (LKB1), $Ca^{2+}$/calmodulin–dependent protein kinase kinase β (CaMKKβ), and transforming growth factor beta-activated kinase 1 [1,15–28]. AMPK activation in cellular energy depletion is primarily mediated by an increase in the AMP/ATP or ADP/ATP ratio [8,29,30]. Thus, AMP or ADP binding to the subunit at cystathionine-beta-synthase repeats results in conformational changes that allows for the phosphorylation at Thr172 by LKB1. This results in an enhancement of AMPK activity by >100-fold [1,8,12,15,31–36]. Moreover, AMP or ADP binding prevents dephosphorylation at Thr172

by protein phosphatases [8,12,37,38]. Additionally, binding of AMP, but not ADP, activates AMPK allosterically [8,11,12,37]. Conversely, ATP binding to the cystathionine-beta-synthase domain results in AMPK dephosphorylation by protein phosphatases [1,8,39].

Besides LKB1-associated regulation of AMPK phosphorylation, an alternative $Ca^{2+}$-involving activation mechanisms independent of AMP exists [6,12,40,41]. Protein kinase CaMKKβ phosphorylates AMPK at Thr172 in response to elevated intracellular $Ca^{2+}$ levels which may be caused by mediators such as thrombin or ghrelin [6,12,23,40,42,43]. Intracellular $Ca^{2+}$ store depletion detected by the $Ca^{2+}$-sensing protein stromal interacting molecule-1 leads to store-operated $Ca^{2+}$ entry (SOCE) involving the $Ca^{2+}$ release-activated $Ca^{2+}$ channel Orai1 [44–49]. Orai1-mediated SOCE impacts on many cellular functions including cell proliferation, differentiation, migration, and cytokine production [44,50–55]. SOCE is involved in a sort of feedback mechanism involving AMPK: SOCE activates AMPK through CaMKKβ. AMPK in turn inhibits SOCE [45]. Moreover, AMPK inhibits SOCE by regulating Orai1 membrane abundance (at least in UMR106 cells) [44,56].

AMPK is a major regulator of whole body energy homeostasis [10,12], impacting on a variety of organs including liver [57–61], skeletal [62–66] and cardiac muscle [67–73], kidney [74–77], and bone [78–80]. In the kidney, AMPK regulates epithelial transport, podocyte function, blood pressure, epithelial-to-mesenchymal transition, autophagy as well as nitric oxide synthesis [75,76,81–83]. Not surprisingly, AMPK is highly relevant for renal pathophysiology, including ischemia, diabetic renal hypertrophy, polycystic kidney disease, chronic kidney disease, and hypertension [40,67,74–76]. This review summarizes the contribution of AMPK to the regulation of renal transport and hence to the final composition of excreted urine. Moreover, pathophysiological implications are discussed.

## 2. AMPK and Renal Tubular Transport

The kidney is particularly relevant for fluid, electrolyte, and acid–base homeostasis. In addition, it is an endocrine organ producing different hormones such as erythropoietin, Klotho, and calcitriol, the active form of vitamin D [84–86]. The kidneys are made up of about 1 million nephrons, their functional elements. A nephron comprises the glomerulus surrounded by the Bowman´s capsule, the proximal tubule, Henle's loop, distal tubule, and the collecting duct. The primary urine is filtered in the glomerulus. Its composition is similar to plasma. In general, large molecules and particularly proteins >6000 Dalton are normally filtered to a low extent, if at all. The renal tubular system modifies the primary urine by reabsorbing or secreting ions and molecules, ultimately yielding the final urine [85–87]. Epithelial transport is mainly dependent on ATP-dependent pumps (primary-active), secondary-or tertiary-active transporters, as well as carriers and channels (passive, facilitated diffusion). Since active transport consumes energy by definition, it is not surprising that it is subject to regulation by AMPK. Moreover, even passive transport involving glucose transporter (GLUT) carriers is controlled by AMPK [74,75].

### 2.1. $Na^+/K^+$-ATPase

The ubiquitously expressed $Na^+/K^+$-ATPase is a primary active ATP-driven pump that mediates the basolateral extrusion of $3Na^+$ in exchange of $2K^+$, thereby establishing a transmembrane $Na^+$ gradient, which is the prerequisite for secondary active $Na^+$-dependent transport (e.g., through $Na^+$-dependent glucose cotransporter 1 and 2 (SGLT1/2), $Na^+/H^+$ exchanger isoform 1 (NHE1), $Na^+$-coupled phosphate transporter (NaPi-IIa), or $Na^+$-$K^+$-$2Cl^-$ cotransporter (NKCC2), as discussed below) [75,88–94]. Almost one-third of the body's energy is consumed by this pump [95]. Therefore, it does make sense that it is regulated by AMPK [74–76,94]: AMPK inhibits $Na^+/K^+$-ATPase in airway epithelial cells by promoting its endocytosis [96–100]. However, AMPK stimulates $Na^+/K^+$-ATPase membrane expression in skeletal muscle cells [101] and in renal epithelia [102], thereby counteracting renal ischemia-induced $Na^+/K^+$-ATPase endocytosis [103]. Interestingly, AMPKβ1 deficiency was found not to alter outcome in an ischemic kidney injury model in mice [104]. Hence, the effect of AMPK on $Na^+/K^+$-ATPase appears to be highly tissue-specific [74,75].

### 2.2. Proximal Tubule

A wide variety of luminal $Na^+$-dependent cotransporters, which are secondary active, are involved in epithelial transport in the proximal tubule. Secondary active transporters utilize the energy of the transmembrane $Na^+$ gradient generated by the primary active ATP-consuming $Na^+/K^+$-ATPase to facilitate transport of a substrate against its concentration gradient [105,106]. These transporters and the basolateral $Na^+/K^+$-ATPase consume substantial amounts of total cellular energy [74,75,107]. Hence, AMPK has been demonstrated to be an important regulator of proximal tubule transport [74,75].

#### 2.2.1. Glucose Transport

Since glucose is freely filtered by the glomerulus, glucose concentration in primary urine is similar to the plasma glucose concentration, whereas excreted urine is usually free of glucose [108–110]. The sugar is reabsorbed in the proximal tubule by the $Na^+$-dependent glucose cotransporter 1 and 2 (SGLT1 and 2), the different expression patterns and properties of which ensure total glucose reabsorption as long as the plasma glucose concentration is not abnormally high [89,108]. SGLT2 has a high transport capacity but low affinity for glucose and is predominantly expressed in the kidney, while SGLT1 is also expressed in other tissues including the small intestine. SGLT2 contributes to the reabsorption of up to 90% of filtered glucose [108,109,111,112]. On the other hand, AMPK-regulated SGLT1 [7,92,113] has a low transport capacity but high affinity for glucose and reabsorbs the remaining glucose [108–110,114,115]. Glucose leaves the basolateral membrane through passive glucose carriers GLUT1 and GLUT2 [108,116–118]. AMPK activates SGLT1-dependent glucose transport, presumably by stimulating membrane insertion of the cotransporter as observed in colorectal Caco-2 cells [92,119]. In line with this, AMPK activation is associated with increased *SGLT1* expression and glucose uptake in cardiomyocytes [113,120]. Although the AMPK-dependent regulation of SGLT1 in the proximal tubule has not explicitly been addressed, it is tempting to speculate that it is similar to other cell types [92,113,119,120]. The regulation of SGLT by AMPK is a doubled-edged sword: on the one hand, SGLT1-dependent reabsorption of glucose in proximal tubular cells requires energy which is generated by β-oxidation of fatty acids to a large extent [121,122]. On the other hand, it prevents the loss of energy-rich glucose [122,123], thereby maintaining the $Na^+/K^+$-ATPase-facilitated $Na^+$ gradient for $Na^+$-dependent transport and many other cellular processes [75,76]. SGLT1-mediated glucose uptake is linked to the GLUT1-dependent efflux at the basolateral side [108,116]. GLUT1 activity is stimulated by AMPK in various cell types [124–131]. Therefore, it is conceivable that renal GLUT1 might also be regulated by AMPK in order to save energy-providing glucose. In line with this, Baldwin et al. (1997) showed enhanced glucose uptake via GLUT1 in baby hamster kidney cells treated with AMPK activator 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) [132]. Moreover, Sokolovska et al. (2010) reported that metformin, another pharmacological AMPK activator, increased *GLUT1* gene expression in rat kidneys [133]. Also, AMPK activation was associated with enhanced activity of GLUT2. These studies, however, found reduced SGLT1 membrane abundance upon AMPK activation, at least in the case of murine intestinal tissue [134,135].

#### 2.2.2. $Na^+/H^+$ Exchanger Isoform 1

The ubiquitous $Na^+/H^+$ exchanger isoform 1 (NHE1) participates in cell volume and pH regulation by extruding one cytosolic $H^+$ in exchange for one extracellular $Na^+$ [136,137]. NHE1 is expressed in all parts of the nephron, including the proximal tubule. However, it cannot be detected in the macula densa and intercalated cells of the distal nephron [136,138,139]. In the proximal tubule, NHE1 is particularly important for $HCO_3^-$ reabsorption [140]. In hypoxia, anaerobic glycolysis is predominant, which results in intracellular accumulation of lactate and $H^+$ [90]. Acidosis, however, inhibits glycolysis [90,141,142] and would jeopardize cellular energy generation. AMPK-dependent stimulation of NHE1 activity in human embryonic kidney (HEK) cells therefore helps cells keep up anaerobic glycolysis in oxygen deficiency, as demonstrated by Rotte et al. (2010) [90]. Given that NHE1

is needed for proximal tubular $HCO_3^-$ reabsorption [140], AMPK may help retain $HCO_3^-$, thereby alleviating acidosis in energy deficiency and hypoxia.

#### 2.2.3. Creatine Transporter

In some organs with high metabolic activity, including skeletal muscle, heart, and brain, creatine is used to refuel cellular ATP levels [143–145]. In the proximal tubule, creatine, a small molecule that is freely filtered, is also reabsorbed through secondary active $Na^+$-dependent creatine transporter (CRT) (SLC6A8) [7,75,143,146]. AMPK has been demonstrated to downregulate CRT activity and apical membrane expression in a polarized mouse S3 proximal tubule cell line, presumably through mammalian target of rapamycin signaling [147]. The AMPK-dependent inhibition of CRT may help reduce unnecessary energy expenditure [75]. Conversely, AMPK stimulates CRT-mediated creatine transport in cardiomyocytes [148,149]. This again demonstrates that AMPK effects are tissue-specific [148].

#### 2.2.4. $Na^+$-Coupled Phosphate Transporter IIa

Inorganic phosphate is mainly reabsorbed by the secondary active $Na^+$-coupled phosphate transporter (NaPi-IIa) (SLC34A1) in the proximal tubule [93,150–152]. Employing electrophysiological recordings in *Xenopus* oocytes, it was shown that AMPK inhibits NaPi-IIa [93]. Kinetics analysis revealed that AMPK decreases NaPi-IIa membrane expression rather than changing its properties.

The regulation of phosphate metabolism by AMPK is not restricted to NaPi-IIa: Recently, AMPK was demonstrated to control the formation of bone-derived hormone fibroblast growth factor 23 (FGF23) [56], which induces renal phosphate excretion by extracellular-signal regulated kinases 1/2 (ERK1/2)-mediated degradation of membrane NaPi-IIa [150]. AMPK inhibits FGF23 production in cell culture and in mice [56]. Despite markedly elevated FGF23 serum levels in AMPKα1-deficient mice, renal phosphate excretion was not different from wild-type animals [56]. The same holds true for cellular localization of NaPi-IIa and renal ERK1/2 [56]. Thus, it is possible that AMPK deficiency is paralleled with some FGF23 resistance.

### *2.3. Loop of Henle*

#### 2.3.1. $Na^+$-$K^+$-$2Cl^-$ Cotransporter

The $Na^+$-$K^+$-$2Cl^-$ cotransporter (NKCC2), expressed in the thick ascending limb (TAL) of the loop of Henle and macula densa, is required for the generation of a hypertonic medullary interstitium, a mechanism needed for concentrating urine [75,76,88,91]. NKCC2 is a direct substrate of AMPK which phosphorylates it at its stimulatory serine residue Ser-126 [153]. Moreover, exposure of murine macula densa-like cells to low salt leads to AMPK activation and increased NKCC2 phosphorylation [154]. In addition, increased subapical expression (and apparent reduced apical expression) of NKCC2 in the medullary TAL of the loop of Henle along with elevated urinary $Na^+$ excretion in AMPKβ1-deficient mice on a normal salt diet were observed [155]. This is in line with AMPK being an important regulator of NKCC2-mediated salt retention in the medullary TAL of Henle [155]. Efe et al. (2016) recently observed markedly increased outer medullary expression of NKCC2 in rats treated with the AMPK activator metformin [156]. However, according to a recent in vivo study by Udwan et al. (2017), a low salt diet induced upregulation of NKCC2 surface expression in mouse kidneys but left AMPK activity unchanged [157]. Therefore, the exact role of AMPK in stimulating NKCC2 remains to be established.

#### 2.3.2. Renal Outer Medullary $K^+$ Channel

The apical renal outer medullary $K^+$ channel (ROMK) is required for NKCC2 to work properly, as it allows the recirculation of $K^+$ ions taken up by NKCC2 into the lumen [75,88]. AMPK is an inhibitor of ROMK by downregulating both channel activity and membrane abundance of the channel protein in a heterologous expression system using *Xenopus* oocytes [158]. In vivo studies revealed that the AMPK

effect on ROMK is relevant for the renal excretion of $K^+$ after an acute $K^+$ challenge, as upregulation of renal ROMK1 protein expression and the ability of $K^+$ elimination were more pronounced in AMPKα1-deficient than in wild-type mice [158].

### 2.4. Distal Tubule

#### 2.4.1. Cystic Fibrosis Transmembrane Conductance Regulator

The ATP-gated and cyclic AMP (cAMP)-dependent $Cl^-$ channel cystic fibrosis transmembrane conductance regulator (CFTR) participates in $Cl^-$ secretion and is broadly known for its role in cystic fibrosis, the pathophysiology of which is due to channel malfunction [74–76,159]. In the kidney, CFTR contributes to $Cl^-$ secretion in the distal tubule and the principal cells of the cortical and medullary collecting ducts [74,75,160]. AMPK has been demonstrated to inhibit CFTR-dependent $Cl^-$ conductance in *Xenopus* oocytes [159] and to decrease CFTR channel activity in the lung [161,162] and colon [163]. cAMP-stimulated cell proliferation and CFTR-dependent $Cl^-$ secretion play a decisive role for epithelial cyst enlargement in autosomal dominant polycystic kidney disease (ADPKD) [164]. In line with this, AMPK activation inhibits CFTR in Madin-Darby canine kidney (MDCK) cells [165] as well as decreases cystogenesis in murine models of ADPKD [165,166], suggesting a potential role for pharmacological AMPK activation in the treatment of ADPKD [165,166].

#### 2.4.2. $Ca^{2+}$ Transport

Most $Ca^{2+}$ is reabsorbed by passive paracellular diffusion along with other ions and water through tight junctions in the proximal tubule and the more distal parts of the nephron [88,167]. Conversely, only 5–10% of filtered $Ca^{2+}$ is reabsorbed by transcellular transport involving the apical transient receptor potential vanilloid 5 channel TRPV5 in the distal convoluted tubule [88]: $Ca^{2+}$ enters the cell through TRPV5, whereas basolateral $Ca^{2+}$ efflux is accomplished by the $Na^+/Ca^{2+}$ exchanger (NCX) and the $Ca^{2+}$-ATPase [88,167,168]. AMPK has been shown to inhibit NCX and decrease Orai1-mediated SOCE in murine dendritic cells [169]. Therefore, it is tempting to speculate that $Ca^{2+}$ reabsorption may be downregulated in the distal tubule in ATP deficiency [169,170]. Indeed, AMPK downregulates Orai1-dependent SOCE in T-lymphocytes [171], endothelial cells [45], and in osteoblast-like cells [56]. Since renal Orai1 activity contributes to kidney fibrosis [172], AMPK-mediated Orai1 downregulation may also be therapeutically desirable.

### 2.5. Collecting Duct

#### 2.5.1. Epithelial $Na^+$ Channel

In the collecting duct, fine tuning of $Na^+$ and $K^+$ homeostasis is accomplished by epithelial $Na^+$ channel (ENaC) and ROMK $K^+$ channel. Both channels are controlled by the renin-angiotensin-aldosterone system [173–175] regulating extracellular volume and hence arterial blood pressure [173–177]. $Na^+$ reabsorption by ENaC in the late distal convoluted tubule and cortical collecting duct principal cells is a highly energy-demanding process, as it utilizes the electrochemical driving force generated by the basolateral $Na^+/K^+$-ATPase [74–76,176,178]. AMPK inhibits epithelial $Na^+$ transport in various tissues, including lung [96,179], colonic [180], and renal cortical collecting duct cells [180–183]. In line with this, AMPKα1-deficient mice exhibit increased renal ENaC expression [180]. In detail, AMPK downregulates ENaC surface expression by inducing the binding of the ubiquitin ligase neural precursor cell expressed developmentally downregulated protein 4-2 (Nedd4-2) to ENaC subunits, resulting in ENaC ubiquitination with subsequent endocytosis and degradation [177,180,184]. In line with this, activation of AMPK enhances the tubuloglomerular feedback and induces urinary diuresis and $Na^+$ excretion in rats [185]. However, AMPKα1$^{-/-}$ mice with genetic kidney-specific AMPKα2 deletion exhibit a moderate increase in diuresis and natriuresis, possibly because NKCC2

activity is insufficient despite upregulated ENaC activity [186]. Taken together, AMPK activity limits ENaC-dependent energy-consuming $Na^+$ reabsorption [177,180,181,185].

#### 2.5.2. Voltage-Gated $K^+$ Channel

The voltage-gated $K^+$ channel (KCNQ1) is important for the cardiovascular system as well as for electrolyte and fluid homeostasis and is expressed in the distal nephron including the collecting duct [170,187–189]. Its exact role is ill-defined, although a contribution to cell volume regulation is postulated [75,187]. Similar to ENaC, AMPK inhibits KCNQ1 via Nedd4-2, as demonstrated in collecting duct principal cells of rat ex vivo kidney slices [187], MDCK cells [190], and *Xenopus* oocytes [191].

#### 2.5.3. Vacuolar $H^+$-ATPase

The primary active vacuolar $H^+$-ATPase (V-ATPase) is located at the apical membrane of proximal tubule cells and collecting duct type A intercalated cells. It contributes to the regulation of acid–base homeostasis by secreting $H^+$ ions into the tubular lumen [76,192,193]. AMPK inhibits the protein kinase A (PKA)-dependent membrane expression of V-ATPase in collecting duct intercalated cells of rat ex vivo kidney slices [193]. Moreover, epididymal proton-secreting clear cells, developmentally related to intercalated cells, exhibit reduced apical membrane abundance of V-ATPase after in vivo perfusion with the AMPK activator 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) into rats [194]. It appears to be likely that energy deficiency limits highly energy-consuming primary active $H^+$ excretion in the proximal tubule, whereas secondary active NHE1-dependent $H^+$ secretion is maintained, thereby keeping up at least anaerobic glycolysis [192]. The opposing effects of AMPK and PKA on V-ATPase expression and activity in kidney intercalated cells can be explained by different phosphorylation sites, as AMPK and PKA phosphorylate the A subunit at Ser-384 and Ser-175, respectively [195,196]. McGuire and Forgac (2018) further demonstrated that AMPK increases lysosomal V-ATPase assembly and activity in HEK293T cells under conditions of energy depletion [197]. In cells depleted of energy, acidification of autophagic intracellular compartments by V-ATPases enables the lysosomal degradation of proteins and lipids to generate energy substrates for ATP production [197,198]. Thus, it appears to be likely that AMPK-regulated V-ATPase activity depends on its concrete cellular localization and function [197].

#### 2.5.4. Water and Urea Handling

AMPK also regulates renal urea and water handling [76,199]. In the inner medullary collecting duct, osmotic gradients are generated by NKCC2 and urea transporter UT-A1 and water is reabsorbed through aquaporin 2 (AQP2) [76,156,199,200]. The concentration of urine requires the antidiuretic hormone vasopressin, which binds to vasopressin type 2 receptors of collecting duct principal cells, resulting in cAMP-mediated activation of PKA and subsequent phosphorylation and apical membrane insertion of AQP2 and UT-A1 [76,156,199]. Congenital nephrogenic diabetes insipidus (NDI) is a disease primarily caused by mutations of vasopressin type 2 receptors that is characterized by renal resistance to vasopressin and limited urine concentrating capacity [156,201]. According to two in vivo studies using rodent models of congenital NDI, the metformin-stimulated AMPK activation ameliorates the ability of the kidney to concentrate urine by increasing the phosphorylation and apical membrane expression of inner medullary AQP2 and UT-A1 [156,202]. In contrast, an ex vivo treatment of rat kidney slices with AICAR led to reduced apical membrane insertion of AQP2 [203]. Moreover, AMPK antagonizes the desmopressin-induced AQP2 phosphorylation in vitro, thus also suggesting an inhibitory function of AMPK on AQP2 regulation [203]. It appears likely that AMPK-independent effects of the pharmacological AMPK agonists contribute to this discrepancy [156,202,203]. Thus, further studies are clearly required.

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## 3. Conclusions and Perspectives

A growing list of studies indicates the pivotal role of AMPK as a metabolic-sensing regulator of a multitude of transport processes in the kidney [7,74–76,170]. Particularly, AMPK activation under conditions of energy deficiency is expected to differentially modulate renal epithelial ion transport in order to preserve cellular energy homeostasis (Figure 1) [7,74–76,94,170]. Alongside the above discussed function of AMPK in kidney tubular transport, a variety of other transport proteins, which are expressed in the kidney as well, are regulated by AMPK in extrarenal tissues [7,94,170,204] that are reviewed elsewhere [170] and [7] and summarized in Table 1. Future studies are required to focus on the therapeutic value of pharmacological AMPK manipulation to combat kidney disease [74–76,205,206].

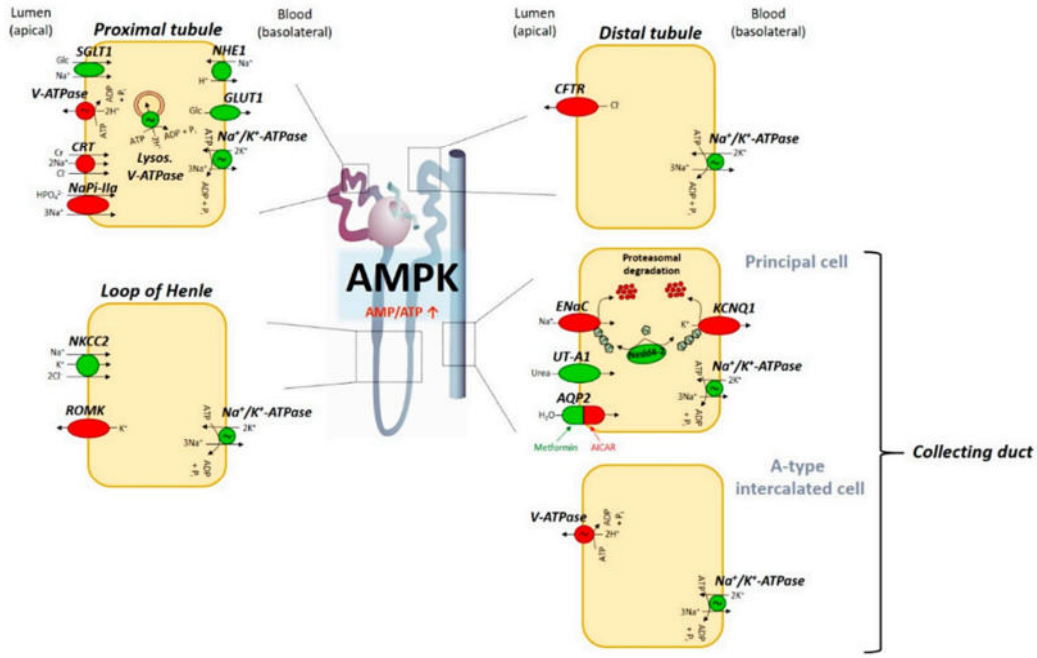


**Figure 1.** Tentative model illustrating AMPK-dependent effects on renal transport along the nephron. Cellular energy depletion (e.g., during hypoxia) leads to an elevated AMP/ATP ratio and subsequent AMPK activation. AMPK in turn regulates a multitude of active and passive epithelial transport processes along the renal tubular system in order to maintain cellular energy homeostasis. Ion channels, transport proteins, and ATPases that are activated upon AMPK stimulation are depicted as green icons, whereas red coloring indicates AMPK-dependent inhibition (see text for details). AMP, 5′-adenosine monophosphate; AMPK, AMP-activated protein kinase; SGLT1, $Na^+$-dependent glucose cotransporter 1; V-ATPase, vacuolar $H^+$-ATPase; CRT, creatine transporter; NaPi-IIa, $Na^+$-coupled phosphate transporter IIa; NHE1, $Na^+/H^+$ exchanger isoform 1; GLUT1, glucose transporter 1; NKCC2, $Na^+$-$K^+$-$2Cl^-$ cotransporter; ROMK, renal outer medullary $K^+$ channel; CFTR, cystic fibrosis transmembrane conductance regulator; ENaC, epithelial $Na^+$ channel; KCNQ1, voltage-gated $K^+$ channel; Nedd4-2, neural precursor cell expressed developmentally downregulated protein 4-2; UT-A1, urea transporter A1; AQP2, aquaporin 2.

**Table 1.** Overview of transport proteins regulated by AMPK in extrarenal tissues and evidence for renal expression.

| Ion Channel/Transporter and Method of Modifying AMPK Activity | AMPK Effect | Cell Type of Studied AMPK Effect/Ref. | Evidence for Renal Expression/Ref. |
|---|---|---|---|
| **Heterologous expression systems** | | | |
| **Kir2.1** | Reduction of channel activity and membrane abundance via Nedd4-2 mediated endocytosis | *Xenopus* oocytes [207] | Human proximal tubular cells [208] |
| **Kv1.5** | Reduction of channel activity and membrane abundance via Nedd4-2 mediated endocytosis | *Xenopus* oocytes [209] | Human kidney biopsies [210] |
| **Kv11.1 (hERG)** | Reduction of channel activity and membrane abundance via Nedd4-2 mediated endocytosis | *Xenopus* oocytes [211] | Human proximal and distal convoluted tubule [212] |
| **SMIT** | Reduction of channel activity | *Xenopus* oocytes [213] | Rat kidney medulla [214] |
| **BGT1** | Reduction of channel activity | *Xenopus* oocytes [213] | Human kidney inner medulla [215] and mouse kidney medulla (basolateral membranes of collecting ducts and TAL of Henle) [216] |
| **EAAT3** | Reduction of channel activity and membrane abundance | *Xenopus* oocytes [217] | Mouse renal proximal tubule [218] |
| **NCX** | Reduction of channel activity and membrane abundance | *Xenopus* oocytes [169] | Rat distal convoluted tubule [219] |
| **$K_{2P}$10.1 (TREK-2)** | Inhibition of channel activity via phosphorylation at Ser-326 and Ser-359 | HEK293 cells [220] | Human proximal tubule [221] |
| **$K_{Ca}$1.1** | Increase in channel activity and membrane abundance | *Xenopus* oocytes [222] | Human clear cell renal cell carcinoma (ccRCC) and healthy kidney cortex [223] |
| **Pharmacological Manipulation** | | | |
| **$K_{Ca}$1.1** | Inhibition of channel activity | Rat carotid body type I cells [224] | |
| **Kir6.2** | Upregulation of channel activity<br>Up- or down-regulation of channel activity | Rat cardiomyocytes [225]<br>Rat pancreatic beta-cells [226,227] | Rat renal tubular epithelial cells [228] |
| **KCa3.1** | Reduction of channel activity | Human airway epithelial cells [229] | Human proximal tubular cells [230] |
| **MCT1 and MCT4** | Upregulation of mRNA expression | Rat skeletal muscle [231] | MCT1: basolateral membrane of mouse proximal tubular epithelial cells [232]<br>MCT4: human ccRCC [233] |
| **PepT1** | Downregulation of channel activity and brush-border membrane abundance | Caco-2 cells [234] | Rat renal proximal tubule [235] |
| **Orai1** | Downregulation of cell membrane abundance and SOCE | Rat UMR106 osteoblast-like cells [56] | Rat glomerular mesangial cells [236] |
| **Genetically Modified Mouse Models** | | | |
| **Orai1** | | Mouse T-lymphocytes [171]<br>Mouse dendritic cells [169] | |

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## Abbreviations

| | |
|---|---|
| ADP | Adenosine diphosphate |
| ADPKD | Autosomal dominant polycystic kidney disease |
| AMPK | 5′-adenosine monophosphate (AMP)–activated protein kinase |
| AQP2 | Aquaporin 2 |
| ATP | Adenosine triphosphate |
| BGT1 | Betaine/γ-aminobutyric acid (GABA) transporter 1 |
| CaMKKβ | $Ca^{2+}$/calmodulin–dependent protein kinase kinase β |
| cAMP | Cyclic adenosine monophosphate |
| ccRCC | Clear cell renal cell carcinoma |
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| CRT | Creatine transporter |
| EAAT3 | Excitatory amino acid transporter 3 |
| ENaC | Epithelial $Na^+$ channel |
| ERK1/2 | Extracellular-signal regulated kinases 1/2 |
| FGF23 | Fibroblast growth factor 23 |
| GLUT | Glucose transporter |
| HEK | Human embryonic kidney cells |
| hERG | Human ether-a-go-go-related gene |
| Kca | $Ca^{2+}$ activated $K^+$ channels |
| KCNQ1 | Voltage-gated $K^+$ channel |
| Kir | Inwardly rectifying $K^+$ channels |
| Kv | Voltage gated $K^+$ channels |
| LKB1 | Liver kinase B1 |
| MCT | Monocarboxylate transporters |
| MDCK | Madin-Darby canine kidney cells |
| NaPi-IIa | $Na^+$-coupled phosphate transporter |
| NCX | $Na^+/Ca^{2+}$ exchanger |
| NDI | Nephrogenic diabetes insipidus |
| Nedd4-2 | Neural precursor cell expressed developmentally down-regulated protein 4-2 |
| NHE1 | $Na^+/H^+$ exchanger isoform 1 |
| NKCC2 | $Na^+$-$K^+$-$2Cl^-$ cotransporter |
| PepT1 | $H^+$-coupled di- and tripeptide transporter 1 |
| PKA | Protein kinase A |
| ROMK | Renal outer medullary $K^+$ channel |
| SGLT | $Na^+$-dependent glucose cotransporter |
| SMIT | $Na^+$ coupled myoinositol transporter |
| SOCE | Store-operated $Ca^{2+}$ entry |
| TAL | Thick ascending limb |
| TREK-2 | Tandem pore domain $K^+$ channel 2 |
| TRPV5 | Transient receptor potential vanilloid 5 channel |
| UT | Urea transporter |
| V-ATPase | Vacuolar $H^+$-ATPase |

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# 4 Discussion

## 4.1 The role of TNFα in driving HFD-induced FGF23 production

Deciphering the complex mechanisms underlying the regulation of FGF23 synthesis comprises a vivid field of ongoing research, yet the knowledge of the interplay of systemic factors and intracellular signaling pathways involved is still fragmentary. Although the exact pathomechanisms remain to be elucidated, a growing body of evidence indicates that inflammation is a major trigger of FGF23 formation, particularly in disease states including CKD [36, 170, 209, 210, 215]. Amongst other inflammation-related markers associated with CKD progression [209, 210, 257], the pro-inflammatory cytokine TNFα is recognized as a potent stimuli of FGF23 synthesis and secretion [36, 170].

Excess body weight in obesity is, along with other determinants such as hypertension and diabetes [258], considered as an important risk factor for the onset and disease progression in CKD [153, 154, 174]. It is commonly accepted that obesity, a feature of the metabolic syndrome [253], is characterized by systemic low-grade inflammation [252, 259–261]. Beyond its classical function as an energy storage depot, adipose tissue (AT) is considered as an endocrine organ, capable to secrete a multitude of bioactive molecules, collectively referred to as adipokines [262]. These factors are involved in diverse biological processes (e.g., glucose metabolism, insulin signaling, appetite and satiety regulation, inflammation, and angiogenesis) exhibiting local (autocrine/paracrine) effects within the AT or endocrine actions as they are secreted into the circulation [261–263]. In obese states of particular excess visceral adiposity, enlargement of adipocytes, owing to inappropriate lipid accumulation and subsequent cell hypertrophy, causes a shift of the adipocyte secretion profile towards an exaggerated production of pro-inflammatory adipokines including TNFα, IL-6, and monocyte chemoattractant protein-1 (MCP-1) [254, 260, 263, 264]. On the contrary, the secretion of anti-inflammatory and insulin-sensitizing adipokines, such as adiponectin, is downregulated in obesity [260, 265, 266]. These pro-inflammatory conditions prime the migration of immune cells, particularly macrophages, to the AT [260, 267]. In turn, infiltrated macrophages synergistically release inflammatory cytokines such as TNFα and IL-6, thereby aggravating the inflammatory microenvironment within the AT that propagates systemically and plays a decisive role in the development of obesity-related comorbidities including type 2 diabetes and cardiovascular disease [252, 253, 260, 262, 263]. In addition, nutrient overload in obesity and accompanied metabolic inflammation is not restricted to the AT. In fact, the liver is another main site of increased TNFα production in obese states [268]. Along those lines, obesity-related hepatic inflammation is considered as an important trigger for the development of nonalcoholic steatohepatitis (NASH) and liver cirrhosis [251, 269].

A dysregulated secretion of pro-inflammatory adipokines as observed in obesity has also been implicated in the progression and severity of CKD, with TNFα being one of the most critical mediators in this context [153, 270–273]. In fact, Roubicek *et al.* (2009) reported higher amounts of subcutaneous and visceral AT *TNFα* mRNA expression levels as well as elevated serum concentrations of TNFα, IL-6, MCP-1, and CRP in pre-obese patients with advanced CKD, namely ESRD, compared to healthy control subjects [272]. Corroborating these findings, Teplan and co-workers (2010) detected increased AT *TNFα* transcripts and increments in circulating TNFα in obese CKD patients versus a non-obese CKD group [273]. Along those lines, the aberrant release of pro-inflammatory cytokines in HFD-induced obesity may promote renal injury and fibrotic remodeling of kidney tissue which contributes to progressive renal failure in CKD development [153, 274]. Moreover, systemic elevations of TNFα, as seen in obesity, are further implicated in the pathogenesis of insulin resistance and type 2 diabetes, both important risk factors promoting CKD [150, 153, 271]. Taken together, both obesity and CKD are suggested to be interlinked by common features of chronic subclinical inflammation, indicating that both pathologies are mutually dependent [270, 273].

Furthermore, several clinical trials have demonstrated positive associations between criteria of the metabolic syndrome, including excess body weight, and increased systemic FGF23 levels [171–173], the latter of which are a hallmark of deteriorating kidney function in CKD progression [117, 159]. In view of the aforementioned remarks, one might suggest a putative key role of subclinical inflammation linking obesity and CKD with elevated FGF23 production. In this regard, the purpose of **Paper 1** was to explore the role of low-grade inflammation-associated TNFα production in HFD-induced FGF23 generation. Likewise to recent observations in rats [275, 276], HFD feeding elicited a substantial surge in the serum FGF23 concentration in wild-type ($tnf^{+/+}$) mice. These findings prompted the question whether obesity-related inflammation, as it is reported in mice after HFD administration [268, 277–279], triggered the upregulation of FGF23 formation in $tnf^{+/+}$ mice. To this end, HFD treatment indeed significantly elevated liver and, at least by tendency, AT *Tnfα* mRNA expression levels in $tnf^{+/+}$ animals (**Paper 1**), reflecting the induction of inflammatory processes, resembling a hallmark feature associated with the metabolic syndrome [252, 253]. These findings were somewhat remarkable as HFD-induced inflammation was evident after a relatively short period of only three weeks of HFD feeding. In contrast, other investigations using similar murine models observed features of HFD-associated hepatic and AT inflammation along with increments in circulating TNFα levels only after a longer duration of respective dietary intervention (e.g., after at least 16 weeks) [268, 274, 277].

Next, **Paper 1** revealed that low concentrations of TNFα (0.5-10 ng/ml), mimicking low-grade inflammatory conditions as apparent in obesity [261, 274, 277], elicited a dose-dependent upregulation of *Fgf23* transcription in rat UMR106 osteoblast-like cells.

Corroborating this, Ito *et al.* (2015) observed similar stimulating effects of low-dose TNFα and other pro-inflammatory cytokines on *Fgf23* mRNA expression in mouse IDG-SW3 osteocyte-like cells [36]. TNFα is a known activator and likewise target gene of the inflammation-related transcription factor NF-κB [217, 218], whose intact signaling has been shown to be essential for triggering TNFα-induced *Fgf23* gene expression in the aforementioned study [36]. In addition, it has recently been demonstrated that the upregulation of *Fgf23* transcription in UMR106 cells is mediated by NF-κB-sensitive SOCE, a pivotal mechanism regulating FGF23 formation, particularly facilitated by the CRAC channel protein Orai1 [226]. Hence, it is tempting to speculate that the surge in *Fgf23* transcript levels observed in TNFα-treated UMR106 cells was, at least in part, facilitated by NF-κB-mediated Orai1-dependent SOCE. Therefore, it appears plausible to address this putative mechanism in future investigations.

It is further reported that pro-inflammatory stimuli, including TNFα, IL-1β, and LPS, are capable to substantially induce *Fgf23* expression in IDG-SW3 cells by concomitant downregulation of negative transcriptional regulators of *Fgf23*, namely *Phex*, *Dmp1,* and *Enpp1* [36]. Consistent with this, a recent study employing primary cultures of murine osteocytes also confirmed that TNFα is a potent trigger of *Fgf23* mRNA expression, at least partly, by blunting the expression of the *Fgf23*-suppressor *Dmp1* [170]. According to these data, it is conceivable that the TNFα-mediated upregulation of *Fgf23* transcripts in UMR106 cells (**Paper 1**) was potentially attributed to inhibitory effects of TNFα on transcriptional suppressors of *Fgf23*. It is further worth to mention that it has not been analyzed in this thesis whether the stimulation of *Fgf23* transcription upon incubation with TNFα translated into enhanced FGF23 protein formation in UMR106 cells. However, TNFα is expected to increase FGF23 protein expression. This notion is supported by Ito *et al.* (2015) who detected amplified C-terminal FGF23 protein secretion in the supernatant of TNFα-treated IDG-SW3 cells, whereas intact FGF23 was unaffected due to increased cleavage by furin-like proprotein convertases [36]. Notably, TNFα triggered *Galnt3* mRNA expression in this setting and in primary murine osteocytes, thus rather suggesting facilitated *O*-glycosylation and thereby stabilization of intact FGF23 than increased degradation [36, 170]. Along those lines, wild-type mice were shown to exhibit increased plasma intact FGF23 concentrations when injected with TNFα [170]. In view of the discrepancies between the aforementioned studies, further research is warranted to clarify the conceivably differential role of TNFα in the transcriptional and post-translational regulation of FGF23 synthesis *in vitro* and *in vivo*.

The pivotal role of TNFα in HFD-induced FGF23 generation postulated in **Paper 1** was further corroborated utilizing gene-targeted TNFα-deficient ($tnf^{-/-}$) mice. Indeed, it could be demonstrated that the stimulatory effect of HFD feeding on FGF23 production was markedly suppressed in $tnf^{-/-}$ mice compared to $tnf^{+/+}$ animals, indicating the relevance of TNFα in HFD-mediated FGF23 formation. However, a significant surge in the serum FGF23 levels of HFD-

fed TNFα-deficient mice compared to the control situation could also be observed. Hence, the underlying mechanisms driving the HFD-induced FGF23 generation in these genetically modified mice appeared not only to be TNFα-mediated. Therefore, apart from TNFα, a number of other mediators implicated in obesity-related low-grade inflammation might also be involved in this context. In this regard, recent evidence from both *in vitro* and *in vivo* studies suggests that the pro-inflammatory cytokines IL-6 and IL-1β, which are upregulated in inflammatory disorders such as HFD-induced obesity and CKD [209, 210, 262–264, 277, 278], are potent stimulators of FGF23 formation [36, 213]. Moreover, HFD exposure to mice and rats is reported to result in the formation of AGEs [280, 281], which are non-enzymatic glycation products of proteins or lipids implicated in aging and the pathogenesis of metabolic diseases such as diabetes [214, 280]. According to Li *et al.* (2005), HFD-induced AGE-generation is further accompanied by enhanced cardiac and hepatic p38MAPK activation [280]. Intriguingly, both AGEs and p38MAPK have recently been described as stimulators of FGF23 formation, putatively mediated by the pro-inflammatory transcription factor NF-κB [214, 220]. These data thus indicate another possible link by which HFD feeding conferred FGF23 upregulation in $tnf^{-/-}$ mice (**Paper 1**).

In addition, insights from several *in vivo* investigations suggest that the kidneys are susceptible to the detrimental effects of high-caloric HFD administration as displayed by the induction of renal inflammation and fibrotic remodeling [271, 274, 282–284]. In more detail, HFD feeding has been shown to be associated with renal upregulation of the pro-fibrotic marker TGF-β1, which is also regarded as a pro-inflammatory adipokine [263, 285], along with decreased activity of the cellular energy sensor AMPK [274, 282, 284]. As a matter of fact, the isoform TGF-β2 has lately been revealed to induce FGF23 synthesis in UMR106 cells [227], and within the scope of this thesis, AMPK suppression has been identified as a novel mechanism triggering FGF23 production (**Paper 2**). Given these data, it appears to be likely that pro-fibrotic mechanisms (possibly mediated by TGF-β1 similar to TGF-β2 [227]), pro-inflammatory signaling pathways, and detrimental effects on AMPK activity synergized in the HFD-induced upregulation of FGF23 formation in $tnf^{+/+}$ mice and particularly in $tnf^{-/-}$ animals as observed in **Paper 1**. However, further investigations addressing these potential intertwined mechanisms are necessary.

The results obtained from **Paper 1** demonstrated the significance of TNFα and the respective induction of inflammation in the augmented FGF23 production triggered by an energy-dense HFD. While there is solid experimental evidence that inflammation is a major trigger of FGF23 formation [36, 170, 209], recent data indicate that FGF23 itself is capable to induce pro-inflammatory cytokine production from hepatocytes [81, 111], cardiomyocytes [195], and immune cells [83, 112]. In detail, Mattinzolli *et al.* (2018) observed increased mRNA expression of *TNFα* and *IL-6* in cultured hepatocytes in response to FGF23 administration

[111], an effect preferably mediated *via* the FGFR4-PLCγ-calcineurin-NFAT signaling pathway independent of α-Klotho [81]. It has further been shown that FGF23 promotes TNFα production in macrophages [83, 112]. Along those lines, liver tissue contains specialized resident phagocytic macrophages called Kupffer cells capable to secrete pro-inflammatory cytokines such as TNFα [113]. Furthermore, nutrient overload in obesity – as mimicked by HFD feeding in **Paper 1** – is reported to lead to hepatic macrophage recruitment along with increased TNFα production [251, 268, 286]. Although it has yet to be determined, it could be hypothesized that the surge in liver *Tnfα* transcripts displayed in HFD-fed tnf$^{+/+}$ mice (**Paper 1**) may not only be attributed to the high-caloric dietary intervention, but also, at least to some extent, secondarily mediated by the observed elevated FGF23 generation, that would have, in turn, potentially provoked hepatocyte- and macrophage-derived TNFα production in the liver of the respective animals. Vice versa, increased hepatic TNFα formation is expected to accelerate osseous FGF23 secretion, implicating a vicious cycle that aggravates both systemic inflammation and excess FGF23 levels as seen in patients with obesity and CKD [81, 171, 175]. Furthermore, Mattinzolli *et al.* (2018) showed that FGF23 is expressed in the liver but not secreted [111], thus likely playing an autocrine and/or paracrine role in hepatic tissue acting *via* the FGFR4-PLCγ-calcineurin-NFAT signaling pathway as discussed above [81] and similar to what has been suggested for myocardiac tissue [108]. Notably, intact calcineurin-NFAT signaling is reported to be crucial for FGF23 synthesis, at least in UMR106 cells [221]. Although it has to be explored in future studies, it can be speculated that FGF23 production in the liver, once initially triggered by local inflammatory stimuli such as TNFα (**Paper 1**), might promote its own synthesis in a potential paracrine feed-forward loop involving the calcineurin-NFAT signaling pathway and thereby would likewise affect hepatic TNFα production.

As outlined above, body fat accumulation in obesity is accompanied by pro-inflammatory cytokine secretion by both adipocytes and macrophages infiltrated in the AT [260, 267, 279]. Intriguingly, there is evidence that FGF23 is expressed by both adipocytes [287] and pro-inflammatory macrophages [83] and is additionally capable to induce TNFα production in macrophages [83, 112]. Similar to what has been afore postulated for the liver, it appears reasonable that the HFD-induced tendency upon elevated AT *Tnfα* transcripts in tnf$^{+/+}$ mice (**Paper 1**) was possibly attributed to, at least in part, reciprocal effects of upregulated FGF23 formation towards stimulated TNFα production in both adipocytes and macrophages within the AT. Whether there are interregulatory autocrine and/or paracrine effects of FGF23 contributing to potential pro-inflammatory feed-forward loops involving different cell types within the AT of HFD-fed mice, and possibly in obese subjects, is currently unknown and thus needs to be addressed in future studies.

It is well documented that FGF23 acts as a suppressor of circulating calcitriol levels by controlling renal expression of key enzymes of calcitriol synthesis and degradation [34, 47, 48].

As expected, and similar to what has recently been observed in HFD-fed rats [275, 276], HFD administration resulted in significantly lower serum calcitriol concentrations in both genotypes compared to mice on control diet (**Paper 1**). This finding could be most likely explained by a reduction in calcitriol synthesis secondary to the increased FGF23 generation triggered by HFD feeding [275, 276]. Considering the high prevalence of vitamin D deficiency in obesity and associated deleterious health effects particularly on the cardiovascular system [288–290], the modulation of circulating calcitriol levels by HFD-stimulated FGF23 formation may be of high medical relevance [291].

Given that FGF23 induces phosphaturia and is therefore expected to elicit hypophosphatemia [34, 48, 49, 51], it is remarkable that HFD administration in both genotypes increased circulating FGF23 levels without altering serum phosphate levels (**Paper 1**). Similarly, Raya *et al.* (2016) also revealed no changes in urinary phosphate excretion and serum phosphate concentrations despite increments in circulating FGF23 levels in rats received a HFD [275]. Accordingly, the authors detected reduced protein expression of α-Klotho, the obligate co-receptor for FGF23, in the kidneys of HFD-exposed rats, suggesting renal resistance to the phosphaturic action of FGF23 elicited by HFD feeding, and thus explaining the absence of hypophosphatemia [275]. Given these data, it is conceivable that similar mechanisms were responsible for the lack of hypophosphatemia observed in the present study (**Paper 1**). However, additional investigations by means of assessing functional parameters related to the phosphaturic action of FGF23, such as determining urinary phosphate excretion as well as detecting renal protein abundance of NaPi-IIa and α-Klotho in HFD-fed mice, are warranted to provide additional mechanistic insights into the tight regulation of phosphate homeostasis in view of the HFD-stimulated FGF23 upregulation demonstrated in **Paper 1**. Future research should also contemplate other animal models with CKD-associated features or rather consider clinical trials in order to expand the relevance of HFD-induced FGF23 production in the context of progressive renal failure in CKD [292].

It is important to mention that HFD feeding for only 3 weeks caused a significant but – compared to other studies that used longer periods of HFD exposure [268, 274] – moderate increase in total body weight (**Paper 1**). Furthermore, the weight gain in $tnf^{+/+}$ mice was more pronounced than in gene-targeted mice devoid of TNFα. Intriguingly, the body weight of $tnf^{-/-}$ mice was almost unchanged after three weeks of HFD feeding as the animals displayed a relative weight gain by only some 0.7%. It is therefore remarkable that an energy-dense HFD was sufficient to substantially increase the formation of FGF23 in $tnf^{-/-}$ mice despite the absence of clear indicators of obesity. It appears essential to consider these somewhat contradictory findings along with the dietary composition of the HFD (purchased from Altromin, Lage, Germany; C1090-70; https://altromin.com/pdf/en/C1090-70foao) used in the present study. In fact, this commercially available HFD contains 70% energy from fat primarily derived

from saturated fatty acids (SFAs) such as palmitic and stearic acid. These lipid compounds, particularly palmitic acid, are known activators of NF-κB as demonstrated in AT, adipocytes, and macrophages, thereby fostering the production of pro-inflammatory cytokines, such as IL-6 [293–295], which is, in turn, a potent stimulator of FGF23 synthesis [213]. Future examinations should therefore aim to assess circulating IL-6 levels in HFD-fed tnf$^{-/-}$ mice.

Another possible explanation by which the SFA palmitic acid may have contributed to the exaggerated FGF23 production in HFD-fed tnf$^{-/-}$ mice might be considered in the context of its impact on the anti-inflammatory and insulin-sensitizing adipokine adiponectin [260, 263]. In fact, palmitic acid inhibits adiponectin gene and protein expression in cultured adipocytes [296, 297]. Corroborating this, diets rich in SFAs are reported to lower circulating adiponectin levels both in rodents and tendentially in humans [296, 298, 299]. In light of the fact that adiponectin is known to activate the cellular energy sensor AMPK [300, 301], which has been revealed as a negative regulator of FGF23 synthesis within the scope of this thesis (**Paper 2**), it is tempting to speculate that the elevated serum FGF23 levels observed in HFD-fed tnf$^{-/-}$ mice (**Paper 1**) might be, at least in part, due to the repression of adiponectin production induced by palmitic acid present in the diet. Assuming this, reduced adiponectin secretion would then be expected to attenuate AMPK activation, which would, in turn, lead to enhanced FGF23 formation. Nonetheless, it is a task for future studies to better characterize the impact of distinct nutritional compounds, particularly dietary fatty acids, in the regulation of FGF23 formation.

Taken together, the data presented in **Paper 1** confirmed the crucial role of TNFα and the respective induction of low-grade inflammatory processes in the elevated FGF23 production induced by HFD feeding. These novel insights strengthen the concept that the increments in circulating FGF23 levels in subjects displaying pathologic derangements associated with chronic inflammation as observed in obesity and CKD [170–172, 210] are in large part attributed to the pro-inflammatory cytokine TNFα. In light of its pathophysiological implications in renal, metabolic, and cardiovascular diseases [155, 172], monitoring of circulating FGF23 concentrations in obese subjects may serve as a reasonable clinical indicator in the risk assessment of obesity and its related complications including CKD [153, 171].

## 4.2 AMPK – a novel regulator of FGF23 production

AMPK is an ubiquitously expressed serin/threonine protein kinase and serves as a master sensor of cellular energy homeostasis [236, 237]. Under conditions of energy deficiency – indicated by an increased intracellular AMP/ATP and ADP/ATP ratio – AMPK is activated to restore cellular energy balance by promoting catabolic processes associated with

ATP production (e.g., glycolysis and fatty acid oxidation) [236, 238–241, 302]. Vice versa, anabolic and energy-consuming pathways, such as gluconeogenesis, lipogenesis, and protein synthesis, are downregulated upon AMPK activation [238, 242, 243]. Besides its pivotal role in the regulation of energy metabolism, AMPK is highly relevant in various pathologies including CKD [255, 256, 284], cardiac hypertrophy [303–305], and diabetes [306], to name a few, and thus appears as a promising pharmacological target in various disease settings [255, 306, 307].

With regard to kidney pathophysiology, growing evidence from experimental CKD models indicates that renal AMPK activity is downregulated in CKD [256, 308, 309]. Considering the fact that obesity represents a major risk factor for CKD [153], it is suggested that nutrient overload causes detrimental effects on AMPK activity in renal and extrarenal tissues [307, 310]. Along those lines, *in vivo* studies using animal models of obesity-induced kidney injury have demonstrated fibrotic and inflammatory remodeling accompanied with suppressed AMPK activity in the kidneys of HFD-fed mice [284, 311]. Vice versa, pharmacological activation of AMPK by metformin exerts renoprotective effects and ameliorates renal fibrosis and inflammation induced by HFD administration [311]. Based on the findings that energy excess in terms of HFD feeding markedly stimulated FGF23 formation in mice (**Paper 1**) and given that elevated FGF23 levels are a clear indicator of declining kidney function in CKD [100, 119], the second study presented in this thesis sought to investigate the role of AMPK in the regulation of FGF23 synthesis and its pathophysiological implications in the context of CKD.

As a prerequisite to study the influence of AMPK on FGF23 formation *in vitro*, it could be demonstrated that rat UMR106 osteoblast-like cells express functional heterotrimeric AMPK as determined by a distinct subunit expression pattern (**Paper 2**). Based on this, pharmacological activation of AMPK by 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) [312] markedly decreased *Fgf23* gene expression, whereas AMPK inhibition by compound C [310] as well as joint siRNA-mediated gene silencing of the catalytic subunits *Ampkα1* and *Ampkα2* significantly increased *Fgf23* transcription (**Paper 2**). Corroborating these *in vitro* findings, it could be revealed that gene-targeted mice devoid of functional *Ampkα1* (ampk$^{-/-}$ mice) display substantially higher bone *Fgf23* transcript levels compared to their wild-type (ampk$^{+/+}$) counterparts. As the bone represents the major source of circulating FGF23 [75], elevated osseous *Fgf23* gene expression in ampk$^{-/-}$ mice consequently translated into significantly higher serum concentrations of C-terminal and intact FGF23 compared to ampk$^{+/+}$ mice. These findings clearly demonstrate that AMPK functions as a hitherto unknown regulator of FGF23 formation both *in vitro* and *in vivo*.

In an attempt to elucidate the underlying mechanism involved in the AMPK-mediated downregulation of *Fgf23* gene expression in UMR106 cells, it was explored whether AMPK

stimulation affects Orai1-sensitive SOCE, a mechanism that has been verified as an essential feature in the regulation of FGF23 in multiple studies [196, 207, 226, 227]. Similar to previous observations in other cell types [249, 313, 314], AMPK activation by AICAR significantly attenuated SOCE as estimated by Fura-2-dependent intracellular $Ca^{2+}$ imaging (**Paper 2**). As described earlier, the cellular machinery accomplishing SOCE involves oligomerization of the ER $Ca^{2+}$ depletion-sensing protein STIM1 and subsequent activation of the cell membrane-located CRAC channel protein Orai1 [228, 231]. As a matter of fact, cell membrane abundance of Orai1 was diminished in UMR106 cells stimulated with the AMPK activator AICAR (**Paper 2**). Confirming this, T-lymphocytes and dendritic cells from $ampk^{-/-}$ mice are reported to exhibit significantly higher cell surface expression of Orai1 along with increased SOCE compared to cells isolated from $ampk^{+/+}$ mice [313, 314].

Membrane-bound Orai1 is known to be subjected to ubiquitination and subsequent proteasomal degradation facilitated by the ubiquitin ligase neural precursor cell expressed developmentally down-regulated protein 4-2 (Nedd4-2) [315, 316]. Along those lines, AMPK has been shown to stimulate Nedd4-2 and thereby fosters the degradation of various ion channels including the epithelial $Na^+$ channel (ENaC) [317, 318] and the voltage-gated potassium ($K^+$) channel KCNQ1 ($K^+$ voltage-gated channel subfamily Q member 1) [319]. In light of these data, it is conceivable that AMPK enhanced Nedd4-2-mediated endocytic retrieval of Orai1 from the plasma membrane and was thereby effective to reduce SOCE in UMR106 cells. Further *in vitro* approaches employing siRNA-targeted gene silencing of endogenous *Nedd4-2* and subsequent assessment of SOCE-dependent $Ca^{2+}$ influx upon treatment with AICAR would help to verify this hypothesis. In addition, **Paper 2** clarified that pharmacological blockage of SOCE by 2-aminoethoxydiphenyl borate (2-APB) and *N*-propylargylnitrendipine (MRS 1845) as well as specific Orai1 inhibition by AnCoA4 significantly blunted the stimulatory effect of compound C-mediated AMPK inhibition on *Fgf23* transcription. These data clearly demonstrate that the inhibitory effect of AMPK on *Fgf23* gene expression in UMR106 cells is in large part achieved by attenuation of Orai1-sensitive SOCE. Confirming this, activation of the anti-inflammatory transcription factor peroxisome proliferator-activated receptor α (PPARα) by fenofibrate has most recently been shown to suppress *Fgf23* gene expression in UMR106 cells *via* AMPK-mediated downregulation of SOCE [320].

Although it has not been addressed in the present study, it can be hypothesized that the pro-inflammatory transcription factor NF-κB might participate as a molecular link mediating the inhibitory effect of AMPK on *Fgf23* gene transcription. In fact, NF-κB activation contributes to inflammatory cytokine-induced FGF23 formation in IDG-SW3 osteocytes [36]. Moreover, NF-κB is reported to stimulate the gene expression of *Orai1* and its activator *STIM1* [321]. Along those lines, *Fgf23* gene expression in UMR106 cells is upregulated by NF-κB-sensitive Orai1-dependent SOCE [226]. As AMPK is a known suppressor of NF-κB [322, 323], it can be

speculated that the inhibitory effect of AMPK on Orai1-sensitive SOCE and the concomitant downregulation of FGF23 formation in AICAR-treated UMR106 cells was, at least in part, due to AMPK-mediated suppression of NF-κB.

Cytosolic $Ca^{2+}$ influx *via* SOCE is related to calcineurin activation and downstream dephosphorylation and nuclear translocation of the transcription factor NFAT [231, 235]. Of note, inhibition of calcineurin-NFAT signaling has recently been shown to decrease FGF23 production in UMR106 cells [221], indicating that calcineurin serves as a stimulatory regulator of FGF23 formation. Furthermore, calcineurin is reported to suppress AMPK in cardiomyocytes [324]. Along those lines, inhibition of AMPK by pharmacological manipulation, siRNA-mediated gene silencing, as well as *Ampkα1* ablation in genetically modified mice induced FGF23 formation both *in vitro* and *in vivo* (**Paper 2**). Hence, decreased intracellular $Ca^{2+}$ influx *via* AMPK-mediated downregulation of Orai1-dependent SOCE and subsequent suppression of *Fgf23* gene expression in UMR106 cells might, at least theoretically, be associated with attenuated calcineurin activation, which would, in turn, be expected to decrease FGF23 formation [221]. This notion would suggest a putative crosstalk between AMPK and calcineurin-NFAT signaling involving a negative feedback loop regulating FGF23 synthesis based on Orai1-sensitive SOCE. Notably, this hypothesis is supported by recent *in vivo* data from Choi *et al.* (2018) who observed that Orai1-deficient mice display significantly decreased gene expression of osteocytic *Fgf23* and concomitantly reduced nuclear localization and transcriptional activity of NFAT1c in corresponding primary calvarial osteoblasts [325]. Nevertheless, additional studies are necessary to confirm a potential interaction of AMPK and the calcineurin-NFAT axis in the regulation of FGF23 synthesis.

The notion that AMPK may participate in a negative feedback regulation of cytosolic $Ca^{2+}$ activity [313] is further corroborated by findings of Sundivakkam and co-workers (2013) [249]. In this study, SOCE activation in endothelial cells caused AMPK activation and subsequent phosphorylation of ER-localized STIM1, which, in turn, terminated SOCE [249]. Consistent with this, AICAR-stimulated AMPK activation downregulated SOCE in UMR106 cells (**Paper 2**). As AMPK is activated upon cellular energy stress [236, 238], it fits the concept that AMPK limits cytosolic $Ca^{2+}$ influx in states of energy deprivation. Along those lines, AMPK-mediated inhibition of SOCE is thus predicted to counteract intracellular $Ca^{2+}$ store depletion that would occur in terms of energy deficiency due to impaired $Ca^{2+}$ removal from the cytosol into intracellular stores caused by compromised activity of ATP-consuming SERCA [326]. In that respect, it seems not surprising that energy-consuming FGF23 synthesis [189] is diminished during energy depletion as determined by AMPK activation according to the data obtained from **Paper 2**. In line with this, Vidal *et al.* (2020) most recently demonstrated the pivotal role of energy availability in the regulation of FGF23 production [291]. In fact, energy intake significantly correlated with plasma FGF23 levels in rats fed diets with different caloric

content [291], an effect consistent with the HFD-induced upregulation of FGF23 observed in **Paper 1**. Intriguingly, further experiments with UMR106 cells revealed that inhibition of the mechanistic target of rapamycin (mTOR) signaling pathway, which mimics caloric restriction [327], reduced *Fgf23* gene expression similarly to values observed in cells cultured in low glucose medium [291]. Since cellular ATP depletion results in AMPK activation and downstream inhibition of mTOR signaling [328], the observations of Vidal *et al.* (2020) reinforce the contention that the AMPK-mTOR axis appears as an essential mechanistic link integrating the inhibitory effects of energy deprivation on FGF23 synthesis [291].

In addition, **Paper 2** provides novel insights concerning the role of AMPK-dependent FGF23 regulation in renal phosphate handling in the context of CKD. Confirming the *in vitro* findings, genetic deletion of *Ampkα1* resulted in elevated FGF23 formation in ampk$^{-/-}$ mice compared to ampk$^{+/+}$ mice. Although increased FGF23 production is expected to impact on parameters of phosphate metabolism [100, 119, 160], serum phosphate levels, urinary phosphate excretion, as well as circulating concentrations of calcitriol and PTH did not significantly differ between ampk$^{-/-}$ and ampk$^{+/+}$ mice. However, these findings resemble the clinical observations in early CKD stages to that extent as increments in circulating FGF23 levels precede determinants of declining kidney function such as hyperphosphatemia and elevated PTH secretion [92, 100, 101, 302]. In addition to increased FGF23 production, there is evidence indicating that renal AMPK activity is suppressed in CKD [255, 256, 302, 308]. Hence, ampk$^{-/-}$ mice displaying elevated FGF23 levels appear as a suitable animal model to study pathophysiological alterations resembling those seen in patients with moderate CKD [302]. Along those lines, it can be speculated that the increased FGF23 production in CKD is not only a compensatory response for declining kidney function to maintain renal phosphate elimination and thus phosphate homeostasis [5, 100, 160], but might also, at least in part, be attributed to suppressed AMPK activity as seen in CKD [255, 256].

Targeting the proximal tubule, the phosphaturic and thus serum phosphate lowering action of FGF23 is mainly accomplished by α-Klotho/ERK1/2-dependent inhibition of the secondary active phosphate transporter NaPi-IIa [33, 49, 51, 61, 329]. Despite substantially higher FGF23 levels in ampk$^{-/-}$ mice than in ampk$^{+/+}$ mice, *Ampkα1* deficiency did not affect either renal α-Klotho/ERK1/2 signaling, cellular localization of proximal tubular NaPi-IIa or functional determinants of phosphate homeostasis (**Paper 2**), thus indicating some degree of renal resistance to FGF23. Notably, AMPK is reported to downregulate both the membrane expression and electrogenic activity of NaPi-IIa, thereby limiting energy-consuming renal tubular $Na^+$/phosphate reabsorption during energy depletion [330]. Hence, it can be speculated that the assumed phosphate-reclamating impact of *Ampkα1* deficiency likely outweighed the phosphaturic effect of concomitantly increased FGF23 production in ampk$^{-/-}$ mice, thereby possibly explaining the tendentially higher serum phosphate levels in ampk$^{-/-}$ mice compared

to ampk$^{+/+}$ mice under high phosphate diet. Moreover, it should be kept in mind that high phosphate diet feeding caused a dramatical surge in the serum concentration of C-terminal FGF23 in ampk$^{-/-}$ mice compared to ampk$^{+/+}$ mice. These findings suggest increased cleavage of biologically active full-length FGF23, resulting in increased secretion of inactive C-terminal FGF23 fragments. Given the similarity to the clinical picture of HFTC as defined by a primary deficiency of intact FGF23 accompanied by hyperphosphatemia due to inactivating *GALNT3* mutations and subsequent increased FGF23 degradation [56, 145, 149], future studies are needed to clarify whether alterations in the post-translational regulation of FGF23 synthesis contributed, at least in part, to the exaggerated C-terminal FGF23 levels and, in turn, promoted the tendency of hyperphosphatemia in ampk$^{-/-}$ mice under high phosphate diet. Nevertheless, in light of the proposed tendency of renal FGF23 resistance in ampk$^{-/-}$ mice, it appears reasonable that the increased FGF23 production in ampk$^{-/-}$ mice could not compensate for the dietary phosphate loading, ultimately leading to enhanced phosphate retention as it is expected for renal *Ampkα1* deficiency [330].

Notably, **Paper 2** revealed that elevated FGF23 levels in *Ampkα1* deficiency are paralleled by indicators of cardiac hypertrophy, a finding resembling a severe sequela of CKD which contributes to the augmented cardiovascular mortality in these patients [38, 118, 155, 331]. In fact, ampk$^{-/-}$ mice exhibited significantly higher heart weight and increased myocyte diameter than ampk$^{+/+}$ mice. These findings are corroborated by data from other studies reporting that FGF23 induces pathologic cardiac remodeling, in particular LVH, *via* activation of the PLCγ-calcineurin-NFAT signaling axis [80, 109]. In the present study, however, cardiac PLCγ activity was not found to be different between the genotypes, indicating that other mechanisms might have been more relevant accounting for the observed moderate cardiac hypertrophy in ampk$^{-/-}$ mice.

According to the results from Zhang *et al.* (2008) and Zarrinpashneh *et al.* (2008), AMPK, in particular Ampkα2, exerts a cardioprotective effect as it antagonizes the development of LVH and myocardial fibrosis by attenuating the activation of mTOR signaling [303, 304], which is implicated in promoting cell growth and myocardial hypertrophy [332, 333]. Vice versa, genetic ablation of *Ampkα2* exacerbates pathologic cardiac remodeling in mouse models of experimental LVH [303, 304]. It should be taken into account that the *in vivo* data obtained from **Paper 2** refer to AMPKα1-deficient mice. In light of the aforementioned studies, it is, however, tempting to speculate that Ampkα1 counteracts cardiac hypertrophy similarly to Ampkα2 in a mTOR-dependent manner. Nonetheless, it requires further study to assess the pathophysiological relevance of moderate heart hypertrophy in ampk$^{-/-}$ mice in relation to functional parameters such as echocardiographic or hemodynamic data.

Although high FGF23 levels in ampk$^{-/-}$ mice appeared to play only a minor role in the context of cardiac hypertrophy (**Paper 2**), it is of note that, opposite directional, LVH itself is a

stimulator of myocardial FGF23 expression [334]. Confirming this, Leifheit-Nestler *et al.* (2016) showed that LVH is associated with enhanced cardiac *FGF23* expression and activation of FGFR4-mediated calcineurin-NFAT signaling in CKD patients [38]. According to these data, it needs to be addressed in future studies whether hypertrophic cardiomyocytes of ampk$^{-/-}$ mice are a source of augmented FGF23 production and may therefore – on a long term basis – potentially contribute to the development of LVH in a paracrine manner involving the calcineurin-NFAT signaling axis [38].

In summary, **Paper 2** unveiled that the cellular energy sensor AMPK functions as a negative regulator of FGF23 synthesis both *in vitro* and *in vivo*. Given its inhibitory role in FGF23 regulation, pharmacological activation of AMPK appears as a promising therapeutic approach to combat elevated FGF23 production in CKD and associated cardiovascular complications including LVH [108, 155]. According to data from experimental studies and clinical trials, it is reported that metformin, an established insulin-sensitizing drug known to activate AMPK, may exert beneficial effects in CKD as it ameliorates renal inflammation and fibrosis [307, 309, 311, 335] and attenuates cardiac hypertrophy [336]. In light of the fact that inflammation [36, 170, 213, 226], the pro-fibrotic factor TGF-β2 [227] as well as cardiac hypertrophy [334] are triggers of FGF23 production, it is a task for future studies to elucidate whether the reno- and cardioprotective effects of metformin are potentially related to AMPK-dependent downregulation of FGF23 [335].

## 4.3 AMPK – a major regulator of renal tubular transport and potential interactions with FGF23

As demonstrated in **Paper 2**, AMPK has been unveiled as a negative regulator of the phosphaturic hormone FGF23. In light of the fact that FGF23 mainly targets the kidney and participates in the homeostatic regulation of calcitriol and mineral metabolism [33, 47, 48, 51, 78, 79], **Paper 3** sought to address the question whether AMPK is directly involved in renal tubular transport. To this end, comprehensive data from experimental *in vitro* and *in vivo* approaches were analyzed and summarized in a review article.

A major function of the kidney is the reabsorption and secretion of electrolytes and ions, thereby ensuring whole body mineral and acid-base homeostasis [337, 338]. Renal transepithelial transport is mainly facilitated by ATP-dependent transport proteins, which account for the large energy consumption of the kidneys [339, 340]. Therefore, the energy sensor AMPK is primarily implicated to limit ATP-requiring renal tubular transport to conserve cellular energy in states of metabolic stress [255, 339, 341]. In particular, AMPK is reported to inhibit secondary active $Na^+$-dependent transporters along all nephron segments including the

creatine transporter (CRT) [342], NaPi-IIa [330], and ENaC [318, 343, 344]. On the other hand, AMPK is suggested to enhance renal glucose reabsorption [339, 345, 346], thereby ensuring the reclamation of energy-providing glucose in order to maintain the transmembrane $Na^+$ gradient generated by ATP-demanding $Na^+/K^+$-ATPase [339].

Given that both FGF23 and AMPK differentially impact on renal tubular transport [33, 255, 339], it seems plausible that there might be interrelations between AMPK and FGF23 regarding the regulation of renal mineral balance. Therefore, renal function parameters of $ampk^{+/+}$ and $ampk^{-/-}$ mice obtained from **Paper 2** need to be considered in more detail. As delineated in **Paper 3**, AMPK is reported to reduce renal tubular $Na^+$ reabsorption presumably by decreasing the expression and activity of ENaC located at the apical plasma membrane in renal cortical collecting duct cells [318, 339, 343, 344, 347]. Hence, activation of AMPK fosters urinary $Na^+$ excretion [348]. However, serum $Na^+$ concentration as well as urinary $Na^+$ excretion were not significantly different between $ampk^{+/+}$ and $ampk^{-/-}$ mice (**Paper 2**). These findings were somewhat surprising in light of the fact that *Ampkα1* deficiency would be expected to result in increased renal ENaC expression [318], which would entail decreased urinary $Na^+$ excretion, and, in turn, increased renal $Na^+$ reabsorption followed by elevated serum $Na^+$ levels. In addition, renal $Na^+$ reclamation and serum $Na^+$ concentration would be assumed to be synergistically increased in $ampk^{-/-}$ mice due to the observed increments in circulating FGF23, the latter of which is reported to facilitate ERK1/2-mediated distal tubular $Na^+$ uptake *via* augmented NCC expression and activity [79]. The lack of hypernatremia in *Ampkα1* deficiency may, at least in part, be explained by the fact that $ampk^{-/-}$ mice displayed some degree of renal resistance to FGF23 as FGF23-downstream activation of renal ERK1/2 signaling [33] appeared to be similar in $ampk^{-/-}$ and $ampk^{+/+}$ mice despite significantly elevated FGF23 levels in $ampk^{-/-}$ mice (**Paper 2**). Inhibition of distal tubular ERK1/2 signaling would, in turn, lead to reduced NCC activation [79], followed by attenuated renal $Na^+$ reabsorption, thereby potentially counteracting hypernatremia in $ampk^{-/-}$ mice.

Moreover, there is evidence indicating that AMPK stimulates the expression and activity of the $Na^+$-$K^+$-$2Cl^-$ cotransporter (NKCC2), which fosters salt retention in the thick ascending limb of the loop of Henle [349–352]. Vice versa, it can be speculated that NKCC2-mediated $Na^+$ retention was reduced in $ampk^{-/-}$ mice, indicating another possible compensatory mechanism to prevent perturbations of mineral metabolism in *Ampkα1* deficiency. Nonetheless, it is a task for future studies to elaborate on the hypothetical regulation of the aforediscussed renal ion transport proteins in $ampk^{-/-}$ and $ampk^{+/+}$ mice in order to elucidate potential interdependent interactions between FGF23 and AMPK in the homeostatic regulation of mineral metabolism.

## 4.4 Conclusion

FGF23 is nowadays not only considered as a phosphaturic hormone but also as a key player in the pathogenesis of inflammatory diseases including CKD and related cardiovascular comorbidities [47, 152, 155, 209]. Unravelling the underlying mechanisms involved in the regulation of FGF23 synthesis in health and disease setttings is of particular scientific interest as it provides the molecular basis for the development of innovative therapeutic approaches aimed at the treatment of FGF23-related pathologies. To this end, the present study sought to elucidate novel regulators of FGF23 production in the context of HFD-induced inflammation, the metabolic sensor protein AMPK, and potential implications in renal tubular transport.

Based on the three publications presented in this thesis, the main objectives of this work were substantially answered:

**I) What is the relevance of HFD-induced inflammation on FGF23 production?**

In an animal model of metabolic syndrome-associated low-grade inflammation, it could be demonstrated that HFD feeding stimulates the production of FGF23 in wild-type mice, an effect in large part attributed to hepatic induction of the pro-inflammatory cytokine TNFα. It was also shown that low concentrations of TNFα induce *Fgf23* gene expression in UMR106 osteoblast-like cells. In addition, it could be revealed that the surge in serum FGF23 following HFD feeding was more pronounced in wild-type mice than in $tnf^{-/-}$ mice, confirming the significance of HFD-induced TNFα formation in FGF23 upregulation. In light of the data obtained from **Paper 1**, future clinical investigations are needed to prove whether caloric restriction and weight loss, respectively, might appear as useful non-pharmacological interventions to lower FGF23 levels by means of reducing the risk for CKD in overweight and/or obese subjects.

**II) What is the impact of AMPK in the regulation of FGF23 synthesis and what are the underlying mechanisms involved?**

The cellular energy sensor AMPK has been revealed as a novel inhibitory regulator of FGF23 formation both *in vitro* and *in vivo*. Specifically, AMPK has been proven to downregulate *Fgf23* gene expression in UMR106 cells *via* inhibition of Orai1-mediated SOCE. In line with the *in vitro* findings, it was verified that osseous *Fgf23* expression as well as circulating FGF23 levels are markedly increased in AMPKα1-deficient mice compared to wild-type counterparts. Moreover, the concurrent appearance of elevated FGF23 serum levels, moderate cardiac hypertrophy but unaltered phosphate metabolism and gross normal renal function in AMPKα1 deficiency conforms, to some extent, the clinical features of early CKD stages. In conclusion, the findings of **Paper 2** indicate that AMPK appears as a putative molecular target linking whole

body energy homeostasis with the regulation of FGF23 synthesis and accompanied pathologies including CKD and cardiovascular comorbidities. Given that renal AMPK activity is reported to be reduced in CKD, it is of high clinical interest and thus a task for future studies to investigate whether pharmacological AMPK activation might serve as a promising therapeutic strategy in the treatment of exaggerated FGF23 production in CKD.

### III) Is there a possible interrelation between AMPK and FGF23 concerning the homeostatic regulation of renal mineral handling?

As extensively delineated in **Paper 3**, AMPK acts not only as an ubiquitously expressed sensor of cellular energy status, but also participates in a multitude of renal tubular ion transport processes. Along those lines, AMPK appears as a decisive molecular target linking the maintenance of cellular energy homeostasis with whole body mineral balance. Considering that FGF23 is a well-established regulator of renal phosphate and $Na^+$ handling, which is putatively under the control of AMPK as well, it is tempting to speculate that AMPK and FGF23 are mutually connected in terms of renal mineral handling. Given that AMPK has been identified as a novel regulator of FGF23 within the scope of this thesis, future investigations should particularly focus on the importance of the renal AMPK-FGF23 axis in disease settings where mineral homeostasis is dysregulated as it is observed in CKD.

# 5 Summary

The bone-derived proteohormone fibroblast growth factor 23 (FGF23) is a pivotal regulator of phosphate homeostasis and vitamin D metabolism. FGF23 mainly targets the kidney where it suppresses tubular phosphate reabsorption and the formation of active vitamin D [$1,25(OH)_2D_3$ or calcitriol] in concert with its co-receptor α-Klotho. Extrarenal actions of FGF23 are targeted at the parathyroid glands, heart, liver, and immune cells. FGF23 has gained broad clinical attention as it is not only implicated in severe hereditary phosphate-wasting diseases, but is also considered as an early detectable biomarker in a wide range of acute and chronic disorders including renal, cardiovascular, and inflammatory diseases. In this regard, excess circulating FGF23 concentrations, as found in patients with chronic kidney disease (CKD), are closely linked to increased cardiovascular morbidity and mortality.

The formation of FGF23 as well as its pleiotropic cellular actions are regulated by a multifaceted network of endocrine feedback loops and intracellular signaling pathways. During the last years, numerous systemic and molecular factors involved in the transcriptional and post-translational regulation of FGF23 have been identified. However, the current knowledge about the complex regulatory mechanisms of FGF23 synthesis in health and particularly in disease settings is still fragmentary. The studies presented as part of this thesis therefore sought to provide novel insights into the regulation of FGF23 production utilizing versatile *in vitro* and *in vivo* approaches. Along those lines, the main task of this dissertation was to study the putative differential regulation of FGF23 formation in terms of excess energy intake – as mimicked by high-fat diet (HFD) feeding – and in respect of the cellular energy sensor 5´-adenosine monophosphate (AMP)-activated protein kinase (AMPK), which is activated upon energy deficiency. Furthermore, current data referring to the functional role of AMPK in the regulation of renal tubular transport processes were summarized and discussed in a systematic review.

It could be demonstrated that energy-dense HFD feeding is capable to stimulate FGF23 production in mice *via* increased formation of the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα). In addition, TNFα was confirmed to be a positive regulator of *Fgf23* gene expression in UMR106 osteoblast-like cells. These findings not only corroborate the notion that inflammation is a major trigger of FGF23 formation, but also provide evidence that high energy availability in terms of hypercaloric diets and chronic low-grade inflammation may contribute to the reported positive association between obesity and elevated systemic FGF23 levels.

Moreover, it could be shown that AMPK functions as a hitherto unknown negative regulator of FGF23 production as revealed by versatile murine *in vitro* and *in vivo* approaches. In particular, fluorescence optics in UMR106 cells confirmed that AMPK mediates its inhibitory effect on *Fgf23* gene transcription in large part *via* downregulation of store-operated $Ca^{2+}$ entry

(SOCE) through lowering the cell surface expression of the $Ca^{2+}$ release-activated $Ca^{2+}$ (CRAC) channel protein Orai1. Given that FGF23 is a phosphaturic hormone, it is of note that phosphate metabolism was not affected in AMPKα1-deficient mice displaying increased FGF23 levels and moderate cardiac hypertrophy, resembling the clinical characteristics observed in individuals in early stages of CKD. Considering that renal AMPK activity is suppressed in CKD as reported by previous studies, lowering circulating FGF23 levels by pharmacological AMPK activation may be considered as a beneficial therapeutic strategy to decelerate CKD disease progression and to improve the cardiovascular outcome of CKD patients.

Referring to current data on the impact of AMPK on renal tubular transport, it can be concluded that AMPK activation in states of cellular energy depletion is largely suggested to limit energy-consuming ion transport processes in the kidney, indicating a particular role of AMPK in the regulation of renal phosphate and $Na^+$ handling. Considering the suppressive effect of AMPK on the phosphaturic and $Na^+$-conserving hormone FGF23, it is a subject for further research to investigate potential molecular interactions between AMPK and FGF23 regarding a differential regulation of renal tubular ion transport proteins and thus of homeostatic mineral balance.

Taken together, HFD-induced TNFα formation and the cellular energy sensor AMPK were identified as powerful regulators of FGF23 production. The data presented in this thesis thus provide novel physiological and pathological insights regarding the complex interrelations between energy metabolism and inflammation in the regulation of FGF23 formation and mineral homeostasis.

# 6 Zusammenfassung

Das vorwiegend im Knochen gebildete Proteohormon Fibroblasten-Wachstumsfaktor 23 (*fibroblast growth factor 23*, FGF23) ist ein zentraler Regulator der Phosphat-Homöostase und des Vitamin D-Stoffwechsels. Eines der Hauptzielorgane von FGF23 ist die Niere, wo es zusammen mit seinem Korezeptor α-Klotho die tubuläre Phosphatrückresorption sowie die Bildung des aktiven Vitamin D [$1,25(OH)_2D_3$ oder Calcitriol] hemmt. Daneben vermittelt FGF23 extrarenale Effekte unter anderem in den Nebenschilddrüsen, dem Herzen, der Leber sowie Immunzellen. FGF23 ist insbesondere klinisch relevant, da es bei schweren erblichen Erkrankungen, die durch hohe Phosphatverluste gekennzeichnet sind, eine wichtige Rolle spielt. Daneben gilt es als früh nachweisbarer Biomarker bei einer Vielzahl von akuten und chronischen Krankheitsbildern wie Nieren-, Herz-Kreislauf- und entzündlichen Erkrankungen. So sind stark erhöhte FGF23-Werte in der Zirkulation bei Patienten mit chronischem Nierenversagen mit erhöhter kardiovaskulärer Morbidität und Mortalität assoziiert.

Die Bildung von FGF23 sowie seine vielfältigen zellulären Wirkungen unterliegen der Regulation komplexer hormoneller Rückkopplungsschleifen sowie intrazellulärer Signalwege. Obwohl in den letzten Jahren zahlreiche systemische und molekulare Faktoren der trankriptionellen und post-translationalen FGF23-Regulation identifiziert wurden, ist das Verständnis über die komplexen Mechanismen der FGF23-Synthese sowohl unter physiologischen als auch pathophysiologischen Gesichtspunkten nach wie vor lückenhaft. Demnach bestand das Ziel der im Rahmen dieser Arbeit dargelegten Studien darin, neue Einblicke in die Regulation der FGF23-Produktion unter Verwendung vielseitiger *in vitro* und *in vivo*-Ansätze zu gewinnen. Diesbezüglich wurde der Einfluss einer überschüssigen Energieaufnahme auf die Bildung von FGF23 im Sinne einer Fütterungsstudie mit fettreicher Diät (*high-fat diet*, HFD) untersucht. Zusätzlich wurde der Frage nachgegangen, inwiefern die bei zellulärem Energiemangel aktivierte 5´-Adenosinmonophosphat-aktivierte Proteinkinase (*5´-adenosine monophosphate (AMP)-activated protein kinase*, AMPK) an der Regulation der FGF23-Synthese beteiligt ist. Zudem wurde die aktuelle Datenlage zur funktionellen Bedeutung der AMPK hinsichtlich tubulärer Transportprozesse in der Niere in Form einer systematischen Übersichtsarbeit zusammengefasst und diskutiert.

Es konnte gezeigt werden, dass die gesteigerte FGF23-Synthese in Mäusen, denen eine energiedichte HFD gefüttert wurde, durch die vermehrte Bildung des pro-entzündlichen Zytokins Tumornekrosefaktor-α (TNFα) vermittelt wird. Zudem wurde TNFα als positiver Regulator der *Fgf23*-Genexpression in osteoblasten-ähnlichen UMR106-Zellen bestätigt. Diese Ergebnisse bekräftigen die Auffassung, dass Entzündungsreaktionen maßgebliche Auslöser einer erhöhten FGF23-Bildung sind. Zudem verdeutlichen die Daten, dass eine hohe Energieverfügbarkeit hinsichtlich der Zufuhr hochkalorischer Diäten sowie chronische,

niedriggradige Entzündungsprozesse möglicherweise dazu beitragen, dass das Auftreten einer Adipositas mit systemisch erhöhten FGF23-Werten assoziiert ist.

Mit Hilfe vielfältiger muriner *in vitro* und *in vivo*-Experimente konnte ferner die AMPK als bislang unbekannter negativer Regulator von FGF23 identifiziert werden. Demnach konnten Fluoreszenzmessungen in UMR106-Zellen aufklären, dass die inhibitorische Wirkung der AMPK auf die *Fgf23*-Transkription im Wesentlichen auf einen verminderten speichergesteuerten $Ca^{2+}$-Einstrom (*store-operated* $Ca^{2+}$ *entry*, SOCE) bedingt durch eine reduzierte Zellmembranexpression des $Ca^{2+}$-Freisetzung aktivierten Calciumkanalproteins (*Ca*$^{2+}$ *release-activated Ca*$^{2+}$, CRAC *channel*) Orai1 zurückzuführen ist. Angesichts der Tatsache, dass FGF23 ein phosphaturisches Hormon ist, ist es bemerkenswert, dass der Phosphathaushalt in AMPKα1-defizienten Mäusen trotz erhöhter FGF23-Werte nicht beeinträchtigt war. Zudem konnte bei den Tieren eine moderate Herzhypertrophie festgestellt werden, die zusammen mit den hohen FGF23-Werten dem klinischen Bild von Patienten in frühen Stadien des chronischen Nierenversagens ähnelt. In Anbetracht dessen, dass die renale AMPK-Aktivität bei chronischem Nierenversagen supprimiert ist, könnte die durch pharmakologische AMPK-Aktivierung vermittelte Senkung erhöhter FGF23-Spiegel eine sinnvolle Therapiestrategie darstellen, die Progression des chronischen Nierenversagens zu verlangsamen sowie die kardiovaskuläre Prognose betroffener Patienten zu verbessern.

Nach Auswertung der aktuellen Datenlage hinsichtlich der Bedeutung der AMPK auf renale Transportmechanismen lässt sich konstatieren, dass die bei zellulärem Energiemangel vermittelte AMPK-Aktivierung eine Limitierung energieaufwendiger Ionentransportprozesse in der Niere zur Folge hat. In diesem Zusammenhang ist der AMPK insbesondere eine wichtige Funktion in der Regulation des renalen Phosphat- und $Na^+$-Haushaltes zuzuordnen. Angesichts der inhibitorischen Wirkung der AMPK auf die Bildung von FGF23, das die Phosphatausscheidung sowie die $Na^+$-Reabsorption in der Niere stimuliert, sollten sich künftige Studien auf potenzielle molekulare Interaktionen zwischen AMPK und FGF23 fokussieren. Mit Blick auf die homöostatische Regulation des Mineralhaushaltes sei hier eine mögliche differenzielle Regulierung tubulärer Ionentransportproteine in der Niere zu betrachten.

Im Rahmen dieser Arbeit konnten die HFD-induzierte TNFα-Bildung sowie der zelluläre Energiesensor AMPK als potente Regulatoren von FGF23 identifiziert werden. Die dargelegten Ergebnisse liefern demnach sowohl aus physiologischer als auch krankheitsbezogener Perspektive neue Erkenntnisse zu den komplexen Wechselwirkungen zwischen Energiestoffwechsel und entzündlichen Prozessen hinsichtlich der Regulation der FGF23-Bildung und der damit verbundenen Homöostase des Mineralhaushaltes.

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# Curriculum vitae

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## Publications

**Glosse, P.**; Föller, M. AMP-Activated Protein Kinase (AMPK)-Dependent Regulation of Renal Transport. *Int J Mol Sci* **2018**, *19*, 3481, DOI: 10.3390/ijms19113481.

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## Oral Presentations

**Glosse, P.** Vegane Ernährung – Trend und Wirklichkeit. Universitätsklinikum Leipzig AöR, Medizinische Berufsfachschule, Förderverein für medizinische Ausbildung e.V., 25. Fortbildungsveranstaltung für Ernährungstherapie, 16 October 2014, Leipzig, Deutschland.

**Glosse, P.**; Romacho, T.; Schoemaker, M. H.; van Tol, E. A.; Eckel, J. The early nutritional factors EPA and DHA exert a direct beneficial effect on human primary adipocytes. J Pediatr Gastroenterol Nutr 56, Supplement 2. (Abstract), 46th Annual Meeting of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), 08 – 11 May 2013, London, United Kingdom (presented by Dr. Tanya Romacho).

## Poster Presentations

**Glosse, P.**; Fajol, A.; Feger, M.; Lang, F.; Föller, M. Eine fettreiche Diät induziert die Bildung des Fibroblastenwachstumsfaktors 23 durch TNFα. 54. Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung e.V., 01 – 03 March 2017, Christian-Albrechts-Universität zu Kiel, Deutschland.

**Glosse, P.**; Putziger, J.; Kilian, J. Ergebnisse eines ambulanten Ernährungsteams bei der Unterstützung des Darmzentrums am Ev. Diakonissen-Krankenhaus Leipzig – 1. Mitteilung. Aktuel Ernahrungsmed 39 - P20 (Abstract), Ernährung 2014 – Ernährungsmedizin ist Partnerschaft, 13. Dreiländertagung der DGEM e.V. in Kooperation mit der AKE und der GESKES, Jahrestagung 2014 des VDOE e.V., 15. Jahrestagung des BDEM e.V., 26 – 28 June 2014, Ludwigsburg, Deutschland (presented by Dr. med. Jens Putziger).

Romacho, T.; Richter, I.; **Glosse, P.**; Elsen, M.; Schoemaker, M.; van Tol, E. A.; Eckel, J. EPA, DHA and DHA/ARA exert differential direct beneficial effects on human primary adipocytes. Obesity Facts 7 (suppl 1): 1-188. (Abstract), 21st European Congress on Obesity (ECO), European Association for the Study of Obesity (EASO), 28 – 31 May 2014, Sofia, Bulgaria (presented by Dr. Tanya Romacho).

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# Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

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