

# Genome-Wide Identification and Expression Profiling Analysis of Wnt Family Genes Affecting Adipocyte Differentiation in Cattle

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## Research Article

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# Abstract

Wnt is a family of conserved glycoproteins that functions in a variety of crucial biological processes including tissue regeneration, animal development, and cell proliferation and differentiation. For its functional diversity and importance, Wnt gene family has gained considerable research interest in a variety of species. However, comprehensive identification and analysis of Wnt genes in Bovinae is lacking. In this study, we identified the repertoire of Wnt genes in cattle and seven other species of Bovinae and obtained 19 Wnt genes. Protein properties of these Wnt genes were also described. Phylogenetic analysis showed that the 149 Wnt proteins in Bovinae, together with 38 Wnt proteins from human and mouse, were clustered into 12 major clades. The Wnt genes belonging to the same subfamilies shared similar protein motif compositions and exon-intron patterns. Chromosomal distribution and collinearity analysis of Wnt genes among cattle and five species of Bovinae revealed that this gene family was conservative in evolution. RNA-seq data analysis indicated that Wnt genes exhibited tissue-specific expression patterns in cattle. qPCR analysis of Wnt gene family showed that each gene had a unique expression pattern during bovine adipocytes differentiation. And the comprehensive analysis indicated that Wnt2B may regulate adipose differentiation through activation of FZD5, which is worthy of further study. Our study presents the first genome-wide study of Wnt gene family in Bovinae, and lay the foundation for further functional characterization of the Wnt family in bovine adipocytes differentiation.

## Introduction

Wnt proteins, the initiators of the Wnt signaling pathway, comprise a large family of secreted glycoproteins that rich in cysteine[41]. The transduction of Wnt signaling mainly includes three pathways: the canonical Wnt (Wnt/ $\beta$ -catenin) signaling pathway, the non-canonical Wnt/Ca<sup>2+</sup> pathway, and the non-canonical planar cell polarity (PCP) pathway. The three patterns all require to bind to transmembrane receptor Frizzled to regulate intracellular responses. In the canonical Wnt pathway, the activated transmembrane receptor Frizzled (Fzd) lead to a stable accumulation of  $\beta$ -catenin in the cytoplasm and translocation to the karyon. Then  $\beta$ -catenin bind to the transcription factor T-cell factor/lymphoid enhancing factor (LEF1/TCF) family, and activate the transcription of target genes to regulate embryo development, tissue regeneration, and cell proliferation and differentiation[24, 36, 56, 64]. In the non-canonical Wnt/Ca<sup>2+</sup> pathway, the activated receptor Fzd (mainly Fzd2), results in intracellular Ca<sup>2+</sup> release and irritates Ca<sup>2+</sup>-calmodulin-dependent protein kinase II (CamKII) calnexin (CaN), and protein kinase C (PKC), thus regulating cell adhesion and gene transcription[14]. In the non-canonical Wnt/PCP pathway, when the receptor Fzd is stimulated by Wnt proteins (e.g., Wnt5a and Wnt11), signals transmission are through Disheveled (Dvl) to trimeric G proteins. This is followed by activating downstream target genes Rho-associated kinase (Rock) and Jun N-terminal serine/threonine kinase (JNK), thereby regulating cytoskeletal actin and cell polarity[2, 63].

Furthermore, studies have shown that Wnt signaling plays an important role in maintaining the undifferentiated state of precursor adipocytes and inhibiting adipogenesis. In 3T3-L1 preadipocytes, *Wnt1* ectopic expression stabilizes  $\beta$ -catenin, thereby activating TCF-dependent gene transcription and blocking adipogenesis[7, 52]. Glucagon Like Peptide-1 (*GLP-1*) promotes adipogenesis through the up-regulation of the *Wnt4* and  $\beta$ -catenin in the canonical Wnt signaling pathway[38]. KDM5A interacts with C/EBP $\beta$  and cooperatively inhibits the transcription of *Wnt6*, leading to the inhibition of Wnt/ $\beta$ -catenin pathway and promotion of 3T3-L1 preadipocyte differentiation[22]. In human bone marrow stromal (mesenchymal) stem cell (hMSC), the treatment with *Wnt3A* activates Wnt/ $\beta$ -catenin signaling pathway, thus decreasing in adipogenesis and increasing in osteogenesis[50, 51]. An activation of *Wnt10B* activates the Wnt signaling cascade and prevents the 3T3-L1 preadipocytes normal differentiation by inhibiting expression of *C/EBP $\alpha$*  and *PPAR $\gamma$* [23, 26, 52]. In porcine adipose-derived mesenchymal stem cells (AMSCs), *Wnt3A* inhibits the potential of adipogenic differentiation and alters the cell fate from adipocytes to osteoblasts through Wnt/ $\beta$ -catenin signaling pathway[34]. In murine embryonic mesenchymal cell line C3H10T1/2, Wnt3-Fz1 chimera is an inhibitor of differentiation into the adipocyte lineage and a potent activator of differentiation into osteoblasts[8]. It has been reported Wnt gene family also functions via non-canonical Wnt signaling pathway in addition to its role as a canonical Wnt ligand. In hMSC, an inhibition of *Wnt3A* can suppress the non-canonical Wnt/JNK pathway and enhance adipocyte differentiation whereas its activation enhance osteoblast differentiation[51]. In 3T3-L1 preadipocytes, *Wnt4* and *Wnt5A* positively regulate adipogenesis at the initial stage of the differentiation process by activation of PKC and calcium/calmodulindependent kinase II[44]. *Wnt5B* partially represses canonical Wnt/ $\beta$ -catenin signaling pathway

and enhances adipogenesis[27]. These findings stimulated our interest and guided us to explore the function of Wnt gene family on bovine adipocyte differentiation.

So far, the Wnt family has been extensively studied in some species (*Drosophila melanogaster*, *Tribolium castaneum*, *Acyrtosiphon pisum*, *Anopheles gambiae*, and *Apis mellifera*, etc. ) [5, 11, 15, 39, 40, 43]. However, there was limited understanding on their expression patterns and regulatory mechanisms in bovine adipocytes differentiation. To investigate the evolution of Wnt family genes in Bovinae and elucidate the function in bovine adipocytes differentiation, we carried out a genome-wide identification and evolutionary analysis of Wnt gene family in eight species of Bovinae. And the expression profiles in different tissues and stages during adipocytes differentiation were also analyzed in cattle based on transcriptome data and qPCR, respectively. Our study provides a basis for understanding the distribution of Wnt genes in Bovinae and is beneficial to further study of potential function of Wnt genes in bovine adipocytes differentiation.

## Results

### The Wnt Gene Repertoire in Bovinae

To identify the Wnt family members, 45 verified Wnt amino acid sequences were used as the query. These belonged to cattle (*Bos taurus*, 7), human (*Homo sapiens*, 19) and mouse (*Mus musculus*, 19). We used query sequences for genome-wide detection of homologous sequences in *Bos taurus*, *Bos indicus*, *Hybrid-Bos taurus*, *Hybrid-Bos Indicus*, *Bos grunniens*, *Bos mutus*, *Bubalus bubalis* and *Bison bison bison*. In *Bos taurus*, 19 non-redundant Wnt protein sequences were identified that included Wnt1-4, Wnt7A-10A, Wnt7B-10B, Wnt2B, Wnt3A, Wnt5A, Wnt5B, Wnt6, Wnt11, and Wnt16 (Table 1). Wnt family proteins were also recognized in *Bos indicus* (17), *Hybrid-Bos taurus* (19), *Hybrid-Bos Indicus* (19), *Bos grunniens* (19), *Bos mutus* (19), *Bubalus bubalis* (19) and *Bison bison bison* (18). (Supplementary info File 1 and 2). Two unannotated genes of the Wnt family, ENSBMUG00000022627 and ENSBMUG00000022624, were identified in *Bos mutus*. Further analysis revealed that they both have the WNT conserved domain, whereas ENSBMUG00000022627 has an incomplete N terminal and ENSBMUG00000022624 has an incomplete C terminal. They showed the highest sequence similarity and a collinearity relationship with Wnt7B, and were named Wnt7B1 and Wnt7B2 preliminary (Supplementary info File 3).

The amino acid lengths of the 19 bovine Wnt proteins ranged from 333 (Wnt8B) to 585 (Wnt4), whereas molecular weight (Mw) ranged from 36.61 to 63.17 kDa, consistent with protein length. Except Wnt3, the rest Wnt family proteins had the isoelectric points (pI) that higher than 8.0, as they contained more basic amino acids than acidic amino acids. Wnt3 was neutral, with a pI of 7.73. All 19 Wnt proteins contained the WNT conserved domain (Supplementary info File 4).

### Structural features of bovine Wnt family members

In order to learn about the structural characteristics of bovine Wnt proteins and genes, we projected the conserved motifs and gene structures based on their phylogenetic relationships (Fig. 1). According to the evolutionary clades, the 19 bovine Wnt family members clustered into six main subfamilies (I-VI). All the Wnt family proteins shared six conserved domains termed motifs 1, 2, 4, 5, 6, and 7 formed by 50, 49, 41, 29, 28 and 9 amino acids, respectively (Supplementary info File 5). Wnt2, Wnt5A and Wnt5B of the first subfamily and Wnt3 of the third subfamily have all ten motifs. Wnt2B, Wnt3A and Wnt4A have nine motifs, since Wnt2B lacks 9, Wnt3A and Wnt4A lacks 10. Wnt7A, Wnt7B, Wnt10A, Wnt10B and Wnt1 (lacking 9 and 10) and Wnt1 (lacking 8 and 10) have eight motifs. Wnt8A, Wnt8B, Wnt11 and Wnt16 comprise seven motifs lacking 8, 9 and 10. Wnt9A and Wnt9B comprise six motifs lacking 3, 8, 9 and 10.

Introns, coding sequences (CDS) and untranslated regions (UTR) were variable among the Wnt gene family. For instance, the length of Wnt genes ranged from 3,084 nt (*Wnt1*) to 64,231 nt (*Wnt7A*), mainly due to intron variation. The number of CDS varied from 3 to 6, and the length and layout of the noncoding areas, 3'UTR and 5'UTR, were also variable. Despite this variability in CDS, introns and UTRs, the Wnt members in the same evolutionary subfamily tend to possess similar patterns in gene structures and conserved motifs.

### Phylogenetic relationship of Wnt proteins in different organisms

Phylogenetic analysis can provide a reference for understanding functional diversification of the Wnt family in Bovinae. So, phylogenetic analysis was conducted, which included eight Bovinae species (*Bos taurus*, *Bos indicus*, *Hybrid-Bos taurus*, *Hybrid-Bos Indicus*, *Bos grunniens*, *Bubalus bubalis*, *Bos mutus*, and *Bison bison bison*) and *Homo sapiens* and *Mus musculus*. Since Wnt proteins in model organisms (human and mouse) have been studied extensively, they were also included in this study. Finally, 186 amino acid sequences from these ten species were aligned to generate a nonrooted Neighbor-Joining (NJ) tree (Fig. 2). It revealed that Wnt family proteins could be subdivided into 12 proposed subfamilies, including Wnt1–11 and Wnt16 subfamily. There were seven subfamilies that contain two Wnt members, they are subfamily 1 (Wnt7A and Wnt7B), 2 (Wnt3 and Wnt3A), 3 (Wnt2 and Wnt2B), 4 (Wnt5A and Wnt5B), 5 (Wnt10A and Wnt10B), 6 (Wnt9A and Wnt9B), 7 (Wnt8A and Wnt8B), respectively.

### Chromosomal distribution and collinearity analysis of Wnt genes

Wnt genes were mapped on nine chromosomes in organisms of Bovinae (Fig. 3). The bovine Wnt genes showed a similar distribution with the other five species. However, the order of *Wnt1* (30832513-30835596 bp) and *Wnt10B* (30841487-30847512 bp) in Chr 5 and *Wnt3A* (3035810-3087361 bp) and *Wnt9A* (3155441-3163239 Mb) in Chr 7 of *Bos taurus* was reversed from that in *Bos grunniens*. In addition, compared with *Bos taurus*, *Wnt9B* and *Wnt16* were lacking in *Bos Indicus*.

Genome collinearity analysis revealed a satisfactory corresponding relationship between chromosomes of *Bos taurus* and *Hybrid-Bos Indicus*, *Hybrid-Bos taurus*, *Bos indicus* and *Bos grunniens*, respectively (Fig. 4a). Although the chromosome number is different between cattle (2 *N* = 60) and buffalo (2 *N* = 50), there is also large chromosome homology between these two species. Also, collinearity modules explain the difference in the position of Wnt gene family between cattle and the other five species in Bovinae. For instance, the position variation of *Wnt2B*, *Wnt11*, *Wnt1* and *Wnt10B* between *Bos taurus* and *Bos grunniens* may be caused by complex intra-chromosomal translocation events (Fig. 4b), whereas *Wnt3* and *Wnt9B* are distributed on different chromosomes between cattle and buffalo (bovine Chr 19 and buffalo Chr 3), which may be caused by inter-chromosomal rupture or fusion during the evolutionary process (Supplementary info File 6).

### Expression analysis of Wnt genes in different tissue

The functionally related genes tend to show a co-expression patterns and these genes often regulate biological processes collaboratively. To explore the expression patterns of the Wnt gene family during adipogenic differentiation, we investigated their expression levels in 163 samples of 60 tissue types. The Wnt genes along with other 13 closely related genes can be classified into four groups (I to IV) (Fig. 5a) according to their differential expression patterns in diverse tissues. Accordingly, the 60 bovine tissue types also clustered into four main clades (a-d) based on the expression patterns of all the 31 genes including Wnt family. The members of Wnt family and its receptors, FZD gene family, displayed similar and overlapping expression patterns in the 60 tissues, suggesting their broad and coordinated regulatory role in life activities.

*PPAR $\gamma$* , a marker gene for adipocyte differentiation, showed high expression in Group a which included omental, intramuscula, subcutaneous and mammary gland fats. And *CTNNB1*, *FZD1*, *FZD5*, *FZD6* and *Wnt2B* clustered and showed the most similar expression pattern with *PPAR $\gamma$* . Further analysis of the five different fat tissues revealed that *CTNNB1*, a core gene of Wnt signaling pathway, showed high expression and similar expression patterns with *PPAR $\gamma$*  (Fig. 5b).

### Isolation and induced differentiation of bovine primary adipocytes

To explore the expression patterns of the Wnt gene family during adipocyte differentiation, primary adipocytes collected from subcutaneous adipose tissue of calves were induced. The results of oil red O staining showed that lipid droplet gathered together in adipocytes induced for 10 days compared to preadipocytes (Fig. 6a). Further analysis revealed that the absorbance of the differentiated adipocytes at 260nm was significantly higher than that of the preadipocytes (Fig. 6b). Also, The adipogenic marker genes (*PPAR $\gamma$* , *CEBP $\beta$* , *FABP4* and *LPL*) were up-regulated (Fig. 6c). These results indicate that an induced differentiation system of primary adipocytes was established successfully, which can be used in the subsequent gene expression analysis.

### Expression analysis of Wnt genes during adipocyte differentiation

A qPCR analysis was conducted to detect the expression of Wnt genes and their receptors (FZD genes) at 0, 2, 6, and 10 days during adipocytes differentiation (Fig. 7). The members of *Wnt8B*, *Wnt11*, *Wnt16* and their receptors *Fzd1*, *Fzd2*, *Fzd3*, *Fzd4*, *Fzd6* showed a relatively high expression in preadipocytes and then reduced with the process of induced differentiation, suggesting that they may collectively play a role in keeping adipocytes undifferentiated. The members of *Wnt2*, *Wnt6*, *Wnt9B*, *Wnt10A* and their receptors *Fzd9*, *Fzd10* showed an up-regulated tendency, indicating that they may play a regulatory role during adipocyte differentiation. Furthermore, *Wnt2B*, *Wnt4*, *Wnt8A* and *Fzd5*, *Fzd8* reached the lowest expression on the second day and displayed a similar overall trend of expression.

## Discussion

### Structural features of bovine Wnt family proteins and genes

The proteins' core motifs and domains determine their function and activity [57]. Gene families usually encode a group of proteins that possess similar active sites and play a regulatory role synergistically, and they tend to have similar conserved motifs[19]. There are six conserved amino acid sequences (Motifs 1, 2, 4, 5, 6 and 7) in all the 19 bovine Wnt family members, pointing to a common functional site. Four Wnt family members (*Wnt2*, *Wnt3*, *Wnt5A*, and *Wnt5A*) have all of the ten motifs, whereas the other 15 members lack of 1 to 4 motifs. It is likely that these four motifs (Motifs 3, 8, 9 or 10) are not located at the core of the Wnt proteins domain.

As the introns and UTRs vary in length and layout, the CDSs distribution of Wnt genes is also variable. Whereas their amino acid sequences and motif patterns, especially in conserved motifs, were similar, and they all have the WNT conserved domain, which play essential roles in keeping their three-dimensional structure and binding function.

### Phylogenetic relationship of Wnt family proteins

Phylogenetic analysis provides a credible way to explore the relationship between acid sequence similarity and function of proteins in the same family[54]. By focusing on the phylogenetic relationship of Wnt family proteins in previous studies of multicellular eukaryotes, it showed that they can be divided into 13 subfamilies. For instance, a total of 11 (*Zhikong scallop*), 12 (*Yesso scallop*, *Pacific oyster*), 13 (*Lingula anatine*, *Plathynereis dumerlii*, *Lottia gigantean*, *Crassostrea gigas*, etc.) subfamilies were identified, respectively[39]. In Bovinae, Wnt family proteins were classified into 12 sunfamilies lacking of WntA. It is consistent with the result that vertebrates have reserved all subfamilies except WntA[21, 49]. Although the Wnt family is highly conserved in molecular function, large-scale analysis showed that several Wnt members have been lost in many species after the complete set of Wnt genes emerged in cnidarians[31, 33]. Among the eight species in Bovinae, Wnt7B is missing in *Bison*, and Wnt9B and Wnt16 is missing in *Bos indicus*. The Wnt9 subfamily specific to *Bilateria* was also found missing in *Chlamys farreri* [39]. It remains unclear whether these genes were failed to identify due to genome assembly limitation or they have been lost during species evolution. In the phylogenetic relationship, two genes from distinct species located in the same clade were defined as orthologs[29]. The orthologous gene pairs among cattle and the other five bovine species were identified based on the homologous relationships. Results showed that the orthologous Wnt members first clustered in one clade, indicating that they were conserved among different species.

### Collinearity analysis of Wnts in Bovidae

The gene duplication events including tandem duplication and segmental duplication can cause gene family expansion in genome evolution[32]. Chromosome distribution and collinearity analysis indicated that both tandem duplication and segmental duplication were the reasons for the expansion of Wnt family in Bovinae (Fig. 3-4). The members of the Wnt gene family distributed in nine chromosomes in the six selected species. Due to the different starting point of chromosome annotation among species, the arrangement of genes may be totally reversed. For instance, the order of Wnt6, Wnt10A and Wnt4 in Chr2, Wnt2 and Wnt16 in Chr4, and Wnt3A, Wnt9A and Wnt8A in Chr 7 was totally opposite between *Bos taurus* and *Hybrid-Bos taurus*. Besides, intra-chromosomal translocation and rearrangement during species evolution also lead to the changes in gene arrangement[45, 61]. For example, the position of Wnt2B between *Bos taurus* and *Bos grunniens* altered due to the inversion of large segments within the chromosome (Fig. 4B). It lead to the positions where Wnt genes located change from collinear

(conserved in the same order) to syntenic (not necessarily in the same order) between two species [37]. In addition, buffalo Chr 1-5 are collinear with bovine Chr 27 and Chr 1, Chr 23 and Chr 2, Chr 19 and Chr 8, Chr 5 and Chr 28, and Chr 16 and Chr 29, respectively (Supplementary info File 6). It is speculated to be caused by inter-chromosomal rupture or fusion during the evolutionary process. In a word, genome-wide collinearity analysis of Wnts provided rich perspectives for studying the function and evolution of genes in Bovidae.

### Wnt genes affecting adipocyte differentiation

Wnt family proteins can activate their receptors, Fzd family proteins, and regulate adipocyte differentiation through the canonical and/or non-canonical Wnt signaling pathway. As it's known to all, functionally related genes usually exhibit similar expression patterns and gene expression clustering analysis could group together genes of similar function efficiently [17]. Previous studies have shown that Wnt is selective in recognizing its Fzd receptors [58]. For instance, Wnt3 formed a chimera with Fzd1 to regulate the canonical Wnt signaling [8]. To dissect the expression pattern of this family, the expression of 19 Wnt members and 10 Fzd members in 60 tissue types was analyzed. The overlapping in expression patterns may suggest their coordinated and selective regulatory role (Fig. 5). Group Ⅱ (*PPARG*, *CTNNB1*, *FZD1*, *FZD5*, *FZD6* and *WNT2B*) were highly expressed in four fat tissues (omental, intramuscular, subcutaneous and mammary gland). Meanwhile, the studies in human showed that Wnt2B and FZD5 have physical interaction and co-expression relationship (Table 2). Furthermore, they displayed similar expression tendency during the differentiation of bovine adipocytes (Fig. 7). As *PPARG* is a marker gene for adipocyte differentiation and *CTNNB1* is a core gene for canonical Wnt signaling pathway, it is hypothesized that *WNT2B* may bind to its receptor *FZD5* and regulate adipogenic differentiation through canonical Wnt signaling pathway.

Adipogenic differentiation is a well-organized and complicated process regulated by various genes. Analysis of the interaction relationship of Wnt and Fzd is essential to explore their roles. An integrated network of the Wnt and Fzd gene family and their interacting genes was constructed by STRING (<https://string-db.org/>) and visualized by Cytoscape (Supplementary info File 7) [53]. In order to ensure the accuracy of the interaction network, only the evidences of literature mining and experimental verification are selected. Results showed there are extensive and complex direct or indirect relationships between Wnt and Fzd gene family. Since there are limited study on the interaction between Wnt gene and FZD gene to regulate Wnt signaling pathway in cattle, GeneMANIA (<https://genemania.org/>) was used to mine their relationship in human. Results displayed the bias of Wnt family members in recognizing their transmembrane receptors, Fzd family proteins (Table 2) [9, 58], which provided reference on the study of cattle. Overall, these results revealed that the Wnt genes interact with Fzd genes, activating the canonical and/or non-canonical Wnt signaling pathway, and collectively regulating adipocyte differentiation. It laid the foundation for further research on Wnt genes regulating adipocyte differentiation in cattle.

## Methods

### Ethics statement

Animal experiments were conducted in accordance with the requirement of the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, 2004). It is authorized by the Animal Ethics Committee of Ningxia University (permit number NXUC20200521). The calf used in the experiment was electric shocked before released and the primary adipocytes was isolated immediately, making all efforts to minimize its suffering. It also conforms to the requirements of American Veterinary Medical Association (AVMA) Guidelines. The reporting in the study follows the recommendations in the ARRIVE guidelines [46].

### Genome-wide identification of Wnt genes

The genome and annotation of *Bos taurus* (ARS-UCD1.2.101 assembly), *Hybrid-Bos taurus* (*Bos indicus*×*Bos taurus*, UOA\_Angus\_1.101 assembly), *Hybrid-Bos Indicus* (*Bos indicus*×*Bos taurus*, UOA\_Brahman\_1.101 assembly), *Bos grunniens* (LU\_Bosgru\_v3.0.101 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 downloaded from Ensembl database (<http://asia.ensembl.org/index.html>), *Bos indicus* (GCF\_000247795.1 assembly) and *Bubalus bubalis* (ASM312139v1

assembly) are downloaded from NCBI database (<https://www.ncbi.nlm.nih.gov/>). To identify all the possible Wnt genes in Bovinae, both Basic Local Alignment Search Tool (BLAST) and Hidden Markov Model (HMM) search were performed[35]. A total of 45 reviewed Wnts sequences of bovine (7), human (19) and mouse (19) obtained from the UniProt database (<https://www.uniprot.org/>) were used as seeds to query potential Wnt genes via BLASTP with a threshold of  $e\text{-value} = 10^{-5}$ . The HMM of Wnt (PF00110) was downloaded from Pfam (<https://pfam.xfam.org/>)[18] and HMMER 3.3.1 (<http://hmmer.org/>)[48] was conducted to construct HMM profiles in Bovidae to detect Wnt genes with the default setting. Candidate sequences were manually checked to confirm the Wnt homology.

### **Analysis of Wnt Gene Characteristics**

Protein properties of identified Wnt genes, including protein length, molecular weights, and isoelectric points, were estimated using ExPASy (<https://web.expasy.org/protparam/>)[4]. The conserved motifs were detected in MEME 5.0[6]. The minimum and maximum number of amino acids in each motif was 6 and 50, respectively. Exon-intron structures and motif patterns of Wnt family were displayed using TBtools software [12]. NCBI-CDD [42] was used to identify the conservative domain compositions of 19 Wnt proteins, and the results were visualized by the TBtools software [12]. Multiple sequence alignments of Wnts and a phylogenetic Neighbor-Joining tree was constructed using the MEGA 7.0 software [30].

### **Phylogenetic Tree Construction, Chromosomal distribution, and Collinearity analysis**

To investigate the phylogenetic relationship of Wnt genes, the protein sequences were aligned using ClustalW. And a Neighbor-Joining tree was constructed in MEGA 7.0[30] using the following parameters: bootstrap method (1000 replicates), Poisson model, and complete deletion. FigTree software (version 1.4.3) was used to adjust and beautify the evolutionary tree. Chromosomal locations of Wnt genes in cattle and other five species in Bovinae were obtained from general feature format (GFF3) files. Gene Location Visualize from GFF [12] was used to map the distribution of Wnt genes. Collinearity analysis of ortholog genes between *Bos taurus* and the other five bovine species was performed using the MCScanX toolkit[59]. Subsequently, genome collinearity results and orthologous Wnts were visualized by Dual System Plot for MCscanX [12].

### **Expression Profiles of Wnt Genes in RNA-Seq**

RNA-Seq data of 163 tissue samples were collected from the Ruminant Genome Database (<http://animal.nwsuaf.edu.cn/code/index.php/Ruminantia>) [13] and all the raw data was deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (Supplementary info File 8). The sequencing quality was checked using FastQC[62]. Quality control of raw sequence data was performed using the trimmomatic-0.36 [10]. Clean reads were then mapped to the *Bos taurus* genome reference using STAR [16] and Hisat2 [28]. FPKMs (Fragments Per Kilobase per Million mapped reads) of the genes in each sample were computed by Ballgown (Version 2.2.0) [20, 47]. The heat map was constructed using the TBtools software [12].

### **Isolation, culture and induction differentiation of bovine primary adipocytes**

Primary adipocytes were isolated and cultured from subcutaneous adipose tissue of the calf in the Zerui ecological breeding farm. The primary adipocytes were isolated and cultivated by Type I collagenase digestion method. The induction of primary adipocytes differentiation [25] and oil red O staining [60] were performed as previous described. The substance extracted from adipocytes induced at day 0 day and 10 day were also measured at 510nm absorbance with isopropanol as control.

### **RNA extraction and quantitative RT-PCR (qPCR)**

The primers of the Wnt genes were designed using Primer Premier 5.0 software (Supplementary info File 9). Total RNA was extracted by the phenol-chloroform method using TRIzol (9109, Takara) and samples with an OD260/OD280 absorbance ratio between 1.8 and 2.0 were used in the subsequent experiments. Then, a total of 1000 ng 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). The qPCR reaction procedure was 40 cycles of pre-denaturation for 3 min (95 °C), denaturation for 10 s (95 °C),

annealing for 20 s (60 °C), extension for 30 s (72 °C). Relative expression was calculated using the 2- $\Delta\Delta C_t$  method[1, 3] with  $\beta$ -actin as the reference. Three replicates were performed for each test. Statistical significance determined using Graphpad Prism 7.0 software.

## Declarations

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**Authors' contributions** CLP and SZW made the same contribution to the work. Conceived and designed the research: YM and CLP, Analyzed the data and conducted the experiment: CLP, SZW and CLH, Wrote the paper: CLP, Modified manuscript: YM, CYY, HS, XSX, HHH, ZXL and MLY. All authors read and approved the final manuscript.

### Competing interests

The authors declare no competing interests.

### Availability of data and materials

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

### Consent for publication

Not applicable.

### Ethics approval and consent to participate

The Animal Ethics Committees of Ningxia University approved the experimental design and animal sample collection (permit number NXUC20200521). We also obtained the informed consent to participate from the owners and the Ethics Committees of Zerui ecological breeding farm. All the 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). The reporting in the study follows the recommendations in the ARRIVE guidelines.

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Tables

**Table 1.** Characteristics of Genome-wide identified Wnt family members in *Bos taurus*

Gene Name	Gene ID	Transcript ID	pI	Mw/kDa	Amino acids	description
WNT1	ENSBTAG000000015364	ENSBTAT000000020414	9.24	41.04	370	Wnt family member 1
WNT2	ENSBTAG000000008097	ENSBTAT000000010650	9.21	40.64	360	Wnt family member 2
WNT2B	ENSBTAG000000014291	ENSBTAT000000018985	9.32	44.17	394	Wnt family member 2B
WNT3	ENSBTAG000000016012	ENSBTAT000000021312	7.73	39.78	355	Wnt family member 3
WNT3A	ENSBTAG000000039397	ENSBTAT000000056888	8.37	38.42	346	Wnt family member 3A
WNT4	ENSBTAG000000051083	ENSBTAT000000085976	9.11	63.17	585	Wnt family member 4
WNT5A	ENSBTAG000000020221	ENSBTAT000000026930	8.88	42.42	380	Wnt family member 5A
WNT5B	ENSBTAG000000054367	ENSBTAT000000068978	8.99	40.05	358	Wnt family member 5B
WNT6	ENSBTAG000000013990	ENSBTAT000000018592	9.19	39.69	365	Wnt family member 6
WNT7A	ENSBTAG000000001668	ENSBTAT000000002188	9.05	38.90	349	Wnt family member 7A
WNT7B	ENSBTAG000000048365	ENSBTAT000000083229	9.87	52.40	472	Wnt family member 7B
WNT8A	ENSBTAG000000005570	ENSBTAT000000035270	8.25	39.06	351	Wnt family member 8A
WNT8B	ENSBTAG000000012914	ENSBTAT000000017163	8.88	36.61	333	Wnt family member 8B
WNT9A	ENSBTAG000000020267	ENSBTAT000000081055	8.88	39.85	362	Wnt family member 9A
WNT9B	ENSBTAG000000002664	ENSBTAT000000086580	9.19	38.74	353	Wnt family member 9B
WNT10A	ENSBTAG000000009217	ENSBTAT000000012148	9.36	46.34	417	Wnt family member 10A
WNT10B	ENSBTAG000000015347	ENSBTAT000000020402	9.22	42.96	391	Wnt family member 10B
WNT11	ENSBTAG000000010820	ENSBTAT000000014355	9.14	39.24	354	Wnt family member 11
WNT16	ENSBTAG000000002940	ENSBTAT000000003825	9.04	40.42	362	Wnt family member 16

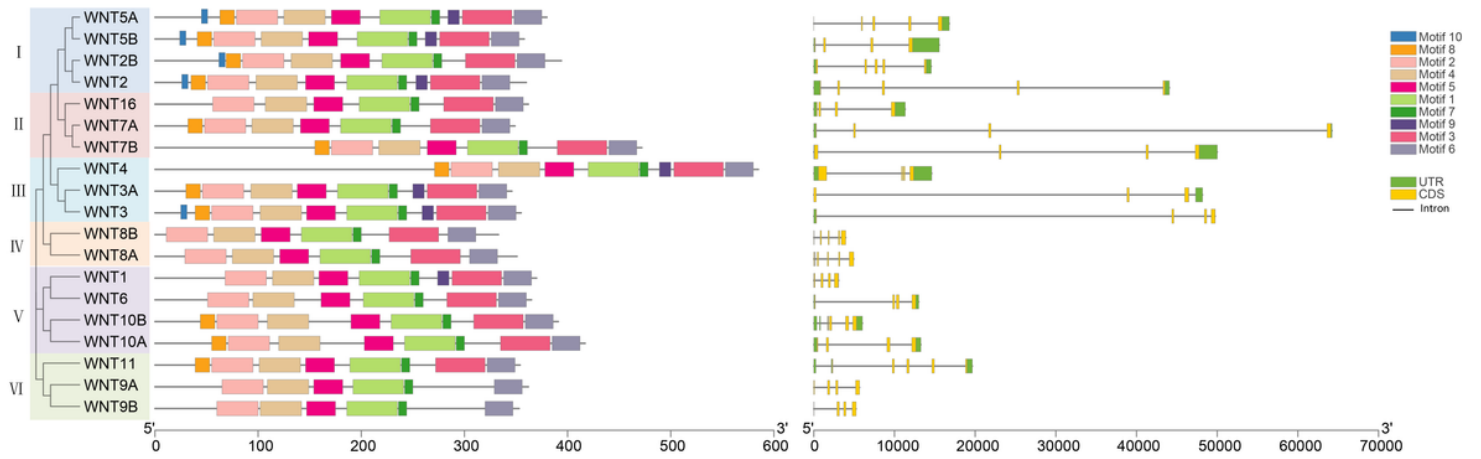
**Mw:** molecular weight, **pI:** isoelectric point

**Table 2.** Interaction relationship between *Wnt* and *Fzd* family genes in Human

Gene Symbol	Interaction types with Fzd family genes				
	pI				
	Mw/kDa				
	Amino acids				
	description				
	Physical interaction	Co-expression	Predicted	Co- localization	Pathway
WNT1	Fzd8	-	Fzd1,Fzd3,Fzd5,Fzd8	-	Fzd1,Fzd3,Fzd5,Fzd8
WNT2	Fzd1,Fzd9	Fzd1,Fzd2	-	-	Fzd1,Fzd2,Fzd9
WNT2B	Fzd5	Fzd5	-	-	Fzd2,Fzd5
WNT3	Fzd1,Fzd7	-	-	-	Fzd1,Fzd2,Fzd7,Fzd8
WNT3A	Fzd1,Fzd2,Fzd8	-	-	-	Fzd1,Fzd2,Fzd5,Fzd8
WNT4	-	-	Fzd1,Fzd5,Fzd8	-	Fzd1,Fzd2,Fzd5,Fzd8
WNT5A	Fzd1,Fzd2,Fzd5	Fzd1,Fzd2,Fzd10	-	Fzd5	Fzd1,Fzd2,Fzd4,Fzd5,Fzd7,Fzd10
WNT5B	-	Fzd2	-	-	Fzd2
WNT6	-	Fzd2	-	-	Fzd1,Fzd2
WNT7A	Fzd5,Fzd9	Fzd9	-	-	Fzd2,Fzd5,Fzd9
WNT7B	-	Fzd3	Fzd1,Fzd5,Fzd8	-	Fzd1,Fzd2,Fzd3,Fzd5,Fzd8,Fzd10
WNT8A	-	-	-	-	Fzd2
WNT8B	-	Fzd3	-	-	Fzd2,Fzd3,Fzd8
WNT9A	-	-	-	-	Fzd2,Fzd8
WNT9B	-	-	-	-	Fzd2,Fzd8
WNT10A	-	Fzd9	-	-	Fzd2,Fzd8,Fzd9
WNT10B	-	Fzd10	-	-	Fzd2,Fzd8,Fzd10
WNT11	-	Fzd4	-	-	Fzd2,Fzd4
WNT16	-	-	-	-	Fzd2

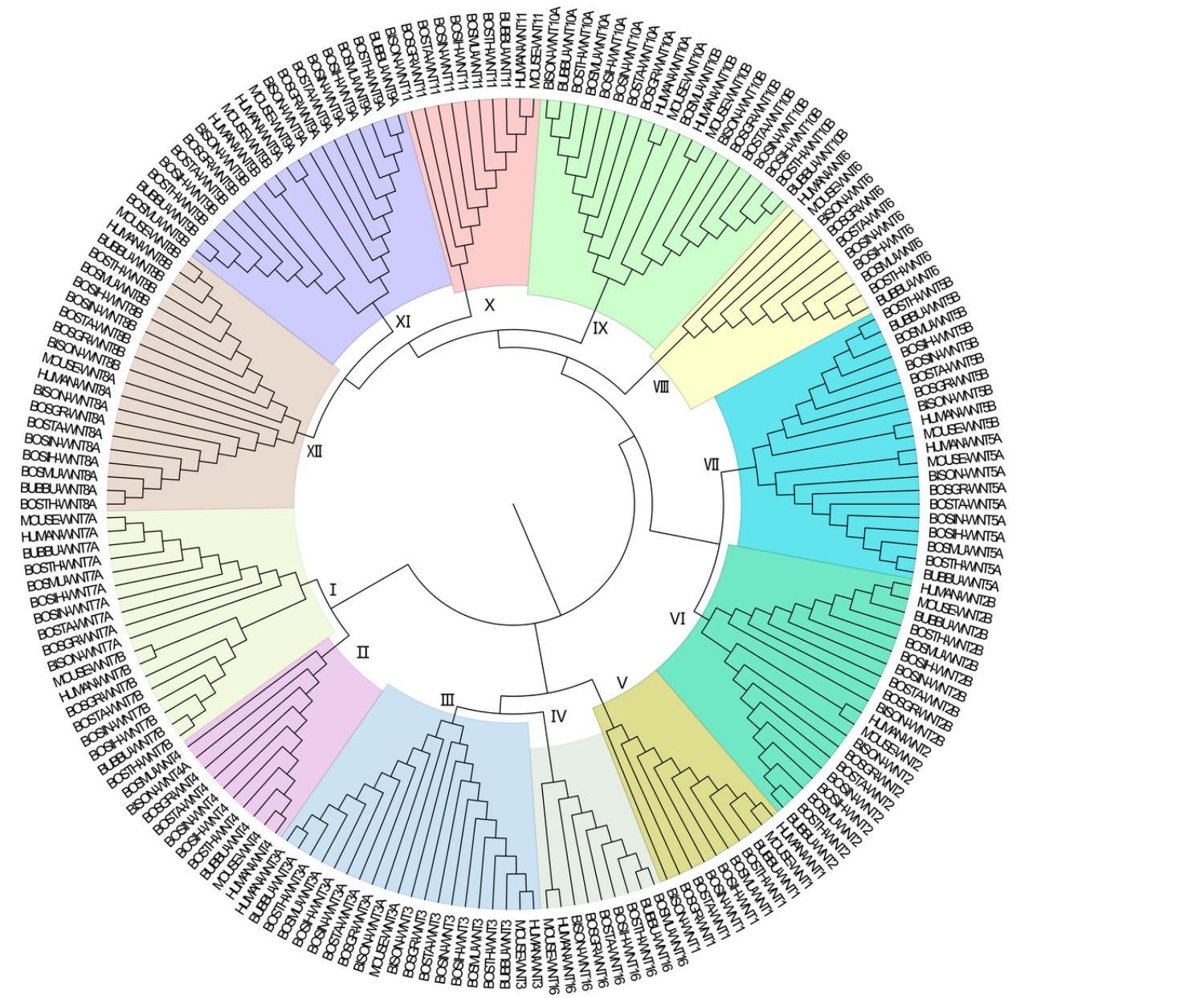
“-” represents the absence of an interacting *Fzd*.

## Figures



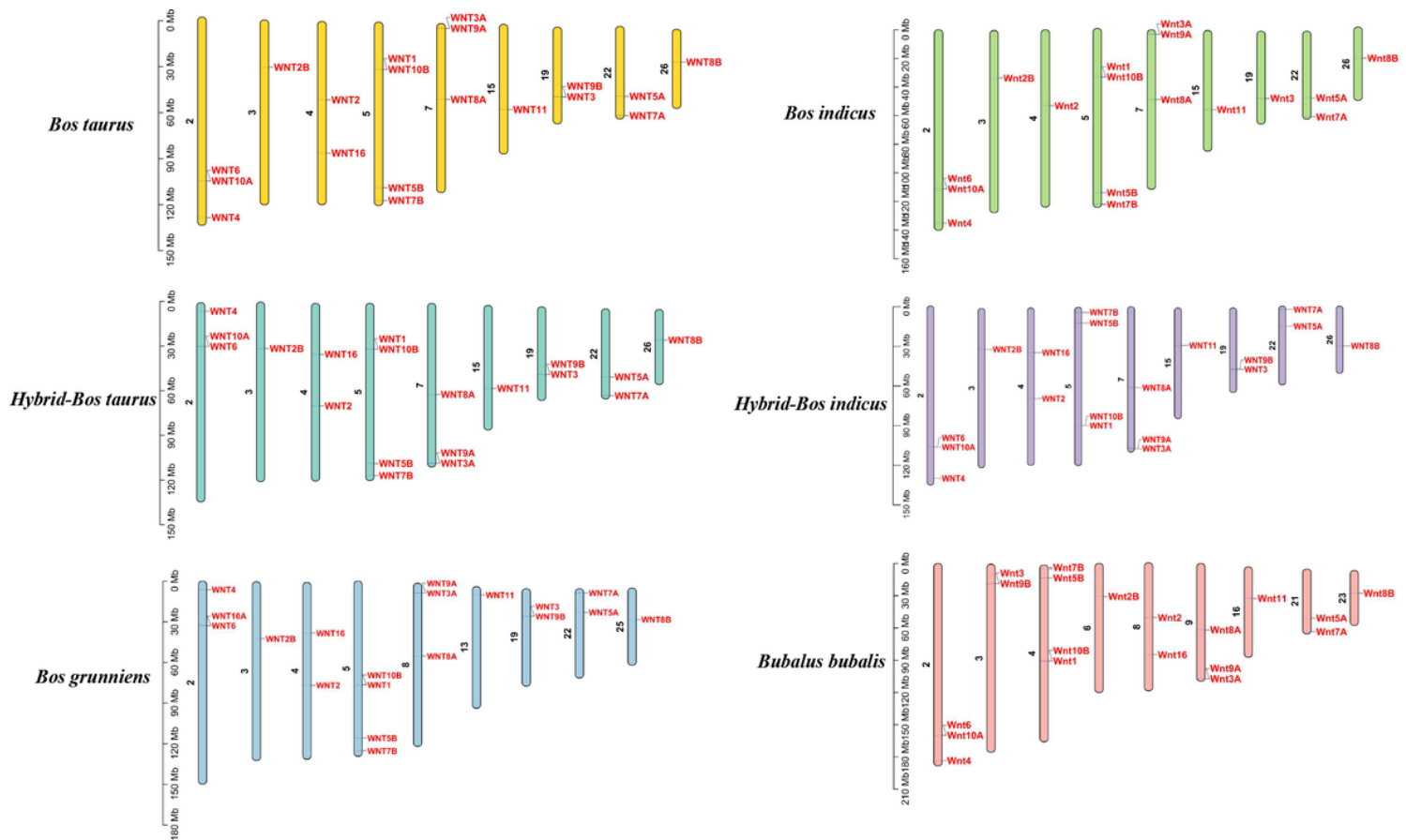
**Figure 1**

Characterization of the identified Wnt proteins and genes in *Bos taurus*. The phylogenetic tree (left) was constructed by the Neighbor Joining method. Motifs of amino acid sequences (middle), where conserved motifs are indicated as rectangles with different colors. Gene structure map (right), where yellow rectangle, black line and green rectangle represent CDS, intron and UTR, respectively.



**Figure 2**

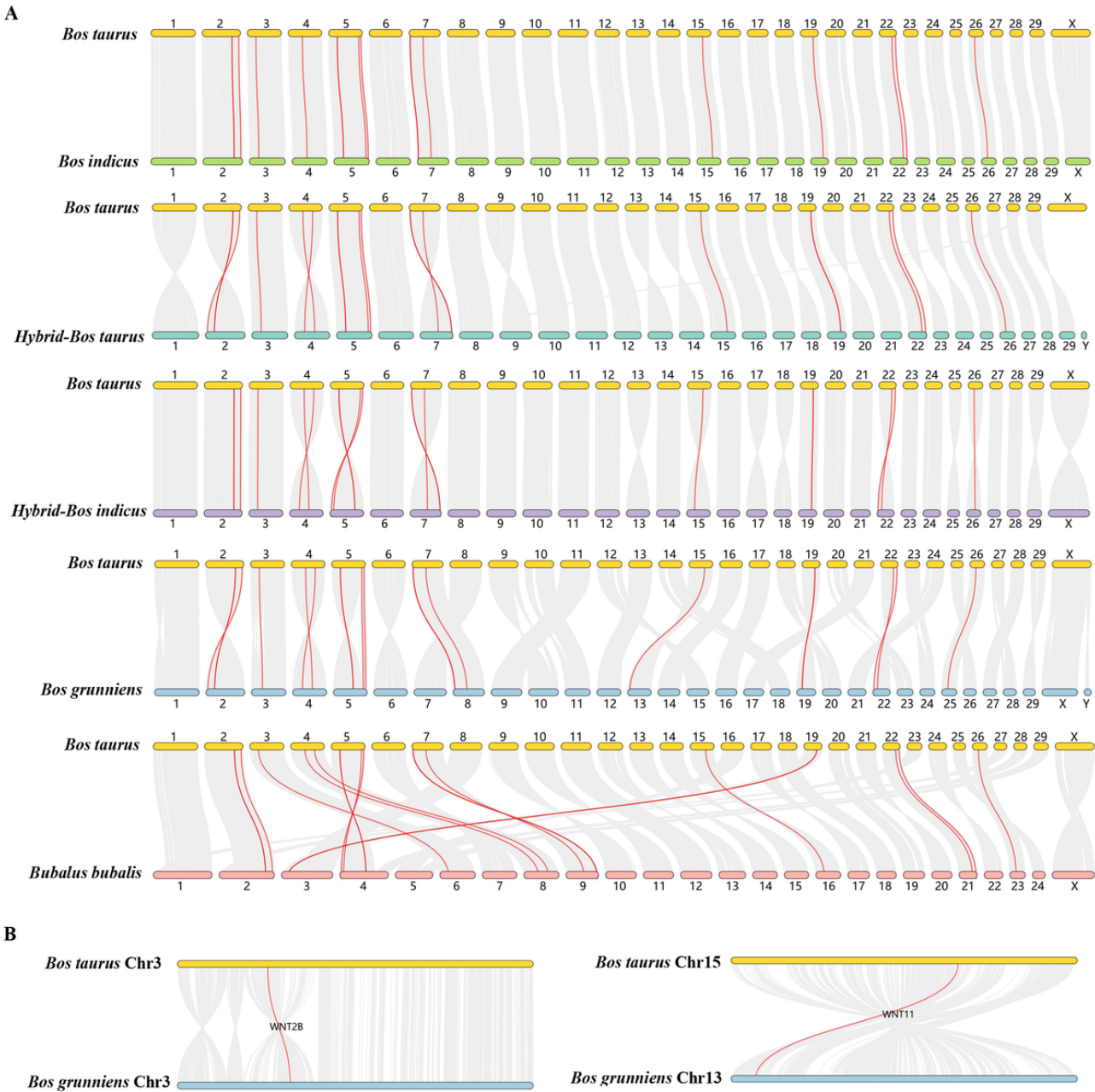
Phylogenetic Neighbor-Joining (NJ) tree of Wnt proteins from ten organisms. Identified Wnts in *Bos taurus* (BOSTA), *Bos grunniens* (BOSGR), Hybrid-Bos Indicus (BOSIH), Hybrid-Bos taurus (BOSTH), *Bos mutus* (BOSMU), *Bison bison bison* (BISOM), *Bos indicus* (BOSIN) and *Bubalus bubalis* (BUBBU) together with verified Wnts from *Homo sapiens* (HUMAN) and *Mus musculus* (MOUSE). Wnt proteins are grouped into 12 clusters (I–XII) shown as different colors.



**Figure 3**

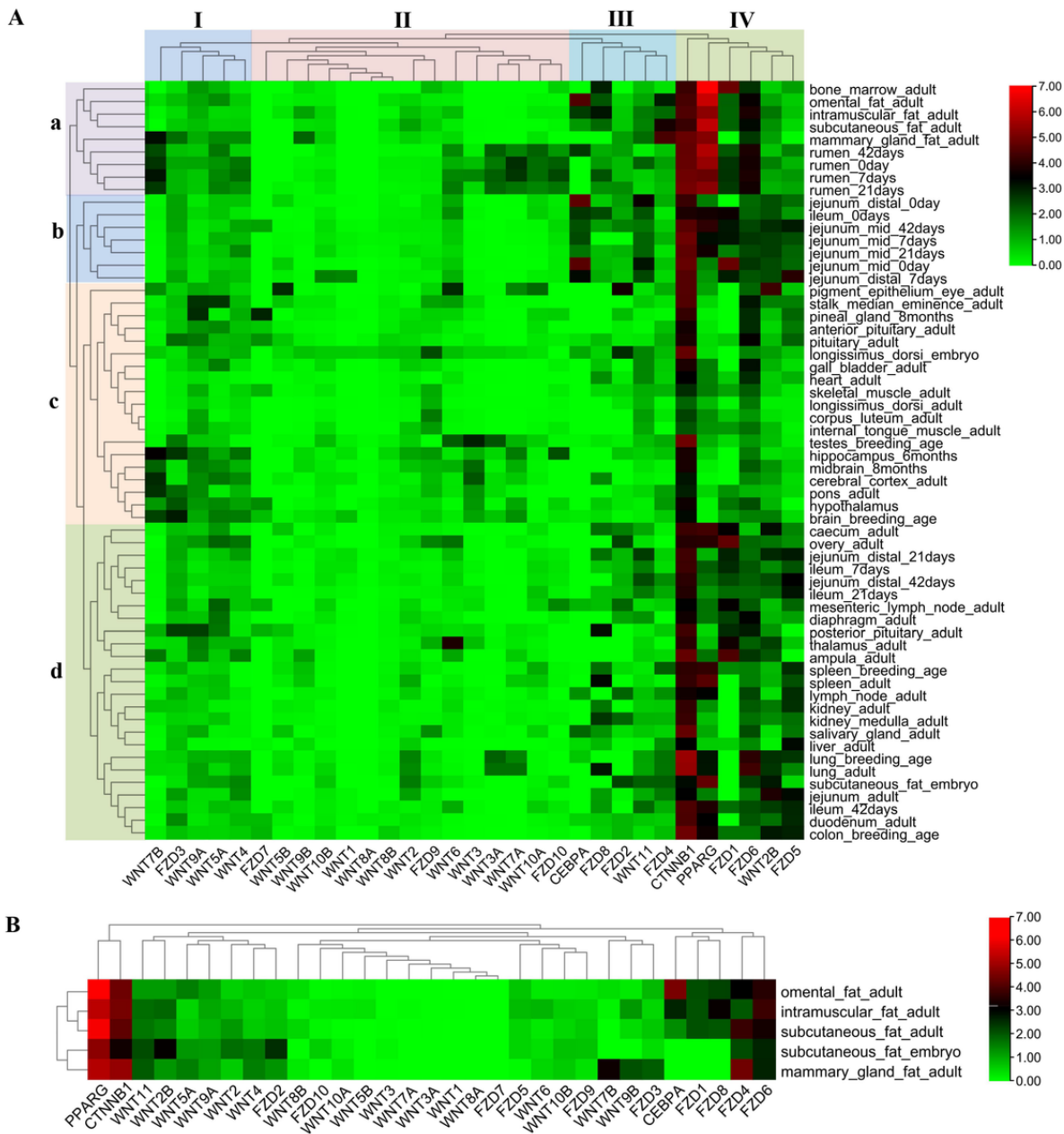
Chromosomal distribution of Wnt genes. The black font on the left represents chromosome numbers and the red font on the right represents Wnt genes.





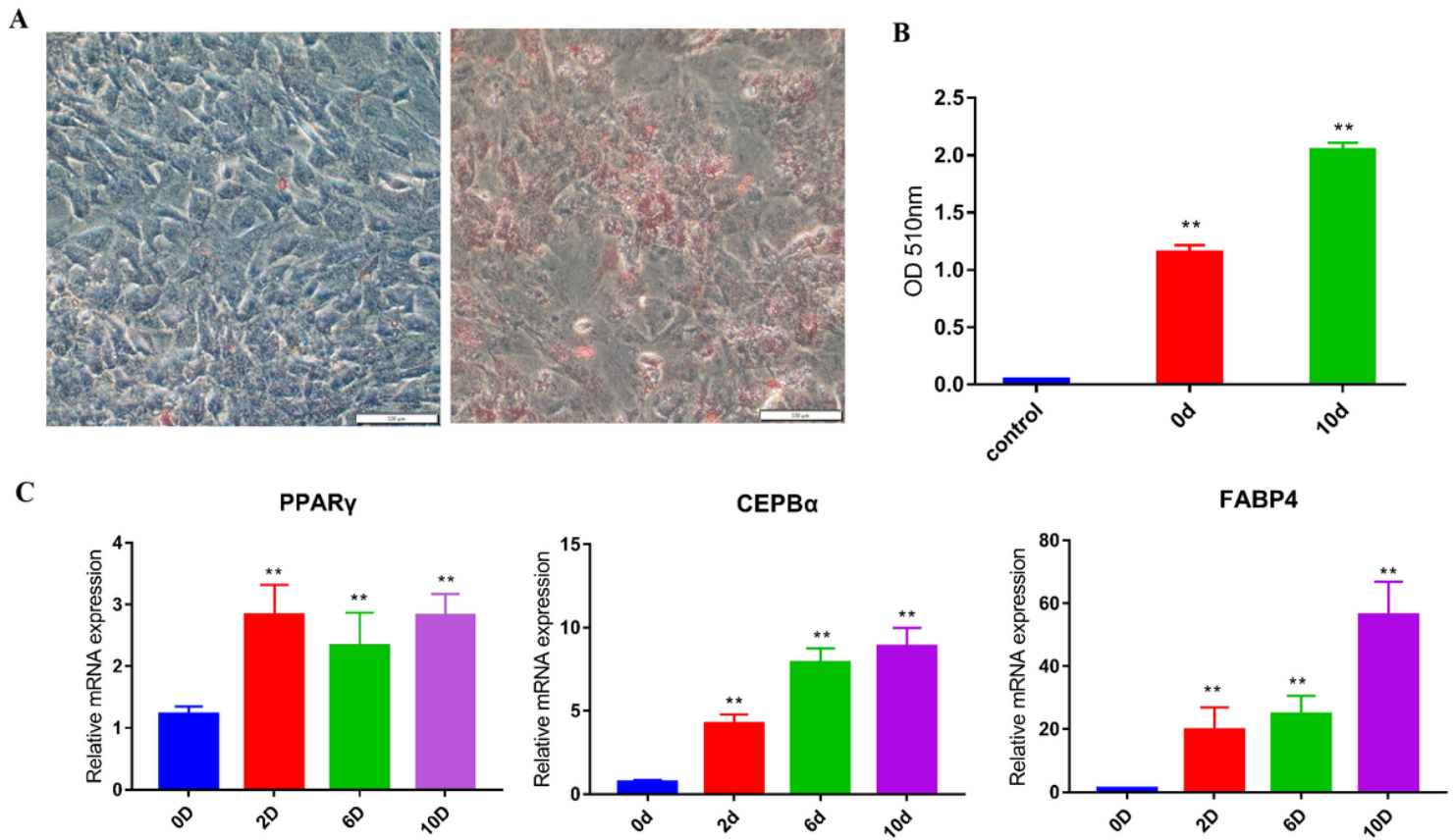
**Figure 4**

Collinearity analysis of Wnt genes between cattle and other organisms. Syntenic genes pairs are linked by grey lines whereas syntenic Wnt genes are shown as red lines.



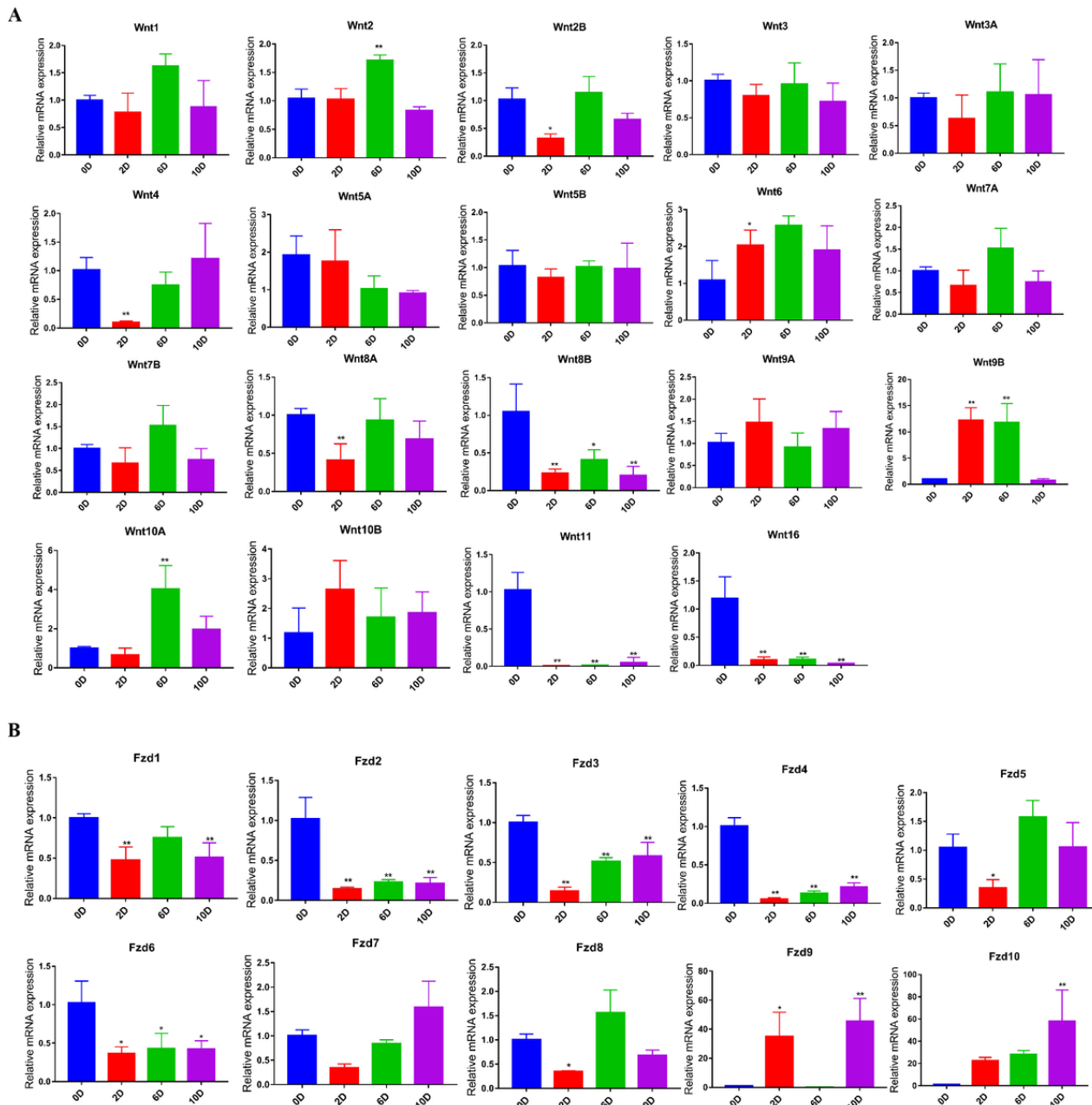
**Figure 5**

Expression analysis of the Wnt gene family in different bovine tissue types. A. Expression analysis of the Wnt gene family in 60 bovine tissues. The tissues were classified into 4 groups (a to d) and the 31 genes were also classified into 4 groups (I-IV) according to their expression pattern. B. Expression analysis of Wnt gene family in 5 bovine fat tissues. The horizontal axis represents genes and the vertical axis represents different bovine tissues.



**Figure 6**

Induced differentiation of primary adipocytes. A. Oil Red O staining of bovine adipocytes induced at day 0 (left) and day 10 (right) of adipogenic differentiation. B. The measurements of absorbance at 510nm. Control: isopropanol, 0d: substance extracted from adipocytes induced at 0 day, 10d: substance extracted from adipocytes induced at 10 day. C. The expression of adipogenic marker genes during adipogenic differentiation.



**Figure 7**

qPCR expression analysis of Wnt and its receptors Fzd family genes during adipocyte differentiation. A. The expression of 19 Wnt genes. B. The expression of 10 Fzd genes. Error bars were gained from three measurements. Symbols \* and \*\* above the bars indicate significant differences at a p value of 0.05 and 0.01 respectively.

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