**Molecular structure and differential function of choline kinases CHK α and CHK β in
musculoskeletal system and cancer**

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Abstract

Choline, a hydrophilic cation, has versatile physiological roles throughout the body, including cholinergic neurotransmission, memory consolidation and membrane biosynthesis and metabolism. Choline kinases possess enzyme activity that catalyses the conversion of choline to phosphocholine, which is further converted to cytidine diphosphate-choline (CDP-choline) in the biosynthesis of phosphatidylcholine (PC). PC is a major constituent of the phospholipid bilayer which constitutes the eukaryotic cell membrane, and regulates cell signal transduction. Choline Kinase consists of three isoforms, $\text{CHK}\alpha 1$, $\text{CHK}\alpha 2$ and $\text{CHK}\beta$, encoded by two separate genes ($\text{CHKA}(\text{Human})/\text{Chka}(\text{Mouse})$ and $\text{CHKB}(\text{Human})/\text{Chkb}(\text{Mouse})$). Both isoforms have similar structures and enzyme activity, but display some distinct molecular structural domains and differential tissue expression patterns. Whilst Choline Kinase was discovered in early 1950, its pivotal role in the development of muscular dystrophy, bone deformities, and cancer has only recently been identified. $\text{CHK}\alpha$ has been proposed as a cancer biomarker and its inhibition as an anti-cancer therapy. In contrast, restoration of $\text{CHK}\beta$ deficiency through CDP-choline supplements like citicoline may be beneficial for the treatment of muscular dystrophy, bone metabolic diseases, and cognitive conditions. The molecular structure and expression pattern of Choline Kinase, the differential roles of Choline Kinase isoforms and their potential as novel therapeutic targets for muscular dystrophy, bone deformities, cognitive conditions and cancer are discussed.

Keywords: Choline Kinase, $\text{CHK}\alpha$, $\text{CHK}\beta$, Cancer, Musculoskeletal system

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1. Introduction

Choline, a charged hydrophilic cation, plays an important role in various organs and cells throughout the body, including cholinergic neurotransmission, memory consolidation and as a critical component of cell membranes. Choline is an essential nutrient and can be produced within the body through the degradation of choline containing phospholipids; however, this source is insufficient to maintain choline levels and dietary intake of choline is necessary to prevent choline-deficiency associated liver and muscle damage [1]. Within cells choline is predominantly incorporated as phosphatidylcholine, which is a crucial component of the cell membrane and has secondary roles as a substrate to produce lipid second messengers including phosphatidic acid and diacylglycerol [1].

Choline uptake is a key step in biosynthetic pathways utilising choline (Fig. 1), and is controlled by the activities of non-specific transporters (organic cation transporters; OCT), and high and low affinity choline specific transporters (choline transporter, CHT1; choline transporter like, CTL1) [2]. Following uptake into cells, choline is converted to acetylcholine through the actions of Choline acetyltransferase (ChAT), which is required for cholinergic neurotransmission [3], and failures in this process have been implicated in Alzheimer's disease, brain ischemic events, and aging [2, 4]. As stated above, the principle choline processing pathway in cells is the generation of phosphatidylcholine. In this pathway choline is initially synthesised into phosphocholine through the actions of choline kinase [5]. Phosphocholine is further converted to cytidine diphosphate-choline (also known as citicoline, cytidine 5'-diphosphocholine, or CDP-choline) and subsequently to phosphatidylcholine [1] (Fig. 1). Within this pathway phosphocholine has recently been shown to have functions independent of its role as an intermediate in phosphatidylcholine synthesis [5], and as such the role of Choline Kinase and subsequent phosphocholine production has become of great interest.

Choline Kinase has three isoforms: $\text{CHK}\alpha 1$, $\text{CHK}\alpha 2$ and $\text{CHK}\beta$ which are encoded by $\text{CHKA}(\text{Human})/\text{Chka}(\text{Mouse})$ and $\text{CHKB}(\text{Human})/\text{Chkb}(\text{Mouse})$ genes respectively and was discovered since early 1950, but its biological roles in muscular dystrophy, bone deformities, and cancer have only recently been described [6]. This review discusses recent progresses on the molecular structure and expression pattern of Choline Kinase, the differential roles of Choline Kinase isoforms in muscular dystrophy, bone deformities, cognitive conditions and cancer, as well as the potential as novel therapeutic targets for these disorders.

2. Molecular structure of Choline Kinase: $\text{CHK}\alpha$ and $\text{CHK}\beta$

Choline Kinase exists in at least 3 isoforms; $\text{CHK}\alpha 1$ and $\text{CHK}\alpha 2$ are produced by alternate splicing of the $\text{CHKA}(\text{Human})/\text{Chka}(\text{Mouse})$ gene, whereas $\text{CHK}\beta$ (also called *Chetk*, *Chkl*) is separately encoded by $\text{CHKB}(\text{Human})/\text{Chkb}(\text{Mouse})$ [7]. Sequence alignment shows that mice $\text{CHK}\alpha 1$ shares 48.47% sequence identity with $\text{CHK}\beta$ with 222 amino acids in identical positions (Fig. 2A) and a similar result has also been seen for human $\text{CHK}\alpha 1$ and $\text{CHK}\beta$ isoforms, with human and mouse orthologues sharing greater than 85% sequence similarity. Although $\text{CHK}\beta$ carries an additional N-acetylalanine site at the N-terminal region, all Choline Kinase proteins share a similar functional region containing one substrate binding site, two nucleotide binding sites, three ATP binding sites (Fig. 2B) as well as some analogous potential ligands (Magnesium, ADP, Kanamycin). Moreover, multiple structure rigid-body alignment (Fig. 3) showed a distinct RMSD (The Root-Mean-Standard-Deviation) score (2.906 Å) between $\text{CHK}\alpha$ and $\text{CHK}\beta$, indicating a lower, but still noticeable, degree of similarity, which was further displayed in detail through 3D protein structure analysis (Fig. 4). Functional Choline Kinase exists as a dimer, and can form homodimers or heterodimers with differential dynamics dependent on the tissue type [8]. $\text{CHK}\alpha$ homodimers display higher enzymatic activity than $\text{CHK}\beta$ homodimers and $\text{CHK}\alpha/\beta$ heterodimers [5] with tissues differing in their relative expression of the varying isoforms. BIOGPS analysis reveals that

CHKA(Human)/*Chka*(Mouse) and CHKB(Human)/*Chkb*(Mouse) genes have both common and differential expression patterns in cells and tissues (Fig. 5), which CHK α has highest expression in testis interstitial cells and lowest expression among whole blood, while CHK β expresses strongly in CD14⁺ monocytes but only marginally in ciliary ganglion and atrioventricular node, indicative of their shared and distinct biological functions. These expression patterns are important for the tissue specific function of CHK α and CHK β in both physiological and pathological stages.

Although we now have summarised some mutual characteristics between CHK α and CHK β and further explored their considerable differences on the basis of their gene expression, sequence alignments, three-dimensional tertiary structures, potential cavities and pockets as well as pre-existing studies, etc, we still barely know the connections between the gene expression and their unique biological functions, the discrepant efficiency caused by homodimers or heterodimers and how do those active sites and speculated ligands contribute to their enzymatic functions. We believe that a clearer picture and a more thorough analysis of the molecular structures shall be required for narrowing down those knowledge gaps and thus leading us to drug discoveries.

3. The Biological Functions of Choline Kinase: CHK α and CHK β

3.1. The role of CHK α

Mice lacking the *Chka* gene die at an early stage of embryogenesis, pointing to an important role in organ development [9]. Evidence suggests that the phosphocholine produced by the actions of CHK α is an important second messenger in DNA synthesis, hence the essential role of this enzyme during development [10]. CHK α has also been extensively implicated in human tumorigenesis. CHK α 1 positively regulates cell proliferation and survival via activation of PI3K/AKT and MAPK signalling pathways [11]. Its overexpression has been detected in 40-60% of human tumours [12], and high levels of CHK α correlate with poor prognosis [13]. Overexpression of the CHK α protein can transform noncancerous cells into cells with a cancerous phenotype, and conversely,

knockdown of the CHK α protein by siRNA can result in cancer cell death [14]. It has also been shown that siRNA mediated knockdown of CHKA results in upregulation of autophagy, which was further associated with a reduction in cell proliferation in breast cancer cell lines [15]. The precise molecular mechanism underlying this link between choline metabolism and autophagy is unclear.

It has been demonstrated that CHK α activity can be induced by collaborative signalling between the epidermal growth factor receptor (EGFR) and c-Src in breast cancer cells. Evidence suggests a direct interaction between EGFR and CHK α protein, modulated by c-Src phosphorylation [16]. It has been proposed that CHK α is acting as a scaffold in this interaction, and its function is independent of its kinase activity [17]. This was supported by the evidence that inhibition of CHK α catalytic activity using the compound V-11-0711 reduced cellular phosphocholine levels but did not affect cancer cell survival as long as concentrations of CHK α protein were unaffected [17]. At higher concentrations causing reductions in CHK α and phosphatidylcholine, V-11-0711 resulted in a reversible cell growth arrest [14]. Further, CHK α can act as an androgen receptor (AR) chaperone in prostate cancer, suggesting a role of CHK α for AR associated tumour progression [18].

Given its involvement in cancer development, CHK α has been proposed as a diagnostic biomarker for cancer by immunohistochemistry [19] and facilitated the development of radiolabels for positron emission tomography (PET) imaging as well as pharmacodynamic markers for non-invasive magnetic resonance spectroscopy (MRS), since the enzymatic changes of choline transporters, such as CHK α , will give rise to a raise in total choline (tCho) signal which can be detected by ^1H magnetic resonance spectroscopy [20]. Furthermore, real time quantitative PCR has also been employed as a prognostic tool for determination of CHK α 1 expression levels in non-small cell lung cancer [13] and, besides, there are several other reviews that have also been well-covered in the latest information of CHK α relating to tumor metabolism and the diagnosis [21, 22].

Collectively, CHK α has emerged as a promising molecular target for cancer diagnosis and treatment.

3.2. The role of CHK β

A recessive mouse mutation resulting in a 1.6-kb intragenic deletion within the *Chkb* gene was found to cause loss of CHK β activity and was associated with rostrocaudal muscular dystrophy and neonatal forelimb bone deformities in mouse [23]. The muscular dystrophy in the skeletal muscle increased in severity from rostral to caudal tissues [23]. The loss of CHK β resulted in impaired phosphatidylcholine biosynthesis, accompanied by an accumulation of choline and increased phosphatidylcholine catabolism [6, 24]. Mitochondria within the skeletal muscle were found to be abnormally large, concomitant with decreased membrane potential [24]. The enlarged mitochondria are potentially due to compensation for defective mitochondria in *Chkb* mutant cells [24]. The impaired phospholipid metabolism in the mitochondria-associated inner membrane was correlated with the abnormal size and aberrant cellular localisation of muscle mitochondria [25].

A genome-wide association study in humans using 500K SNP microarrays suggest that a genetic variant with decreased *Chkb* expression is associated with narcolepsy [26]. *Chkb* mutations that cause megaconial congenital muscular dystrophy (MCMD) have been found in many human cases from Japanese, Turkish, and British populations. Affected muscle cells from individuals exhibit characteristics of mitochondrial enlargement at the periphery of muscle fibres, concomitant with the loss of mitochondria in the centre of muscle fibres [27]. In addition, several patients with *Chkb* mutations with MCMD showed mitochondrial mitophagy in muscle cells [28]. Interestingly, several patients have been described with mutations in *Chkb* resulting in loss of components of the mitochondrial electron transport chain [29, 30]. These studies indicate that mutation of *Chkb* in mice and humans presents a similar phenotype within the muscle cells, indicating conservation of CHK β function between species.

3.3. Involvement of *CHKβ* in bone and cartilage homeostasis

Recent studies have found that *CHKβ* has a critical role in the normal embryogenic formation of the radius and ulna in mice. *Chkb*^{-/-} mice display expanded hypertrophic zones in the radius and ulna with disrupted proliferative columns in their growth plates, accompanied by delayed formation of primary ossification centers and impaired chondrocyte differentiation [31]. Interestingly these chondrocyte defects appear to be constrained to the forelimb bones during embryogenesis, indicative of a pattern-specific mode of regulation. We further identified *CHKβ* as a novel regulator of bone homeostasis in adult mice throughout the entire axial skeleton [32]. In our study *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis was employed to generate a repertoire of mutants for gene function studies [33]. Phenotypic screening identified a mouse line exhibiting a bone loss phenotype with forelimb bone deformities. The affected locus was identified as the *Chkb* gene through sequencing; an A->T single base substitution in the first codon resulted in no detectable *CHKβ* protein product in affected mice [32]. *Chkb* mutant mice showed elevated osteoclast numbers and activity [32]. The biosynthesis of phosphatidylcholine was impaired in osteoclasts from *Chkb* mutant mice with reduced levels of phosphocholine and CDP-choline [32]. The reduced phosphocholine levels also impacted osteoblast mineralising activity. In line with this finding, mineralisation requires the activity of the phosphatase PHOSPHO1 [34], whose predominant substrate is phosphocholine [35]. The combined enhancement of osteoclast formation and function, and reduction in osteoblast activity result in the observed osteoporosis in the *Chkb* mutant mouse line [32].

The underpinning molecular mechanism by which *CHKβ* impacts bone homeostasis is not clear. As mentioned above, the loss of phosphocholine directly impacts osteoclast resorptive function; however, the exact role of *CHKβ* in osteoclasts remains to be elucidated. Osteoclastic bone

resorption is regulated by RANKL/RANK and down-stream signaling processes; including the formation of F-actin rings mediated by c-src, activation of V-ATPase via coupling with chloride channel [36, 37], and stimulation of calcium influx via calcium channels [38]. RANK activation results in phosphorylation of PI3K [39], which has been shown to regulate the activation of CHK β [40]. Interestingly, recent microarray analysis showed that choline kinase is able to regulate the gene expression of both chloride channels and voltage-dependent calcium channels [10]. In the light of these findings, it is possible that the activation of RANK/PI3K/CHK β might regulate osteoclastic bone resorption via regulating the gene expression of chloride and voltage-dependent calcium channels essential for osteoclast function [36, 37]. Thus, it will be important to define RANK/PI3K/CHK β downstream targets that are involved in osteoclastic bone resorption. Understanding the role of CHK β in bone loss may lead to the discovery of a new molecular target for anti-resorptive drug development.

4. Therapeutic targets of Choline Kinase: CHK α and CHK β

4.1. Compound inhibitors of Choline Kinase

CHK α is overexpressed in various human tumours and has become a key drug or gene target for the treatment of cancers. Thus, inhibition of CHK α with small molecule compounds or targeting the expression of CHK α with RNA interference are potential anticancer chemotherapy approaches resulting in anti-proliferative and anti-tumoral effects. To date, several small molecule compounds have been developed.

MN58b is a well characterized CHK α inhibitor [12]. CHK α protein expression was directly correlated with sensitivity to MN58b, which caused endoplasmic reticulum stress and ultimately resulted in the induction of apoptosis [41]. Consistently, knockdown of *Chka* resulted in resistance to MN58B induced cell death. In addition, gemcitabine-resistant pancreatic ductal adenocarcinoma (PDAC) cells displayed enhanced sensitivity to CHK α inhibition and, *in vitro*,

MN58b had additive or synergistic effects with gemcitabine, 5-fluorouracil, and oxaliplatin, in the treatment of pancreatic ductal adenocarcinoma [42].

Hemicholinium-3 (HC-3), also known as hemicholine can block the reuptake of choline by the high-affinity choline transporter and decreases the synthesis of acetylcholine [43]. HC-3 is also known as an inhibitor of Choline Kinase. The inhibitory actions of HC-3 are likely owing to one of HC-3 oxazinium rings complexed with $\text{CHK}\alpha$ and occupied the choline-binding pocket [44]. The inhibitory effects of HC-3 are much more profound on $\text{CHK}\alpha$ than on $\text{CHK}\beta$, suggesting a specificity of action that could be useful for differential targeting of the Choline Kinase isoforms in human cancer.

CK37, N-(3,5-dimethylphenyl)-2-[[5-(4-ethylphenyl)-1H-1,2,4-triazol-3-yl]sulfanyl] acetamide, a novel selective inhibitor against $\text{CHK}\alpha$ was found to inhibit $\text{CHK}\alpha$ activity, reduce the steady-state concentration of phosphocholine in transformed cells, and selectively suppress the growth of neoplastic cells [45]. Further, CK37 was found to attenuate tumour growth via Ras-AKT/ERK signalling in a T-lymphoma xenograft murine model [46].

Compound V-11-0711 is a novel selective inhibitor of $\text{CHK}\alpha$ that can act at low doses to selectively reduce phosphocholine levels without impacting $\text{CHK}\alpha$ protein levels and phosphatidylcholine concentrations [17]. As opposed to other Choline Kinase inhibitors, V-11-0711 does not target the choline binding pocket of Choline Kinase, but selectively interacts with the catalytic domain of $\text{CHK}\alpha$. Furthermore, V-11-0711 displays an 11-fold higher specificity for $\text{CHK}\alpha$ over $\text{CHK}\beta$, hence this compound might represent a novel potential selective $\text{CHK}\alpha$ inhibitor [14].

Other small molecule inhibitors to $\text{CHK}\alpha$ are also currently being validated for their anti-oncogenic properties [47]. Despite the extensive evidence suggesting an important role for $\text{CHK}\alpha$ in promoting tumour formation, $\text{CHK}\beta$ has not been implicated in this process. Given the similarity of $\text{CHK}\alpha$ and $\text{CHK}\beta$ and the functional activity of homo or heterodimers the design of anti-tumor

drugs selectively targeting the CHK α isoform is challenging. Blockade of CHK β activity might lead to detrimental changes to mitochondrial function and lipid content alteration in muscle cells. Ideally, the novel selective CHK α inhibitors will have high specificity and efficiency with limited side effects due to undesirable actions on CHK β in host cells.

4.2. Supplemental therapies to target Choline Kinase deficiency using CDP-choline

Deletion of *choline kinase* genes leads to abnormal phosphatidylcholine biosynthesis and catabolism. Using various experimental animal models, long term administration of CDP-choline to aged mice was shown to elicit a partial recovery of dopamine and acetylcholine receptor densities and function in the striatum of mice [48], and to mitigate memory impairment resulting from poor environmental conditions in rats [49]. More recently, CDP-choline supplementation was found to improve regeneration and functional restoration of injured sciatic nerves and nerve adherence and separability in rats [50, 51], and to exert neuroprotective effects via stimulating axonal regeneration in peripheral nerve injury in rat [52]. In addition, CDP-choline supplements may be used as a potential therapeutic agent to prevent necrotizing enterocolitis [53], and bronchopulmonary dysplasia in rats [54].

Notably, human studies have shown that CDP-choline (also known as citicoline - an international non-proprietary name) supplements might be beneficial in patients with mild mental deterioration and Alzheimer's disease with APOE genotype [55], and also in patients with non-arteritic ischaemic optic neuropathy [56]. CDP-choline supplements might also be used as a pro-cognitive strategy [57], and evidence is suggestive for a role in cognitive enhancement through a nicotinic cholinergic mechanism in healthy volunteers [58]. Randomized clinical CDP-choline trials have shown improved outcome of ischemic and hemorrhagic clinical stroke [59]. However, more recent clinical trials have revealed no benefits in ischaemic stroke and traumatic brain injury [60]. The efficacy of

CDP-choline in the treatment of neural pathologies remains to be further investigated, however it has an excellent biosafety profile [59].

Given the recent discovery of a predominant role for CHK β in the musculoskeletal system, supplementation with CDP-choline, a regimen that bypasses CHK β deficiency, might be particularly useful to treat CHK β deficiency in musculoskeletal conditions. Indeed, it has been shown that CDP-choline can elicit a repairing effect on the damaged membranes of aged animals [61]. Injection of CDP-choline was able to increase the phosphatidylcholine content of hindlimb muscles and partially rescued the muscle weakness in *Chkb* mutant mice [24]. A recent study revealed that a human patient with a *Chkb* mutation with loss of function also had changes in mitochondria in their cardiac muscle [62]. For patients such as these CDP-choline supplements might be beneficial to reduce the need for heart transplant. Changes in phosphatidylcholine levels in cardiac muscle have been correlated with aberrations in mitochondrial function and subsequently increased risk for heart disease [63], and modification of phosphatidylcholine synthesis pathways via treatment with CDP-choline shows potential as a novel treatment approach for cardiomyocyte dysfunction [64].

Osteoporotic fracture is a highly significant problem as it results in much morbidity and mortality, of particular concern is that osteoporotic fractures in the elderly have been correlated with increased mortality rates [65, 66]. The identification of a role for CHK β in the skeletal system could pave the way for the development of a novel therapeutic regime for this highly widespread medical condition. In *Chkb* mutant mice, treatment with CDP-choline *in vivo* and *in vitro* was able to restore abnormal osteoclast numbers to physiological levels, indicating that CDP-choline supplements might also be beneficial against osteoporosis [32].

5. Conclusion

The functions of Choline Kinase in the production of phosphatidylcholine have been known for nearly 70 years, however we still do not completely understand the mechanisms by which the cell specific activities of this kinase are controlled. The three isoforms of Choline Kinase, $\text{CHK}\alpha 1$, $\text{CHK}\alpha 2$ and $\text{CHK}\beta$, share similar structures and enzyme activity, but display some distinct molecular structural domains and differential tissue expression patterns. The importance of $\text{CHK}\alpha$ in human cancer development and progression together with the emerging understanding of the importance of $\text{CHK}\beta$ in musculoskeletal tissues, and the potential to specifically target these molecules in pathological processes will support significant further research into these essential kinases.

Disclosure

All authors state that they have no conflicts of interest.

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References

- [1] Li Z, Vance DE. Phosphatidylcholine and choline homeostasis. *J Lipid Res.* 2008;49:1187-94.
- [2] Michel V, Yuan Z, Ramsubir S, Bakovic M. Choline transport for phospholipid synthesis. *Exp Biol Med* (Maywood). 2006;231:490-504.
- [3] Okuda T, Haga T. High-affinity choline transporter. *Neurochem Res.* 2003;28:483-8.
- [4] Michel V, Bakovic M. The ubiquitous choline transporter SLC44A1. *Cent Nerv Syst Agents Med Chem.* 2012;12:70-81.
- [5] Aoyama C, Liao H, Ishidate K. Structure and function of choline kinase isoforms in mammalian cells. *Prog Lipid Res.* 2004;43:266-81.
- [6] Wu G, Vance DE. Choline kinase and its function. *Biochem Cell Biol.* 2010;88:559-64.
- [7] Aoyama C, Yamazaki N, Terada H, Ishidate K. Structure and characterization of the genes for murine choline/ethanolamine kinase isozymes alpha and beta. *J Lipid Res.* 2000;41:452-64.
- [8] Liao H, Aoyama C, Ishidate K, Teraoka H. Deletion and alanine mutation analyses for the formation of active homo- or hetero-dimer complexes of mouse choline kinase-alpha and -beta. *Biochim Biophys Acta.* 2006;1761:111-20.
- [9] Wu G, Aoyama C, Young SG, Vance DE. Early embryonic lethality caused by disruption of the gene for choline kinase alpha, the first enzyme in phosphatidylcholine biosynthesis. *J Biol Chem.* 2008;283:1456-62.
- [10] Ramirez de Molina A, Gallego-Ortega D, Sarmentero-Estrada J, Lagares D, Gomez Del Pulgar T, Bandres E, et al. Choline kinase as a link connecting phospholipid metabolism and cell cycle regulation: implications in cancer therapy. *Int J Biochem Cell Biol.* 2008;40:1753-63.
- [11] Glunde K, Raman V, Mori N, Bhujwalla ZM. RNA interference-mediated choline kinase suppression in breast cancer cells induces differentiation and reduces proliferation. *Cancer Res.* 2005;65:11034-43.
- [12] Gallego-Ortega D, Gomez del Pulgar T, Valdes-Mora F, Cebrian A, Lacal JC. Involvement of human choline kinase alpha and beta in carcinogenesis: a different role in lipid metabolism and biological functions. *Adv Enzyme Regul.* 2011;51:183-94.
- [13] Ramirez de Molina A, Sarmentero-Estrada J, Belda-Iniesta C, Taron M, Ramirez de Molina V, Cejas P, et al. Expression of choline kinase alpha to predict outcome in patients with early-stage non-small-cell lung cancer: a retrospective study. *Lancet Oncol.* 2007;8:889-97.
- [14] Falcon SC, Hudson CS, Huang Y, Mortimore M, Golec JM, Charlton PA, et al. A non-catalytic role of choline kinase alpha is important in promoting cancer cell survival. *Oncogenesis.* 2013;2:e38.
- [15] Kim HS, Tian L, Jung M, Choi SK, Sun Y, Kim H, et al. Downregulation of Choline Kinase-Alpha Enhances Autophagy in Tamoxifen-Resistant Breast Cancer Cells. *PLoS One.* 2015;10:e0141110.
- [16] Bonora M, Wieckowski MR, Chinopoulos C, Kepp O, Kroemer G, Galluzzi L, et al. Molecular mechanisms of cell death: central implication of ATP synthase in mitochondrial permeability transition. *Oncogene.* 2015;34:1608.
- [17] Mori N, Wildes F, Kakkad S, Jacob D, Solaiyappan M, Glunde K, et al. Choline kinase-alpha protein and phosphatidylcholine but not phosphocholine are required for breast cancer cell survival. *NMR Biomed.* 2015;28:1697-706.
- [18] Asim M, Massie CE, Orafidiya F, Pertega-Gomes N, Warren AY, Esmaeili M, et al. Choline Kinase Alpha as an Androgen Receptor Chaperone and Prostate Cancer Therapeutic Target. *J Natl Cancer Inst.* 2016;108.
- [19] Gallego-Ortega D, Ramirez De Molina A, Gutierrez R, Ramos MA, Sarmentero J, Cejas P, et al. Generation and characterization of monoclonal antibodies against choline kinase alpha and their potential use as diagnostic tools in cancer. *Int J Oncol.* 2006;29:335-40.
- [20] Glunde K, Penet MF, Jiang L, Jacobs MA, Bhujwalla ZM. Choline metabolism-based molecular diagnosis of cancer: an update. *Expert Rev Mol Diagn.* 2015;15:735-47.
- [21] Glunde K, Bhujwalla ZM, Ronen SM. Choline metabolism in malignant transformation. *Nat Rev Cancer.* 2011;11:835-48.
- [22] Arlauckas SP, Popov AV, Delikatny EJ. Choline kinase alpha-Putting the ChoK-hold on tumor metabolism. *Prog Lipid Res.* 2016;63:28-40.
- [23] Sher RB, Aoyama C, Huebsch KA, Ji S, Kerner J, Yang Y, et al. A rostrocaudal muscular dystrophy caused by a defect in choline kinase beta, the first enzyme in phosphatidylcholine biosynthesis. *J Biol Chem.* 2006;281:4938-48.
- [24] Wu G, Sher RB, Cox GA, Vance DE. Understanding the muscular dystrophy caused by deletion of choline kinase beta in mice. *Biochim Biophys Acta.* 2009;1791:347-56.
- [25] Gutierrez Rios P, Kalra AA, Wilson JD, Tanji K, Akman HO, Area Gomez E, et al. Congenital megaconial myopathy due to a novel defect in the choline kinase Beta gene. *Arch Neurol.* 2012;69:657-61.
- [26] Miyagawa T, Kawashima M, Nishida N, Ohashi J, Kimura R, Fujimoto A, et al. Variant between CPT1B and CHKB associated with susceptibility to narcolepsy. *Nat Genet.* 2008;40:1324-8.
- [27] Mitsuhashi S, Nishino I. Megaconial congenital muscular dystrophy due to loss-of-function mutations in choline kinase beta. *Curr Opin Neurol.* 2013;26:536-43.
- [28] Nishino I. [New congenital muscular dystrophy due to CHKB mutations]. *Rinsho Shinkeigaku.* 2013;53:1112-3.
- [29] Castro-Gago M, Dacruz-Alvarez D, Pintos-Martinez E, Beiras-Iglesias A, Delmiro A, Arenas J, et al. Exome sequencing identifies a CHKB mutation in Spanish patient with megaconial congenital muscular dystrophy and mtDNA depletion. *Eur J Paediatr Neurol.* 2014;18:796-800.

- [30] Castro-Gago M, Dacruz-Alvarez D, Pintos-Martinez E, Beiras-Iglesias A, Arenas J, Martin MA, et al. Congenital neurogenic muscular atrophy in megaconial myopathy due to a mutation in CHKB gene. *Brain Dev.* 2016;38:167-72.
- [31] Li Z, Wu G, Sher RB, Khavandgar Z, Hermansson M, Cox GA, et al. Choline kinase beta is required for normal endochondral bone formation. *Biochim Biophys Acta.* 2014;1840:2112-22.
- [32] Kular J, Tickner JC, Pavlos NJ, Viola HM, Abel T, Lim BS, et al. Choline kinase beta mutant mice exhibit reduced phosphocholine, elevated osteoclast activity, and low bone mass. *J Biol Chem.* 2015;290:1729-42.
- [33] Nelms KA, Goodnow CC. Genome-wide ENU mutagenesis to reveal immune regulators. *Immunity.* 2001;15:409-18.
- [34] Roberts S, Narisawa S, Harmey D, Millan JL, Farquharson C. Functional involvement of PHOSPHO1 in matrix vesicle-mediated skeletal mineralization. *J Bone Miner Res.* 2007;22:617-27.
- [35] Roberts SJ, Stewart AJ, Sadler PJ, Farquharson C. Human PHOSPHO1 exhibits high specific phosphoethanolamine and phosphocholine phosphatase activities. *Biochem J.* 2004;382:59-65.
- [36] Okamoto F, Kajiya H, Toh K, Uchida S, Yoshikawa M, Sasaki S, et al. Intracellular CIC-3 chloride channels promote bone resorption in vitro through organelle acidification in mouse osteoclasts. *Am J Physiol Cell Physiol.* 2008;294:C693-701.
- [37] Kornak U, Kasper D, Bosl MR, Kaiser E, Schweizer M, Schulz A, et al. Loss of the CIC-7 chloride channel leads to osteopetrosis in mice and man. *Cell.* 2001;104:205-15.
- [38] Zaidi M, Moonga BS, Huang CL. Calcium sensing and cell signaling processes in the local regulation of osteoclastic bone resorption. *Biol Rev Camb Philos Soc.* 2004;79:79-100.
- [39] Saitoh Y, Koizumi K, Sakurai H, Minami T, Saiki I. RANKL-induced down-regulation of CX3CR1 via PI3K/Akt signaling pathway suppresses Fractalkine/CX3CL1-induced cellular responses in RAW264.7 cells. *Biochem Biophys Res Commun.* 2007;364:417-22.
- [40] Ramirez de Molina A, Penalva V, Lucas L, Lacal JC. Regulation of choline kinase activity by Ras proteins involves Ral-GDS and PI3K. *Oncogene.* 2002;21:937-46.
- [41] Sanchez-Lopez E, Zimmerman T, Gomez del Pulgar T, Moyer MP, Lacal Sanjuan JC, Cebrian A. Choline kinase inhibition induces exacerbated endoplasmic reticulum stress and triggers apoptosis via CHOP in cancer cells. *Cell Death Dis.* 2013;4:e933.
- [42] Mazarico JM, Sanchez-Arevalo Lobo VJ, Favicchio R, Greenhalf W, Costello E, Carrillo-de Santa Pau E, et al. Choline Kinase Alpha (CHKalpha) as a Therapeutic Target in Pancreatic Ductal Adenocarcinoma: Expression, Predictive Value, and Sensitivity to Inhibitors. *Mol Cancer Ther.* 2016;15:323-33.
- [43] Cannon JG. Structure-activity aspects of hemicholinium-3 (HC-3) and its analogs and congeners. *Med Res Rev.* 1994;14:505-31.
- [44] Hong BS, Allali-Hassani A, Tempel W, Finerty PJ, Jr., Mackenzie F, Dimov S, et al. Crystal structures of human choline kinase isoforms in complex with hemicholinium-3: single amino acid near the active site influences inhibitor sensitivity. *J Biol Chem.* 2010;285:16330-40.
- [45] Clem BF, Clem AL, Yalcin A, Goswami U, Arumugam S, Telang S, et al. A novel small molecule antagonist of choline kinase-alpha that simultaneously suppresses MAPK and PI3K/AKT signaling. *Oncogene.* 2011;30:3370-80.
- [46] Xiong J, Bian J, Wang L, Zhou JY, Wang Y, Zhao Y, et al. Dysregulated choline metabolism in T-cell lymphoma: role of choline kinase-alpha and therapeutic targeting. *Blood Cancer J.* 2015;5:287.
- [47] Zech SG, Kohlmann A, Zhou T, Li F, Squillace RM, Parillon LE, et al. Novel Small Molecule Inhibitors of Choline Kinase Identified by Fragment-Based Drug Discovery. *J Med Chem.* 2016;59:671-86.
- [48] Gimenez R, Raich J, Aguilar J. Changes in brain striatum dopamine and acetylcholine receptors induced by chronic CDP-choline treatment of aging mice. *Br J Pharmacol.* 1991;104:575-8.
- [49] Teather LA, Wurtman RJ. Dietary CDP-choline supplementation prevents memory impairment caused by impoverished environmental conditions in rats. *Learn Mem.* 2005;12:39-43.
- [50] Aslan E, Kocaeli H, Bekar A, Tolunay S, Ulus IH. CDP-choline and its endogenous metabolites, cytidine and choline, promote the nerve regeneration and improve the functional recovery of injured rat sciatic nerves. *Neurol Res.* 2011;33:766-73.
- [51] Caner B, Kafa MI, Bekar A, Kurt MA, Karli N, Cansev M, et al. Intraperitoneal administration of CDP-choline or a combination of cytidine plus choline improves nerve regeneration and functional recovery in a rat model of sciatic nerve injury. *Neurol Res.* 2012;34:238-45.
- [52] Gundogdu EB, Bekar A, Turkyilmaz M, Gumus A, Kafa IM, Cansev M. CDP-choline modulates matrix metalloproteinases in rat sciatic injury. *J Surg Res.* 2016;200:655-63.
- [53] Cetinkaya M, Cansev M, Cekmez F, Tayman C, Canpolat FE, Kafa IM, et al. CDP-choline reduces severity of intestinal injury in a neonatal rat model of necrotizing enterocolitis. *J Surg Res.* 2013;183:119-28.
- [54] Cetinkaya M, Cansev M, Kafa IM, Tayman C, Cekmez F, Canpolat FE, et al. Cytidine 5'-diphosphocholine ameliorates hyperoxic lung injury in a neonatal rat model. *Pediatr Res.* 2013;74:26-33.
- [55] Alvarez XA, Mouzo R, Pichel V, Perez P, Laredo M, Fernandez-Novoa L, et al. Double-blind placebo-controlled study with citicoline in APOE genotyped Alzheimer's disease patients. Effects on cognitive performance, brain bioelectrical activity and cerebral perfusion. *Methods Find Exp Clin Pharmacol.* 1999;21:633-44.
- [56] Parisi V, Coppola G, Ziccardi L, Gallinaro G, Falsini B. Cytidine-5'-diphosphocholine (Citicoline): a pilot study in patients with non-arteritic ischaemic optic neuropathy. *Eur J Neurol.* 2008;15:465-74.

- [57] Knott V, Smith D, de la Salle S, Impey D, Choueiry J, Beaudry E, et al. CDP-choline: effects of the procholine supplement on sensory gating and executive function in healthy volunteers stratified for low, medium and high P50 suppression. *J Psychopharmacol.* 2014;28:1095-108.
- [58] Knott V, de la Salle S, Smith D, Choueiry J, Impey D, Smith M, et al. Effects of acute CDP-choline treatment on resting state brain oscillations in healthy volunteers. *Neurosci Lett.* 2015;591:121-5.
- [59] Clark WM. Efficacy of citicoline as an acute stroke treatment. *Expert Opin Pharmacother.* 2009;10:839-46.
- [60] Grieb P. Neuroprotective properties of citicoline: facts, doubts and unresolved issues. *CNS Drugs.* 2014;28:185-93.
- [61] Gimenez R, Soler S, Aguilar J. Cytidine diphosphate choline administration activates brain cytidine triphosphate: phosphocholine cytidylyltransferase in aged rats. *Neurosci Lett.* 1999;273:163-6.
- [62] Vanlander AV, Muino Mosquera L, Panzer J, Deconinck T, Smet J, Seneca S, et al. Megaconial muscular dystrophy caused by mitochondrial membrane homeostasis defect, new insights from skeletal and heart muscle analyses. *Mitochondrion.* 2016;27:32-8.
- [63] Hatch GM. Cell biology of cardiac mitochondrial phospholipids. *Biochem Cell Biol.* 2004;82:99-112.
- [64] Gonzalez-Pacheco H, Mendez-Dominguez A, Hernandez S, Lopez-Marure R, Vazquez-Mellado MJ, Aguilar C, et al. Pre-conditioning with CDP-choline attenuates oxidative stress-induced cardiac myocyte death in a hypoxia/reperfusion model. *ScientificWorldJournal.* 2014;2014:187071.
- [65] Iacovino JR. Mortality outcomes after osteoporotic fractures in men and women. *J Insur Med.* 2001;33:316-20.
- [66] Cummings SR, Bates D, Black DM. Clinical use of bone densitometry: scientific review. *JAMA.* 2002;288:1889-97.

Figure legends:

Fig. 1. (A) Choline as part of the structural composition of phospholipid bilayer that consists of hydrophobic tail and hydrophilic head. (B) Biosynthetic pathways controlling acetylcholine and phosphatidylcholine production from choline. Choline is transported into cells through both active and passive processes via different choline transporters. The phosphatidylcholine biosynthetic process begins with the phosphorylation of choline by Choline Kinase to produce phosphocholine. Phosphocholine is then converted to CDP-choline by CTP:phosphocholine cytidylyltransferase (CT). CDP-choline combines with diacylglycerol under the actions of CDP-choline:1, 2-diacylglycerol cholinephosphotransferase to form phosphatidylcholine (PC). Additionally, choline can be converted to acetylcholine (Ach) by Choline Acetyltransferase, which transfers an acetyl group to choline. PC is hydrolyzed by phospholipase D (PLD) to yield choline and phosphatidic acid (PA). CK, choline kinase; CDP-choline, cytidine diphosphate-choline; CT, CTP:phosphocholine cytidylyltransferase; CTP, cytidine triphosphate; CPT, CDP-choline:1,2-diacylglycerol cholinephosphotransferase, ChAT,; Ach, acetylcholine; PLD, phospholipase D.

Fig. 2. (A) Multiple sequence alignment showed that mouse CHK α protein shares 48.47% sequence identity with CHK β with 222 amino acid with identical positions. (*) indicates identical amino acid; (:) indicates highly conserved residues; (.) indicates conserved residues. (B) Structural similarity between CHK α and CHK β . The CHK α protein consists of one substrate binding site (aa 115-117), two nucleotide binding sites (aa113-119, aa203-209), and three ATP binding sites (aa142, aa304, aa326). CHK β protein consists of one substrate binding site (aa 77-79), two nucleotide binding sites (aa 75-81, aa 146-152), and three ATP binding sites (aa104, aa244, aa264). In addition, CHK β carries an N-acetylalanine site at amino acid residue 2 position.

Fig. 3. Alignment of the protein structures of human CHK α and CHK β . RMSD of the alignment is 2.906 Å.

Fig. 4. Predicted structures of human CHK α and CHK β showing substrate and nucleotide binding regions in association with the ligands Kanamycin (Kan), adenosine diphosphate (ADP), magnesium (Mg) and adenosine triphosphate (ATP). Images obtained from protein prediction software (Raptor X) and are presented using Protein 3D software (DNASTAR).

Fig. 5. BIOGPS analysis reveals that CHKA and CHKB genes have both common and differential expression patterns in different cells and tissues.

Fig. 1

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Fig. 2

Fig. 2

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