Genome-Wide Identification of Cyclin Dependent Kinase (CDK) Family Genes Influencing Adipocyte Differentiation in Cattle

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Abstract

Background: The cyclin dependent kinases (CDKs) are protein kinases regulating important cellular processes such as cell cycle and transcription. A variety of studies have shown that many CDK genes also played a critical role during adipogenic differentiation. However, there is a lack of systematic research on the CDK gene family regulating bovine adipocyte differentiation.Therefore, this study aimed to characterize CDK family genes in bovine and study the expression pattern during adipocyte differentiation.

Results: We performed a genome-wide analysis and identified 25, 25, 22, 21, 22, 24, 22 and 24 CDK genes in *Bos taurus*, *Bos indicus*, *Hybrid-Bos taurus*, *Hybrid bos indicus*, *Bos grunniens*, *Bos mutus, Bison* and *Bubalus bubalis*, respectively. All the CDK genes classified into 8 subfamilies through phylogenetic analysis. Chromosome localization displayed 25 bovine CDK genes distributed on 16 chromosomes. Collinearity analysis revealed that CDK family genes of *Bos taurus* were extensively homologous with *Bos indicus*, *Hybrid-Bos taurus*, *Hybrid bos indicus*, *Bos grunniens* and *Bubalus bubalis*. Tanscriptome analysis showed that several of the CDK family genes had relatively high expression levels in preadipocytes compared with differentiated adipocytes, which is generally similar to qPCR, indicating that it could have a significant function in the growth of the emerging lipid droplets.

Conclusion: We performed a comprehensive analysis for the CDK family genes including identification, phylogenetic classification, structural characterization, chromosomal distribution, collinearity analysis and expression profile analysis by tanscriptome sequencing and qPCR. The results provide a basis for further study to determine the roles of CDK family genes in regulating adipocyte differentiation, which is beneficial for beef quality improvement.

Background

With the improvement of people's living standard, high-quality beef would become the mainstream of consumption in the future.The intramuscular fat (IMF) content of beef is a crucial factor that affects the flavor, freshness, juiciness, tenderness and color, which plays an important role in the improvement of taste and nutritional value. So it is of great scientific significance to further reveal the molecular mechanism of IMF deposition in cattle. Adipogenic differentiation is a complex process regulated by many genes, among which C/EBPα and PPARγ are the key transcription factors in the regulatory network that could regulate the expression of a series of adipogenic phenotypic genes (FABP1, FABP4, LPL, aP2, CAP, Perilipin, etc.) through transcriptional activation[1–3].A number of genes regulate adipocyte differentiation and lipid droplet formation by regulating C/EBPα and PPARγ directly or indirectly. For example, another important member of C/EBP gene family, C/EBPβ, could transactivate the expression of C/EBPα and PPARγ to promote adipocyte terminal differentiation[2]. In addition, C/EBPβ is involved in the regulation of C/EBPα and PPARγ through activating Klf5, SREBP1c, Ebf1, Xbp1 and Atg4b, respectively[2]. C/EBPβ can also activate some other transcriptional factors and inhibit the expression of Wnt10b which is an anti-adipogenic factor that can suppress PPARγ through β-catenin signaling pathway[4, 5]. SREBP-1a, SREBP-1c and SREBP-2 are three members of SREBP gene family regulating de novo synthesis of fatty acids, the differentiation of adipocytes and synthesis of cholesterol, respectively, by targeting fatty acid metabolism-related genes including ACC, SCD, FASD, GPAT,etc.[6]. Besides, there are a variety of gene families that function in adipogenic differentiation such as KLFs, SMADs, RARs, DGATs and so on[7].

CDKs are a large family of serine/threonine protein kinases that were first discovered in the regulation of cell cycle, and they had diverse functions in various of biological processes in eukaryotes including mRNA processing, regulation of transcription[8–11]. Recently, they have been shown to regulate adipocyte differentiation and lipid droplet formation by phosphorylating a series of associated transcription factors or adipocyte-specific genes. CDK6, a member of CDK family genes, was targeted by miR107 to inhibit Notch and its downstream gene Hes1, thereby inhibiting glucose uptake and triglyceride synthesis in adipocytes[12]. MAPK and CDK2/cyclinA sequentially activated C/EBPβ by maintaining the phosphorylated state of Thr188 during the progression of mitotic clonal expansion (MCE) and adipocytes terminal differentiation[13]. Insulin activated the CCND3-CDK4 complex which in turn phosphorylated Ser388 of the insulin receptor IRS2 to maintain the active status of the insulin signaling pathway in adipocyte, eventually promoting de novo lipid synthesis[14]. In addition, CDK4 could phosphorylate Rb to release E2F leading to preadipocyte proliferation as well as phosphorylate PPARγ to regulate the terminal differentiation of adipocytes[15]. CDK5 could reduce the insulin sensitivity of adipocytes by phosphorylating Ser273 of PPARγ, and inhibition the phosphorylation of Ser273 would promote browning and thermogenesis of white adipose tissue[16]. CDK7 complex could inactivate PPARγ through the phosphorylation of PPARγ-S112 to inhibit adipogenesis[17]. CDK8 promotes the ubiquitination and degradation of SREBP-1c by phosphorylating its serine residues, thus inhibiting the adipogenesis[18]. These findings inspired our curiosity to explore the effects of CDK family genes on bovine adipocytes differentiation. However, the expression patterns and regulatory mechanisms of CDKs in bovine adipocytes have not been systematically studied and elucidated.

Therefore, the present study aimed to detect CDK family genes in the bovine genome, and then perform a detailed analysis of the classification, physicochemical properties, phylogenetic analysis, structural features, and functional analysis. Furthermore, the expression pattern analysis by transcriptom and qPCR verification was performed in order to identify essential members of CDK family that affect adipogenic differentiation. Our study provided a deep insight into CDKs that influence adipogenic differentiation, which is essential for future study in improving IMF in the process of bovine breeding.

Results

Identification of the members in the CDK family

To identify the CDK family members, 59 verified CDK amino acid sequences from cattle (*Bos taurus*, 8), human (*Homo sapiens*, 26) and mouse (Mus *musculus*, 25) were used as the query for genome-wide detection of the homologous sequences in *Bos taurus*, *Bos indicus*, *Bos grunniens*, *Hybrid-Bos Indicus*, *Hybrid-Bos taurus*, *Bos mutus*, *Bison bison bison*, and *Bubalus bubalis*. As a result, 25 non-redundant CDK protein sequences including CDK1-10,CDK11B, CDK12-20, CDKL1-5 were identified in *Bos taurus* (Table 1). In parallel, 22, 21, 22, 24, 22, 25 and 24 CDK family proteins were recognized in *Bos grunniens, Hybrid-Bos Indicus, Hybrid-Bos taurus, Bos mutus, Bison bison bison, Bos indicus* and *Bubalus bubalis*, respectively (Additional file 1) and the protein sequences of all the CDKs were provided in Additional file 2. Transcript ENSBIXT00000049337 is a newly identified member of CDK family in *Hybrid-Bos taurus*, which is named *CDK20* according to the sequence similarity and collinearity.

Table 1
Details of Genome-wide identified CDK family members in *Bos taurus*

Protein Name	gene ID	transcript ID	pI	Mw/Da	Amino acids	description
CDK1	ENSBTAG00000010109	ENSBTAT00000013337	8.38	34025.40	297	cyclin dependent kinase 1
CDK2	ENSBTAG00000004021	ENSBTAT00000005252	8.79	33873.46	298	cyclin dependent kinase 2
CDK3	ENSBTAG00000010509	ENSBTAT00000013885	8.13	34805.48	305	cyclin dependent kinase 3
CDK4	ENSBTAG00000007160	ENSBTAT00000009420	6.51	33646.73	303	cyclin dependent kinase 4
CDK5	ENSBTAG00000007766	ENSBTAT00000010212	7.57	33288.47	292	cyclin dependent kinase 5
CDK6	ENSBTAG00000044023	ENSBTAT00000061349	6.22	37014.40	326	cyclin dependent kinase 6
CDK7	ENSBTAG00000011046	ENSBTAT00000014667	8.67	38946.26	346	cyclin dependent kinase 7
CDK8	ENSBTAG00000016737	ENSBTAT00000022252	8.72	53282.71	464	cyclin dependent kinase 8
CDK9	ENSBTAG00000004695	ENSBTAT00000006162	9.04	42747.58	372	cyclin dependent kinase 9
CDK10	ENSBTAG00000033333	ENSBTAT00000047400	9.16	41046.93	361	cyclin dependent kinase 10
CDK11B	ENSBTAG00000010737	ENSBTAT00000014227	5.34	89901.85	771	cyclin dependent kinase 11B
CDK12	ENSBTAG00000013238	ENSBTAT00000002005	9.54	140641.60	1264	cyclin dependent kinase 12
CDK13	ENSBTAG00000001528	ENSBTAT00000002003	9.71	164717.14	1512	cyclin dependent kinase 13
CDK14	ENSBTAG00000048664	ENSBTAT00000068321	9.06	53169.98	470	cyclin dependent kinase 14

Mw: molecular weight, **pI**: isoelectric point

Protein Name	gene ID	transcript ID	pI	Mw/Da	Amino acids	description
CDK15	ENSBTAG00000055073	ENSBTAT00000086547	6.68	45011.42	405	cyclin dependent kinase 15
CDK16	ENSBTAG00000016769	ENSBTAT00000022303	7.23	55758.68	496	cyclin dependent kinase 16
CDK17	ENSBTAG00000001510	ENSBTAT00000077282	9.1	59563.16	523	cyclin dependent kinase 17
CDK18	ENSBTAG00000012673	ENSBTAT00000085187	9.26	54126.19	471	cyclin dependent kinase 18
CDK19	ENSBTAG00000007288	ENSBTAT00000009583	8.66	56685.13	500	cyclin dependent kinase 19
CDK20	ENSBTAG00000015171	ENSBTAT00000020188	6.06	38546.53	346	cyclin dependent kinase 20
CDKL1	ENSBTAG00000004780	ENSBTAT00000036046	9.08	40735.16	352	cyclin dependent kinase like 1
CDKL2	ENSBTAG00000014038	ENSBTAT00000031574	8.76	64289.09	569	cyclin dependent kinase like 2
CDKL3	ENSBTAG00000010979	ENSBTAT00000014574	9.37	67477.82	591	cyclin dependent kinase like 3
CDKL4	ENSBTAG00000024044	ENSBTAT00000033135	8.88	39465.72	342	cyclin dependent kinase like 4
CDKL5	ENSBTAG00000007428	ENSBTAT00000076996	9.56	107236.16	960	cyclin dependent kinase like 5

Mw: molecular weight, **pI**: isoelectric point

The length of amino acid sequences of 25 cattle CDK proteins ranged from 292 (CDK5) to 1512 (CDK13), and their molecular weight (Mw) was 33288.47-164717.14 Da, which correlated well with the protein length. The isoelectric points (pI) of most CDK family proteins was higher than 8.0, which containing more basic amino acids than acidic amino acids, except for 2 neutral proteins(CDK5 and CDK16), whose pI are 7.57 and 7.23, respectively, and 5 acidic proteins (CDK4, CDK6, CDK11B, CDK15 and CDK20), whose pI is between 5.34 and

6.68. Moreover, we detected all the 25 CDK proteins contained the Serine/Threonine Kinase conserved domain (Additional file 3).

Structural features of bovine CDK family members

To explore the structural characteristics of bovine CDK proteins and genes, the conserved motifs and gene structures were projected based on their phylogenetic relationships (Fig. 1). Results showed the CDKs of cattle initially categorized into three main subfamily according to the evolutionary clades. Among 25 bovine CDK family genes, the first subfamily contains 6 members including CDKL1, CDKL2, CDKL3, CDKL4, CDKL5 and CDK20. The second subfamily possesses CDK10 and CDK11B, and the other members belongs to the third subfamily. Six conserved domains(Motif 1, 3, 5, 6, 7, and 9), containing 29, 21, 21, 21, 21, and 21 amino acids respectively, were shared among all the CDK family proteins (Additional file 4). As a small branch in the third subfamily, CDK16, CDK17 and CDK18, have all of the ten motifs. CDK4, CDK15 and CDK20 all consists of eight motifs, while CDK4 lacks of Motif 4 and Motif 10, CDK15 is short for Motif 2 and Motif 10, and CDK20 is without Motif 4 and Motif 10. The rest CDK proteins comprise nine motifs lacking of CDK10, which indicates they all have the same conserved patterns.

The items of introns, coding sequences (CDS) and untranslated region (UTR) were various among CDK family genes, for instance, the gene length *CDKs* ranged from 3599nt (*CDK4*) to 678562nt (*CDK14*), which is mainly due to the variation in intron. The number of CDS varied from 7 to 17 and the length and layout of 3'UTR and 5'UTR were also various in the noncoding areas. Although CDS, introns and UTRs varied greatly, analysis discovered that CDK family members in the same evolutionary branch tend to show similar gene structures and semblable conserved patterns in motifs.

Phylogenetic relationship of CDK proteins in different organisms

To assess evolutionary relationships of CDK proteins between cattle and other organisms, we conducted a phylogenetic analysis of animals in bovinae (*Bos taurus, Bos indicus, Bos grunniens, Hybrid-Bos Indicus, Hybrid-Bos taurus, Bos mutus, Bison bison bison, and Bubalus bubalis*). Besides, CDK proteins in *Homo sapiens* and *Mus musculus* were also included for they have been studied extensively as two model organisms. Accordingly, 236 amino acid sequences from 10 organisms were aligned to generate nonrooted Neighbor-Joining (NJ) tree (Fig. 2). Phylogenetic analyses revealed CDK family proteins were classified into eight major clades. Clade □ contained CDK4 and CDK6 and Clade □ included CDK5, CDK7 and CDK20. Then CDK14 and CDK15 coalesced into a single branch named Clade □, CDK11A, CDK11B, CDK16, CDK17 and CDK18 got together named Clade □, CDK1, CDK2 and CDK3 got together named Clade □, CDK9 of the 10 species merged together named Clade □, all the Cyclin Dependent Kinases Like proteins (CDKL1, CDKL2, CDKL3, CDKL4 and CDKL5) classified into a category named Clade □, and the rest (CDK8, CDK10, CDK12, CDK13 and CDK19) clustered into a branch named Clade □.

Chromosomal distribution and collinearity analysis of CDK genes

CDK family genes were mapped on the chromosomes of six bovinae species (Fig. 3). 25 bovine *CDKs* distribute on 16 chromosomes including Chr 2, Chr 4, Chr 5, Chr 6, Chr 7, Chr 8, Chr 9, Chr 10, Chr 11, Chr 12, Chr 16, Chr 18, Chr 19, Chr 20, Chr 28 and Chr X. Among them, the *CDKs* of cattle have a similar position distribution with *Bos*

indicus, whereas the arrangement of a few genes on chromesomes are different between cattle and the other species. For example, the order of *CDK14* (7.94–8.62 Mb), *CDK6* (9.94–10.19 Mb), *CDK13* (80.95–81.08 Mb) and *CDK5* (113.630-113.634 Mb) in *Bos taurus* Chr 4 was opposite from that in *Hybrid-Bos Indicus, Hybrid-Bos taurus and Bos grunniens*, which is *CDK5*, *CDK13*, *CDK6* and *CDK14*, respectively. *CDK4*, *CDK2* and *CDK7* were three tandem genes in *Bos taurus* at the location of 29.66–29.69 Mb, 29.66–29.69 Mb and 29.72–29.92 Mb on Chr 5, while the arrangement of these three genes were reversed in *Bos grunniens, Hybrid-Bos Indicus* Chr 5 and *Bubalus* Chr 4. In addition, compared with *Bos taurus*, *Bos grunniens* lacks of *CDK7*, *CDK11B* and *CDK20*, *Hybrid-Bos Indicus* lacks of *CDK11B*, *CDK16*, *CDK20* and *CDKL4*, *Hybrid-Bos taurus* is without *CDK11B*, *CDK20*, *CDKL4* and *CDKL5*, and *Bubalus* is short of *CDK11B*. What's more, *CDKL5* is located on chromosome X in *Bos taurus*, while on chromosome Y in *Bos grunniens.*

Collinearity analysis of the genome resulted in the identification of 31,691, 34,495, 33,570, 32,378 and 33,327 pairs of collinear genes between *Bos taurus* and *Bos indicus*, *Hybrid-Bos Indicus*, *Hybrid-Bos taurus*, *Bos grunniens* and *Bubalus bubalis*, respectively(Fig. 4). Results showed that there is a one-to-one correspondence between chromosomes of *Bos taurus* and *Hybrid-Bos Indicus*, *Hybrid-Bos taurus*, *Bos indicus* and *Bos grunniens*. A large chromosome homologous also existed between cattle (2 *N* = 60) and buffalo (2 *N* = 50), although the chromosome number is different of the two species.The syntenic blocks revealed that Chr1 of buffalo appears to be a fusion of cattle Chr1 and Chr 27, buffalo Chr 2 appears to be a combination of cattle Chr2 and Chr 23, buffalo Chr 3 amounts to cattle Chr 8 and Chr 19, buffalo Chr 4 equals cattle Chr 5 and Chr 28, and buffalo Chr 5 equals cattle Chr- 16 and Chr 29. The detailed syntenic relationships of CDK family genes between cattle and the other five species in bovinae was displayed in Table 2.

Table 2
Syntenic relationships of CDK family genes between cattle and the other five species

Gene	*Bos indicus*	*Hybrid-Bos taurus*	*Hybrid-Bos Indicus*	*Bos grunniens*	*Bubalus bubalis*
CDK1	Y	Y	Y	Y	Y
CDK2	Y	Y	Y	Y	Y
CDK3	Y	Y	Y	Y	Y
CDK4	Y	Y	Y	Y	Y
CDK5	Y	Y	Y	Y	Y
CDK6	Y	Y	Y	Y	Y
CDK7	N	Y	Y	-	Y
CDK8	Y	Y	Y	Y	Y
CDK9	Y	Y	Y	Y	Y
CDK10	Y	Y	Y	Y	Y
CDK11B	N	-	-	-	-
CDK12	Y	Y	Y	Y	Y
CDK13	Y	Y	Y	Y	Y
CDK14	Y	Y	Y	Y	Y
CDK15	Y	Y	Y	Y	Y
CDK16	Y	Y	-	Y	Y
CDK17	Y	Y	Y	Y	Y
CDK18	Y	Y	Y	Y	Y
CDK19	Y	Y	Y	Y	Y
CDK20	Y	Y	-	-	Y
CDKL1	Y	Y	Y	Y	Y
CDKL2	Y	Y	Y	Y	Y
CDKL3	Y	Y	Y	Y	Y
CDKL4	Y	-	-	Y	Y
CDKL5	Y	-	Y	Y	Y

'Y' represents the synteny of genes between two species, while 'N' means not and '-' means lacking of the gene.

The expression analysis of CDKs in different tissue

The expression pattern of genes could provide important references for their function. To explore the expression pattern of the CDK gene family during adipogenic differentiation, we investigated the relative expression level in 163 samples of 60 tissue types including heart, liver, spleen, lung, kidney, muscle, fat, etc. The results showed that *CDKs* displayed differential expression patterns in diverse tissues (Fig. 5a), which could be classified into 5 groups (A to E). As a marker gene for adipocyte differentiation, *PPARγ* had a high expression in Group B including omental fat, intramuscular fat, subcutaneous fat and mammary gland fat, indicating that the results is reliable. The 25 *CDKs* could be grouped into 4 categories according to their expression patterns and they all expressed in 60 tissues, suggesting that they may play a broad regulatory role in life activities. Group Ⅰ(*CDK4*, *CDK9* and *CDK11B*) showed the highest expression levels, followed by Group Ⅱ(*CDK3*, *CDK5*, *CDK7*, *CDK8*, *CDK10*, *CDK18*, and *CDK20*) and Group Ⅲ (*CDK1*, *CDK2*, *CDK6*, *CDK12*, *CDK13*, *CDK14*, *CDK16*, and *CDK17*). Group Ⅳ comprised the rest members of *CDKs*, whose expression level was the lowest. Further analysis of the five different fat tissues revealed that *CDK9* was highly expressed in all the fat tissues and its expression pattern was similar to *PPARγ* (Fig. 5b).

Expression analysis of CDKs in preadipocytes and differentiated adipocytes by RNA-seq

Transcriptome analysis of 25 *CDKs* in preadipocytes and differentiated adipocytes revealed that *CDKs* showed a up-regulation trend in preadipocytes compared with differentiated adipocytes except for *CDK1*, *CDK3*, *CDK6*, *CDK19*, *CDKL1* and *CDKL4* (Fig. 6). *CDK7* displayed a significant high expression, whereas *CDK1* showed a significant low expression in preadipocytes within the 95% confidence interval. And *CDK4*, *CDK8*, *CDK9* and *CDK14* all displayed a significant high expression in preadipocytes within the 99% confidence interval.

Expression analysis of CDKs during adipocyte differention by qPCR

To further explore the expression pattern of CDK family genes, preadipocytes collected from perirenal adipose tissue of premature calves were induced differentiation. The results of oil red O staining showed that lipid droplet accumulation was significantly increased in adipocytes induced for 10 days compared to preadipocytes (Additional file 5), indicating that the induction and differentiation was successful. And we conducted qPCR to detect the expression of *CDKs* at 0, 2, 4, 6 and 10 days during adipocytes differentiation(Fig. 7). Results suggested that *CDKs* showed a relatively high expression in preadipocytes and then decreased as differentiation process went on in addition to *CDK1*, *CDK15*, *CDK18*, *CDKL3* and *CDKL5*. The three members, *CDK1*, *CDKL3* and *CDKL5*, all had the highest expression on the second day of differentiation and the lowest expression points were on the 6, 8 and 6 day, respectively. The expression level of *CDK15* and *CDK18* increased with adipocyte differentiation and reached the peak on the fourth day, then decreased.

Discussion

Cattle is known as an important species for supplying meat. The IMF content directly affects the taste and flavor of beef and it is of great scientific significance to reveal the molecular regulation mechanism of IMF deposition

for meat quality improvement. The CDK family genes encoding functional proteins have been well studied in the regulation of transcription, metabolism and cell differentiation[8–10]. However, investigation of *CDKs* in adipocyte differentiation, especially in bovidae, was limited. Since cattle and several species of bovidae were sequenced, the vast amount of genetic resources might serve as references for exploring the evolution and function of CDK gene family and advancing genome science in bovidae.

Structural features of bovine CDK family proteins and genes

The activity of proteins depend on their functional motifs and domains[19]. Six conserved amino acid sequences including Motif 1, Motif 3, Motif 5, Motif 6, Motif 7 and Motif 9 were well-kept among all CDK family members in cattle, indicating the high conservation in motif distribution of CDK family proteins. These highly conserved motifs usually locate at the active sites of the enzymes, which may play essential roles in maintaining the structure, binding to substrate and catalyzing[20, 21]. CDK16, CDK17 and CDK18, a small branch in a subfamily, have all of the ten motifs, meaning some specific functions may exist in the three members. It is speculated that Motif 10 is not located in the core of the catalytic domain due to the other 22 members of CDK family proteins lacking of Motif 10 show kinase activities as well. Several CDK proteins such as CDK4, CDK15 and CDK20 are short of Motif 4, Motif 2 and Motif 8, respectively, in addition to Motif 10, indicating some sequence loss occurred in the evolution. As a newly identified member in *Hybrid-Bos taurus*, CDK20 possesses a conserved STKc domain and nine motifs (Additional file 6). The domain and motifs analysis revealed that it is consistant with the other CDK proteins.

What's more, the gene structural analysis showed that the distribution and number of CDSs, introns, and UTRs were various in *CDKs*. This divergence was mainly caused by the length and layout of introns and UTRs, while the gene coding sequences translated proteins were similar. In other words, the nucleic acid sequences of bovine CDK family members were less conversed compared with amino acid sequences. These results have suggested that the similarity of amino acid sequences, especially that in the conversed motifs, may play essential roles in keeping the kinase functions of CDK proteins.

Phylogenetic relationship of CDK family proteins

The phylogenetic analysis of CDK family proteins in ten species provided an in-deep insight for their evolutionary relationships[22]. The results revealed that CDK family proteins were classified into eight major clades and the same member from different species first clustered in one branch, indicating that they were conserved in sequences among the 10 species. Clade □ was separated out initially while Clade □, □ and □ clustered into a subfamily and the others clustered into another subfamily, manifesting that they have been evolved asymmetrically and the evolutionary relationship between this three subfamilies may be relatively far. Notably, Clade □ includes all the Cyclin Dependent Kinases Like proteins (CDKL1, CDKL2, CDKL3, CDKL4 and CDKL5), which is consistant with the study in human that divided the CDKs into CDK and CDKL[10]. As expected, members of the CDK proteins with a closer relationship tend to have a nearer evolutionary distance, which means that they may cluster together first. For example, *Bos Indicus* CDK1 first clustered with that of *Bubalus bubalis*, and then get together with *Hybrid-Bos taurus*, *Bos taurus, Bos mutus, Hybrid-Bos Indicus*, *Bos grunniens, Bison*, human and mouse in turn.

Collinearity analysis of CDKs in bovidae

The family genes may distribute on different chromosomes or co-locate on the same chromosome, which are generally defined as segmental duplication events in the former and tandem duplication events in the latter[23–25]. Chromosomal distributions of the CDK family genes showed that they located on 13 to 16 chromosomes in six species of bovidae, indicating both segmental and tandem duplication events have occurred for the expansion of CDK genes. The genomes of *Bos taurus*, *Bos grunniens*, *Hybrid-Bos Indicus*, *Hybrid-Bos taurus*, and *Bos indicus* consist of 29 autosomes and a pair sex chromosomes(XX/XY)[26, 27], while *Bubalus bubalis* has 24 autosomes plus a pair sex chromosomes(XX/XY). The collinearity results showed a one-to-one correspondence between chromosomes of *Bos taurus* and *Hybrid-Bos Indicus*, *Hybrid-Bos taurus*, *Bos indicus* and *Bos grunniens*, and large homologous chromosomal regions between *Bos taurus* and *Bubalus*. The arrangement of genes in some syntenic chromosomes may be totally reversed between two species, such as Chr 1, Chr 2, Chr 4, etc. between *bos taurus* and Hybrid-*Bos taurus*, Chr 4, Chr 5, Chr 7, etc. between *Bos taurus* and Hybrid-*Bos taurus*, Chr 2, Chr 4, Chr 6, etc. between *Bos taurus* and *Bos grunniens*, and Chr 9, Chr 10, Chr 11, etc. between *Bos taurus* and *Bubalus bubalis.* The discrepancies may due to the opposite starting point for chromosome annotation. Since all the chromosomes were homologous in bovidae, the positions where *CDKs* located were either collinear (conserved in the same order) or syntenic (not necessarily in the same order) between each two species except for only a few gene pairs[28]. For example, *CDK7* in Chr 20 and *CDK11B* in Chr 16 didn't show synteny between *Bos taurus* and *Bos indicus.*The positions of *CDKL4* and *CDK9* are opposite between *Bos taurus* and *Bos grunniens*, which locate at 21.50-21.55 Mb and 98.46–98.47 Mb in Chr 11 of *Bos taurus* and 41.02–41.08 Mb and 7.13–7.14 Mb in Chr 9 of *Bos grunniens.* The deficiency and discrepancies of CDK genes might be caused by the sequence variation and chromosome rearrangement in the process of evolution[29]. In addition, Chr16 of *Bos taurus* showed syntenic relationship with Chr5 and scaffold NW_020228957.1 of *Bubalus bubalis* (Fig. 4e), suggesting that NW_020228957.1, which hasn't been assembled yet, may be a part of *Bubalus* Chr5. Meanwhile, it was syntenic between CDK18 of *Bos taurus* Chr16 and that in scaffold NW_020228957.1 of *Bubalus.* In a word, the extensive homology provided rich perspectives for studying the function and evolution of CDK family genes in bovidae.

CDK genes affecting adipocyte differentiation

CDK family proteins, as a kind of phosphorylases, could regulate adipocytes differentiation by phosphorylating a series of transcription-related factors or adipocyte-specific genes[12–16, 18]. To dissect the expression pattern of CDK family genes, we analyzed the expression values of the 25 members in 60 tissue types. As a results, *CDK4*, *CDK9* and *CDK11B* showed the highest expression in four types of fat tissues (omental fat, intramuscular fat, subcutaneous fat and mammary gland fat) in relative with other tissues. So we suspected the three genes may play more important and wide-ranging roles in adipose tissue compared with other members of CDK family. Previous studies have revealed that CDK4 could phosphorylate IRS2 and Rb to promote adipogenesis[14, 15]. CDK9, a component of positive transcription elongation factor b (P-TEFb), could phosphorylate the C-terminal domain of RNA polymerase II and regulate the transcription of target genes by facilitating transcriptional elongation[30]. In 3T3-L1 cells, CDK9 increased the adipogenic potential by phosphorylating PPARγ directly and inducing its transcriptional activity[31]. *CDK11B* had similar expression patterns with *CDK4* and *CDK9* by tissue expression analysis, while it has not been reported to be involved in adipogenic differentiation by now. It would be valuable to further explore the function of *CDK11B* in the regulation of adipocytes differentiation.

Adipogenic differentiation is a complicated and well-organized process regulated by multiple genes. Analyzing the expression patterns of CDK family genes during adipocytes differentiation is the basis of exploring their

functions. Results of transcriptome analysis and qPCR validation both revealed that CDK4, CDK7, CDK8 and CDK9 showed significant high expression in preadipocytes. It is speculated that the four members may have important functions in targeting newly generated lipid droplets. The expression of CDK1, CDKL3 and CDKL5 reached the highest in the second day, while CDK15 and CDK18 reached the peak in the fourth day indicating that they may play regulatory roles during adipocyte differentiation in turn. The functions of CDK1, CDK4, CDK7, CDK8 and CDK9 in adipocytes differentiation have been preliminarily studied and need to be further explored[14, 15, 17, 18, 31–35]. Expression analysis revealed that CDK15, CDK18, CDKL3 and CDKL5, whose functions has not been studied, may also play significant roles. In addition, the expression trends of some members were inconsistent between RNA-seq and qPCR validation. For instance, there was no significant differences in the expression of CDK2, CDK6, CDK10, CDK11B, CDK12, CDK16, CDK17, CDK19 and CDKL1 by RNA-seq analysis, but they showed a significant down-regulation in qPCR detection. This discrepancy may caused by different sample sources. The samples for RNA-seq were separated from inguinal subcutaneous fat of two 1 year old male Qinchuan cattle, while samples for qPCR were from perirenal fat of a premature female Holstein calf. In summary, the functions of *CDKs* during adipocytes differentiation is complicated and need to be studied in depth and analyzed comprehensively.

The CDK family genes and the interacted genes constructed an integrative network by literature mining using Agilent Literature Search plug-in of Cytoscape (Additional file 7)[36]. For example, *CDK7* could directly activate *CDK9* to maintain the high expression of *MDM4* and *MDM2*[34, 35]. *MDM2* facilitates adipocyte differentiation through CRTC-mediated activation of *STAT3*[37]. Overall, the results revealed that CDK family genes encoding the enzymes directly or indirectly interact with each other or some other genes, playing non-redundant roles, collectively regulating the life activity including cell cycle, adipocyte differentiation, lipid metabolism etc.

Conclusions

This study conducted a comprehensive genome-wide analysis of CDK family genes in bovidae. A total of 185 CDK genes were identified and grouped into eight distinct clades. Collinearity analysis revealed that CDK family genes were homologous between cattle and other species in bovinae. The expression analysis and functional prediction indicated that *CDKs* may play an significant and complicated role in regulating bovine adipocyte differentiation. The results provided an essential reference for further studies of CDK family genes in the regulation of adipocyte differentiation in cattle.

Methods

Ethics statement

Animal experiments were conducted according to the guidelines of the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, 2004). All animal protocols were approved by Animal Ethics Committee of Ningxia University. A premature female calf of a Holstein pregnant cow used in the experiment was released and the primary adipocyte were isolated immediately. We made all efforts to minimize the calf's suffering.The pregnant cow are not sampled and are still being raised in Zerui ecological breeding farm (yinchuan, China) after a period of recuperation.

Genome-wide identification of CDK genes

The genome and annotation of *Bos taurus* (ARS-UCD1.2.101 assembly), *Bos grunniens* (LU_Bosgru_v3.0.101 assembly), *Hybrid-Bos Indicus* (*Bos indicus* × *Bos taurus*, UOA_Brahman_1.101 assembly), *Hybrid-Bos taurus* (*Bos indicus* × *Bos taurus*, UOA_Angus_1.101 assembly), *Bos mutus* (BosGru_v2.0.101 assembly), *Bison bison bison* (Bison_UMD1.0.101 assembly), *Homo sapiens* (GRCh38.101 assembly) and *Mus musculus* (GRCm38.101 assembly) are from Ensembl database (http://asia.ensembl.org/index.html); *Bos indicus* (GCF_000247795.1 assembly) and *Bubalus bubalis* (ASM312139v1 assembly) are from NCBI database (https://www.ncbi.nlm.nih.gov/). In order to identify all the possible CDKs in bovine, both Hidden Markov Model (HMM) search and Basic Local Alignment Search Tool (BLAST) were performed[38]. The number of 59 reviewed CDKs sequences of bovine *(Bos taurus)*, human (*Homo sapiens*) and mouse (*Mus musculus*) were obtained from UniProt database (https://www.uniprot.org/). These protein sequences were taken as seeds to query the potential candidates of CDK gene family via BLASTP with a threshold of e-value = 10^{-5}. Besides, the HMM of CDKs (Pkinase) was downloaded from Pfam (https://pfam.xfam.org/)[39] and HMMER 3.3.1 (http://hmmer.org/)[40] was used to constructe HMM profiles in bovidae for detection of CDK family genes with the default setting. The candidate sequences obtained from two methods were further manual checked to confirm the CDK homolog sequences. Subsequently, the non-redundant CDK homologs were submitted to NCBI CD-search [41] to verify the presence of the conserved protein domain. The molecular weight and isoelectric point of bovine CDK proteins were calculated by ExPASy (https://web.expasy.org/protparam/)[42].

Phylogenetic analysis

The known CDK amino acid sequences in the *Homo sapiens* and *Mus musculus* were downloaded from UniProt database (https://www.uniprot.org/) (Additional file 2). The identified and known amino acid sequences of CDK in *Bos taurus*, *Bos grunniens*, *Hybrid-Bos Indicus*, *Hybrid-Bos taurus*, *Bos mutus* ,*Bison bison bison*, *Bos indicus* and *Bubalus bubalis*, as well as the known CDKs from *Homo sapiens* and *Mus musculus* were aligned by ClusalW and constructed a Neighbor-Joining tree in MEGA 7.0[43]. The bootstrap was set as 1000 replication. FigTree software (version 1.4.3) was used to adjust and beautify the evolutionary tree.

Structural features analysis

To further evaluate the structural diversity of cattle CDK genes and proteins, a phylogenetic Neighbor-Joining tree was constructed and the conserved motifs were detected in MEME 5.0[44] and visualized in TBtools[45]. The minimum and maximum number of amino acids in each motif were 6 and 50. The motif number of each CDK protein was limited to 10. Also, coding sequences and corresponding genomic sequences of bovine CDKs were loaded into the TBtools to portray the numbers and positions of CDSs and introns graphically.

Chromosomal distribution and collinearity analysis

Positional information of predicted CDK genes of *Bos taurus*, *Bos grunniens*, *Hybrid-Bos Indicus*, *Hybrid-Bos taurus*, *Bos indicus* and *Bubalus bubalis* were extracted from the genomic sequence and annotation files and then were visualized in TBtools[45]. The identified CDKs of each species were mapping on chromosomes. Comparisons between each two genomes were determined by all-against-all BLASTP searches (e-value = 10^{-5}) using the proteome sequences of *Bos taurus* as queries against those of other five bovine species above. The collinearity analysis between *Bos taurus* and other five species for orthologous genes was conducted using MCScanX toolkit[46]. The results of collinearity analysis and orthologous CDKs were visualized by TBtools[45].

Gene expression analysis by transcriptom

The RNA-Seq data of preadipocytes and differentiated adipocytes was downloaded from the National Center for Biotechnology Information (NCBI) Sequence Read Archive(SRR3056892, SRR3064490, SRR3064491, SRR3064492)[47] and transformed into fastaq format by Fastq-dump.The sequencing quality was checked using FastQC[48]. Quality control of raw sequence data, including removal of the adapter sequences and low-quality sequences were performed using the Trim_galore. Clean reads were then mapped to the Bos taurus genome(ARS-UCD1.2.101) using STAR. The RSEM and FeatureCounts was used to calculate the expression of transcripts. Data was normalized by calculating the RPKM for each gene. These results were used to analyze the expression of *CDKs* between preadipocytes and differentiated adipocytes in cattle. The RNA-Seq data of 163 bovine tissue samples were downloaded from Ruminant Genome Database (http://animal.nwsuaf.edu.cn/code/index.php/RGD)[49]. The SRR number and adjusted RPKM values of 163 tissue samples were provided in Additional file 8. The heatmap was performed in R software.

Isolation, culture and induction differentiation of bovine primary adipocytes

Primary adipocyte was isolated and cultured from the perirenal adipose tissue of premature calf in Zerui ecological breeding farm. Type Ⅱ collagenase digestion method was used for the isolation and cultivation of calf preadipocytes. The method described by Huang et al.[50] was adopted in the induction of preadipocytes differentiation, and the method described by Wang et al.[51] was applied for oil red O staining.

Rna Extraction And Quantitative Rt-pcr (qrt-pcr)

RNA extraction and quantitative RT-PCR (qRT-PCR)
According to reference sequence from NCBI, quantitative primers of *CDK* family genes were designed used Primer Premier 5.0 software and the primer sequences were provided in Additional file 9. Total RNA were extracted at 0d, 2d, 4d, 6d and 10d during the differentiation of bovine preadipocytes by phenol-chloroform method using the TRIzol reagent (9109, Takara). RNA samples were measured for absorbance at 260 nm and 280 nm in the multifunctional full-wavelength Multiskan and the samples with an OD260/OD280 ratio between 1.8 and 2.0 was used in the subsequent experiment. Then, 1000 ng total RNA was reverse transcribed using random primers with Moloney murine leukemia virus reverse transcriptase (Takara Bio, Kyoto, Japan). Realtime PCR was carried out in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with SYBR Green Master Mix (Takara Bio, Kyoto, Japan).

Statistical Analysis

All qRT-PCR results were calculated using a $2^{-\Delta\Delta Ct}$ method. Three independent technical repetitions were processed for each test. Statistical significance was examined using Graphpad Prism 7.0 software.

Abbreviations

CDK: cyclin dependent kinases; CDS: coding sequences; HMM: Hidden Markov Model; Chr: Chromosome; IMF: intramuscular fat; pI: isoelectric points; Mw: molecular weight

Declarations

Acknowledgments

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Authors' contributions

CLP and ZXL made the same contribution to the paper. Conceived and designed the research:YM and CLP; Analyzed the data and conducted the experiment: CLP and ZXL; Wrote the paper: CLP; Modified manuscript: YM, LW, ZXL, SZW, XPW, DWW, XYC, ZMLR. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

The Animal Ethics Committees of Ningxia University approved the experimental design and animal sample collection for the present study (permit number NXUC20200618).We obtained the verbal informed consent to participate from the owners and the Ethics Committees of Zerui ecological breeding farm, because our laboratory has a long-term cooperation agreement with the farm about the experiment animals used in experimental research. And animal experiments were conducted strictly followed the guidelines of the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, 2004).

Consent for publication

Not applicable.

Competing interests

The authors declare that we have no competing interests.

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Figures

Figure 1

Characterizations of the identified CDK proteins and genes in Bos taurus. The phylogenetic tree (left) was constructed by Neighbor Joining method. Structure of amino acid sequences (middle), rectangles with different colors represent ten conserved motifs. Gene structure map (right), green rectangle, black line and red rectangle represent CDS, intron and UTR, respectively.

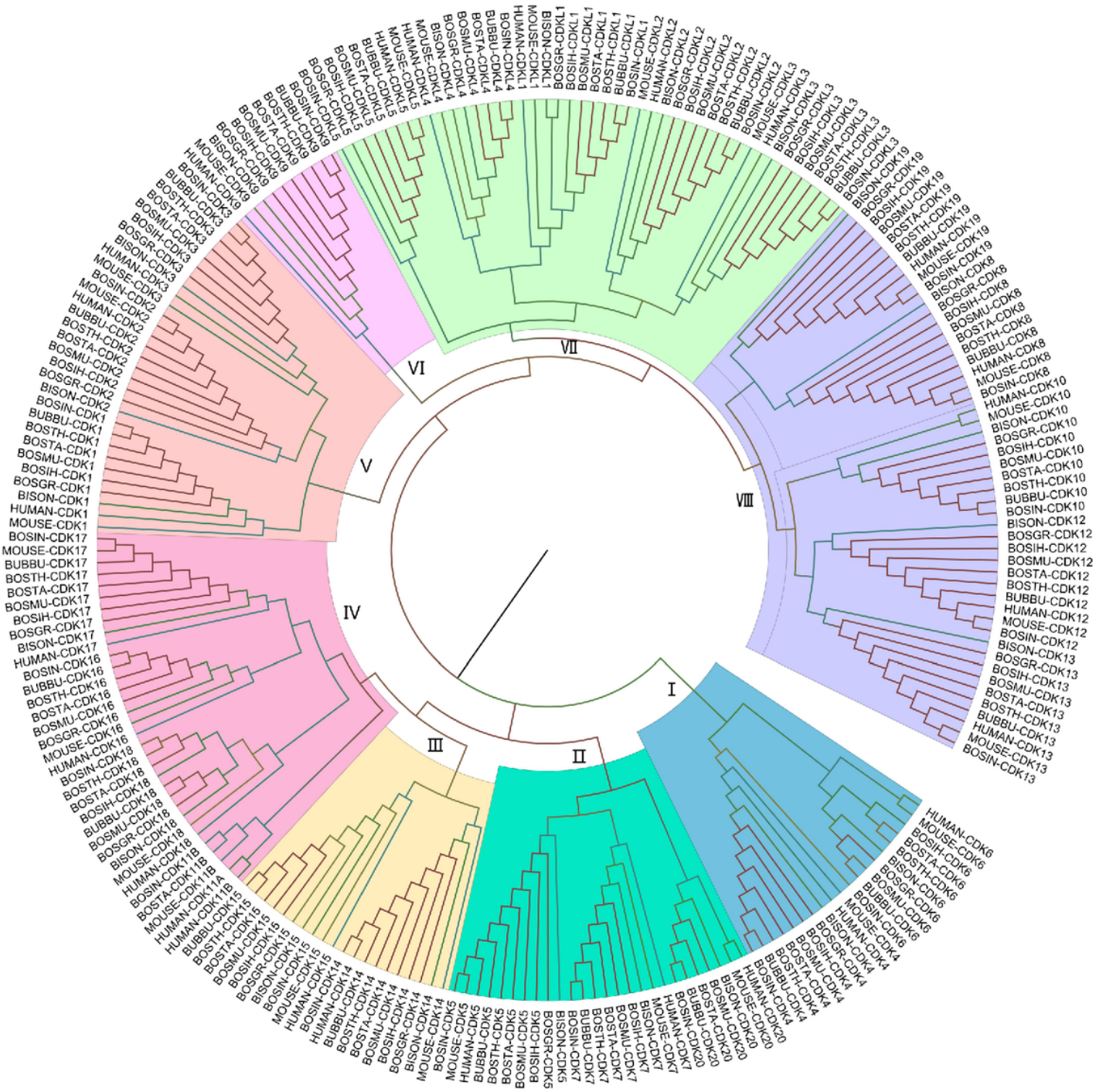

Figure 2

Phylogenetic Neighbor-Joining (NJ) tree of CDK proteins from ten organisms. Identified CDKs in Bos taurus (BOSTA), Bos grunniens (BOSGR), Hybrid-Bos Indicus (BOSIH), Hybrid-Bos taurus (BOSTH), Bos mutus (BOSMU), Bison bison bison (BISOM), Bos indicus (BOSIN) and Bubalus bubalis (BUBBU) together with verified CDKs from Homo sapiens (HUMAN) and Mus musculus (MOUSE) were included in the analyses.The CDK proteins are grouped into eight clusters (Ⅰ-Ⅷ), which are represented by different colors.

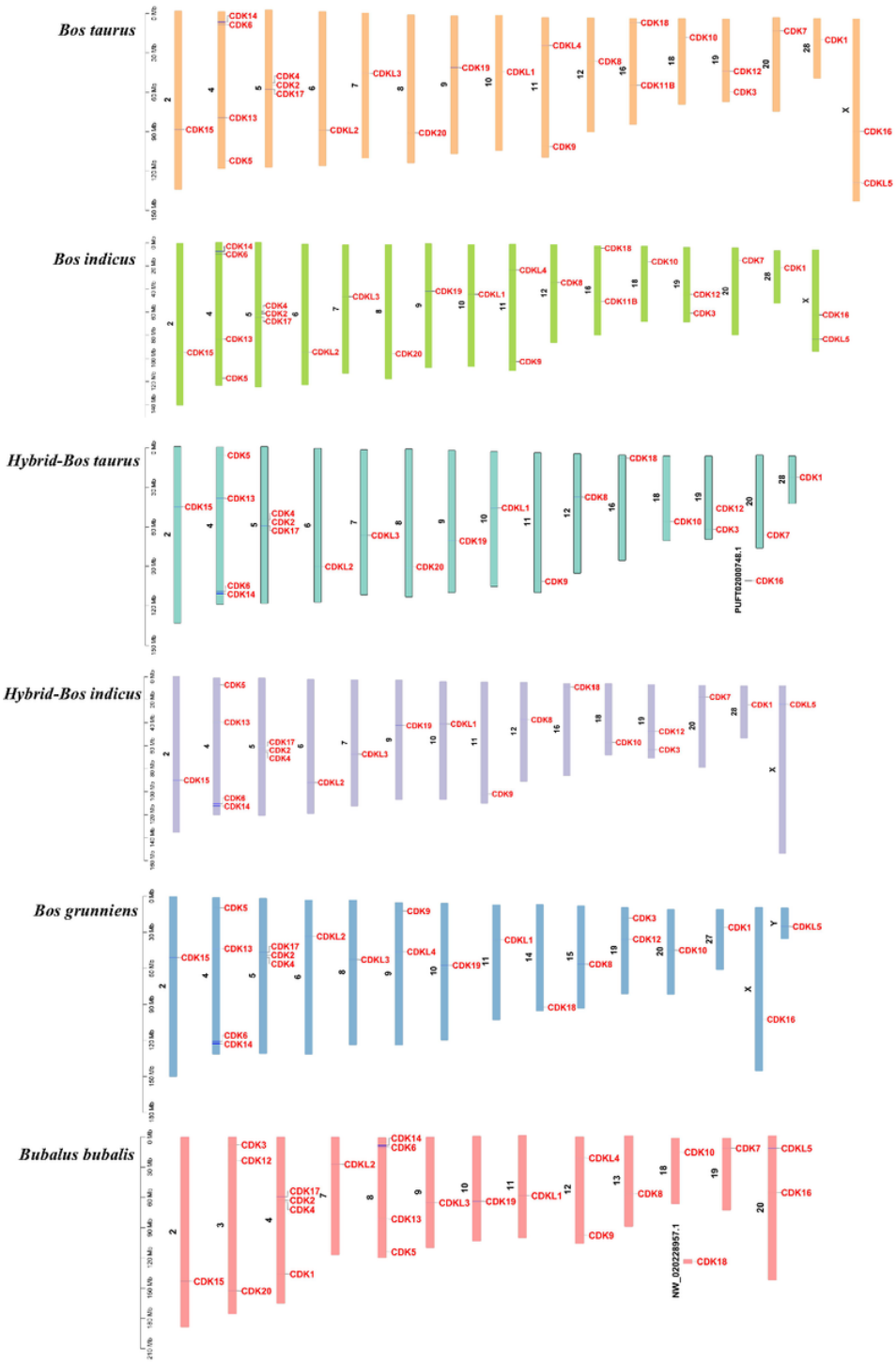

Figure 3

Chromosomal distribution of CDK genes. The black font on the left represents chromosome numbers and the red font on the right represents CDK family genes.

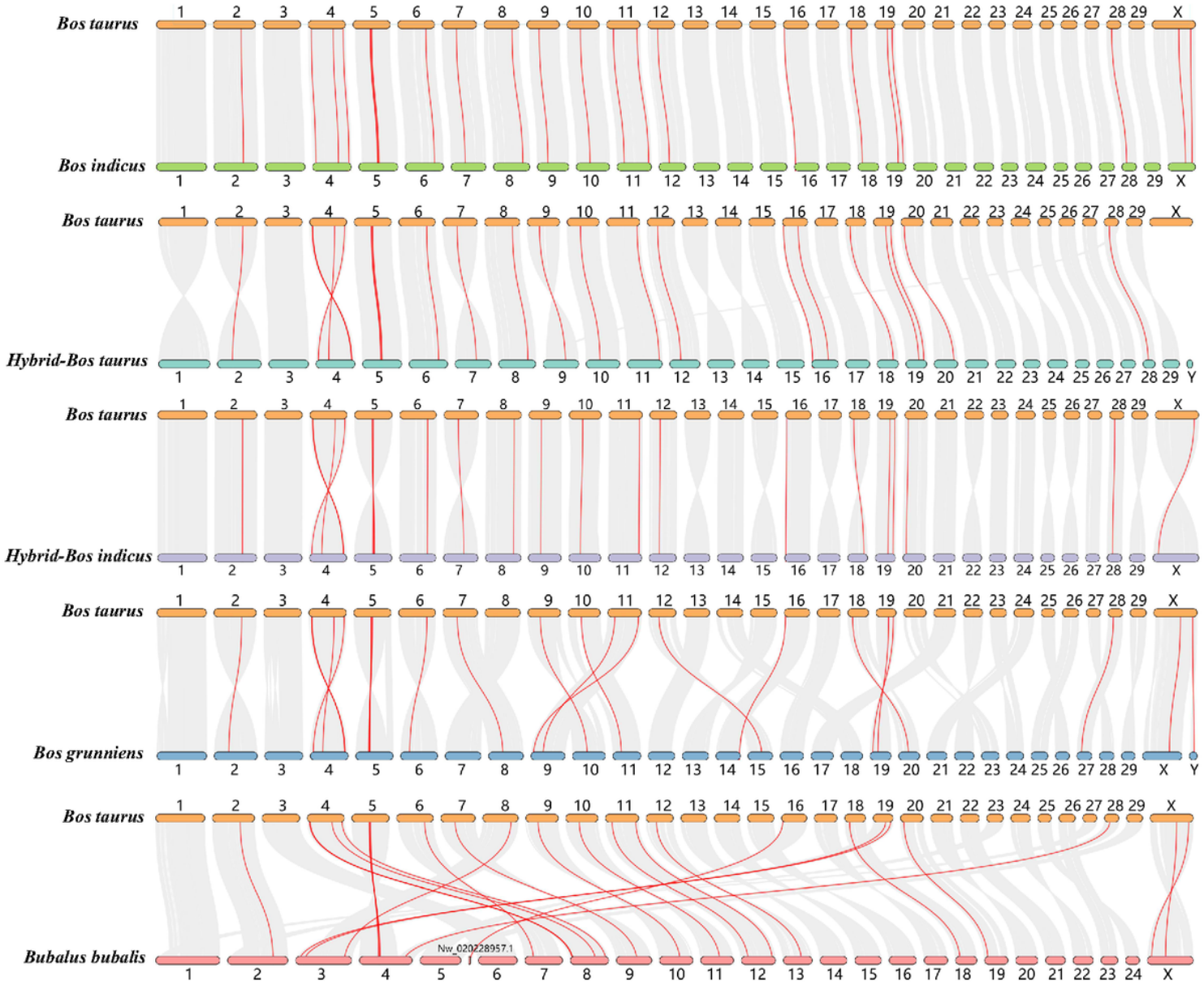

Figure 4

Collinearity analysis of CDK genes between cattle and other organisms. Each pair of linked genes by grey lines are syntenic genes and the red lines represent syntenic CDKs.

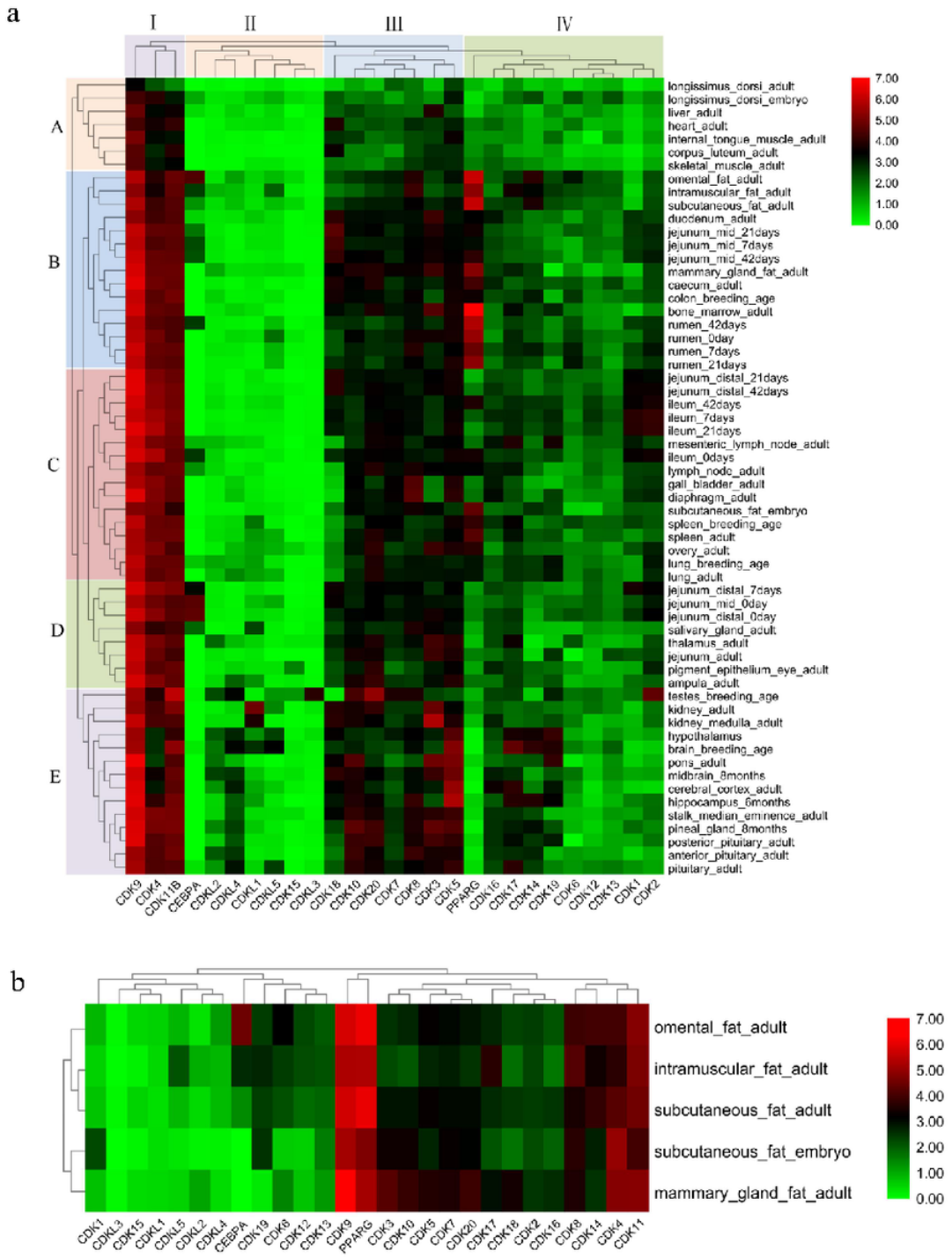

Figure 5

Expression analysis of CDK family genes in different bovine tissue types. a. Expression analysis of CDK family genes in 60 bovine tissues. The tissues were classifed into 5 groups (A to E) and the 27 genes were classifed into 4 groups (□-□) according to their expression pattern. b. Expression analysis of CDK family genes in 5 bovine fat tissues. The horizontal axis represents 25 CDKs and 2 marker genes (PPARG and CEBPA) of adipocyte differentiation. The vertical axis represents different bovine tissues.

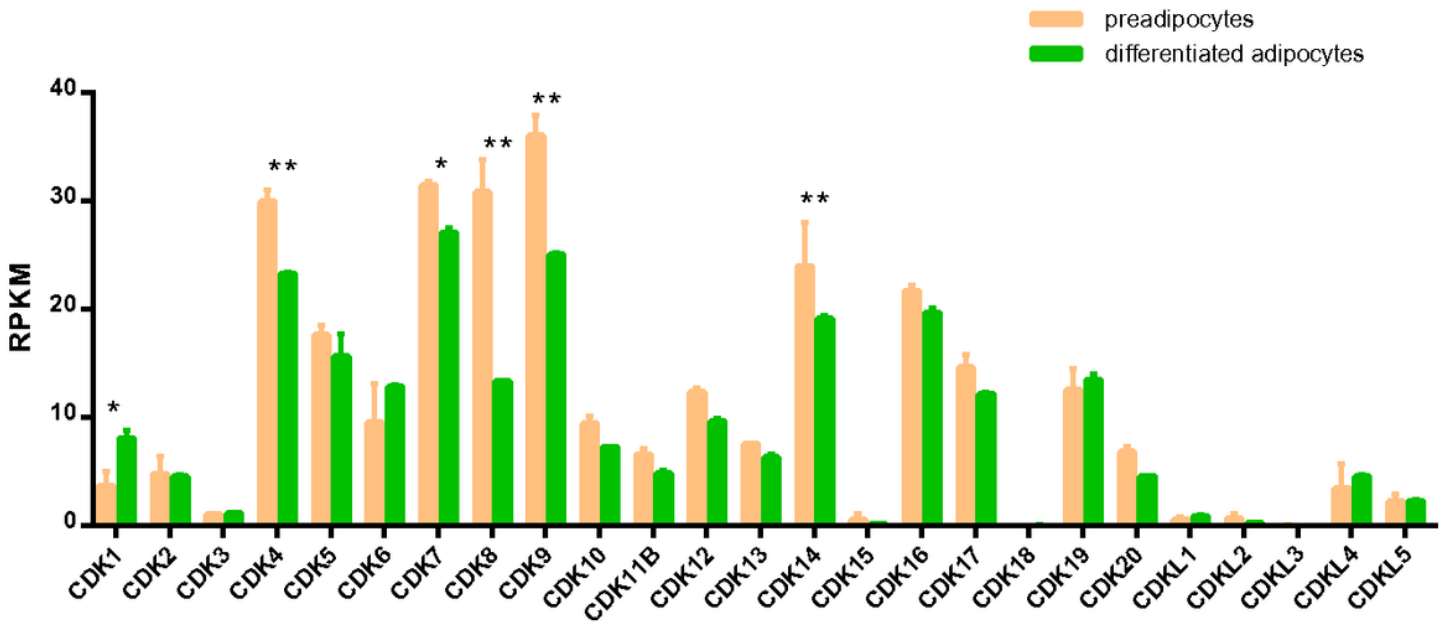

Figure 6

Expression analysis of CDKs in preadipocytes and differentiated adipocytes by RNA-seq. Error bars were obtained from two measurements. '*' and '**' above the bars indicate significant differences at 0.05 and 0.01 level between preadipocytes and differented adipocytes.

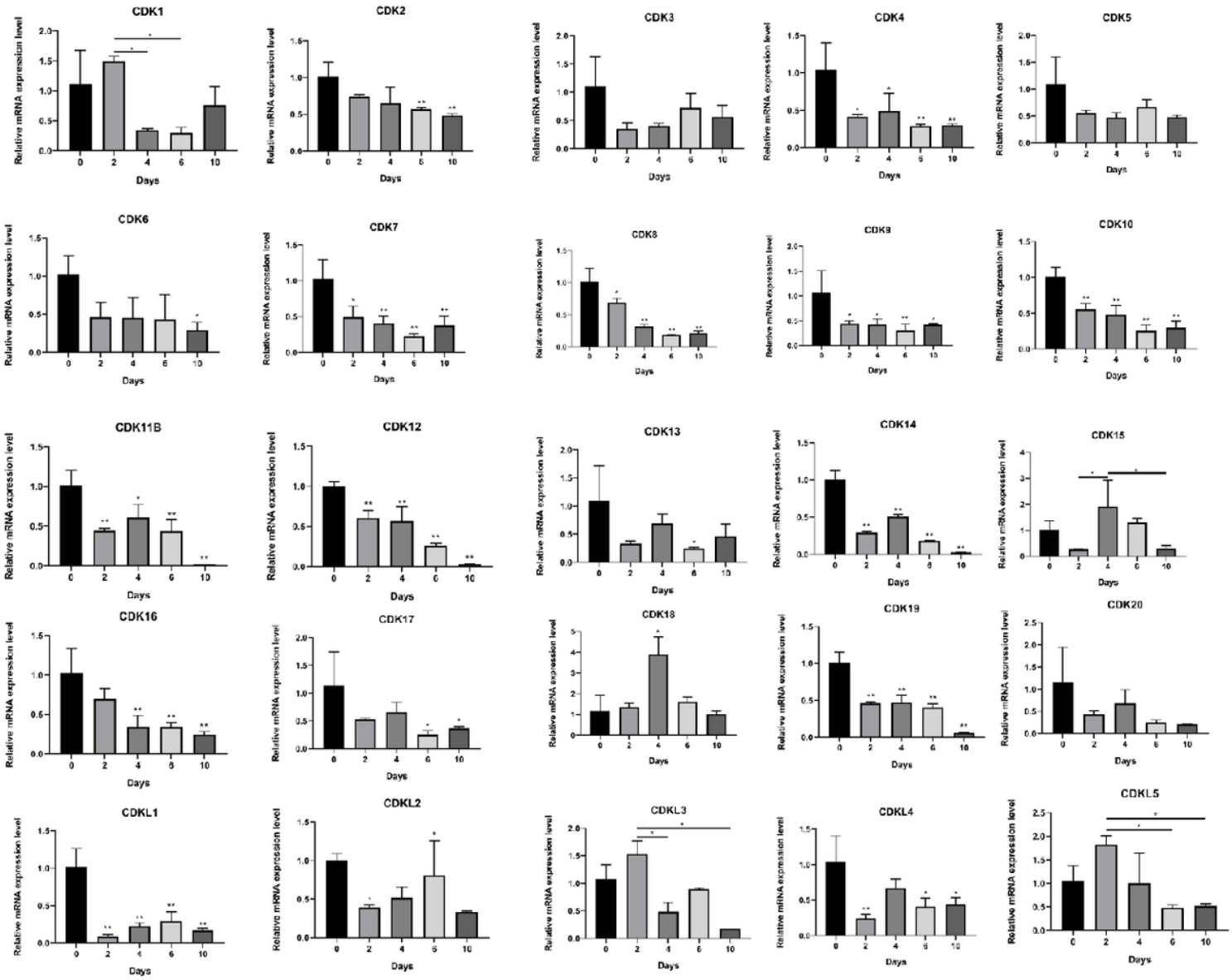

Figure 7

Expression analysis of CDKs during adipocyte differention by qPCR. Error bars were obtained from three measurements. '*' and '**' above the bars indicate significant differences at 0.05 and 0.01 level compared with preadipocytes (0 day). The line with '*' and '**' represents the significant differences between two labeled pillars.

Supplementary Files

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