Discussion
4. Discussion

Identification of WDR13 gene in \textit{H. fossilis}

A contig of zebrafish genomic sequence was identified showing 73\% and 83\% similarity at cDNA and protein level respectively when homology searched with human cDNA of WDR13 gene. Primers designed from conserved regions identified through the Clustal analysis, when used to amplify \textit{H. fossilis} cDNA from brain tissue resulted an amplicon of 800bp. RACE analysis when carried out with primers designed from the amplified region, identified a transcript of 2070bp long which including ORF, 5' and 3' UTRs. This cDNA sequence when compared with other species including human showed 77\% similarity indicating the presence of WDR13 homologue in \textit{H. fossilis}.

Structure of WDR13 gene in \textit{H. fossilis}

In \textit{H. fossilis} the CDS of WDR13 is 2070bp in length and the transcript starts with ‘G’ which is 124bp upstream of the first ATG codon. Presence of another ATG is seen 45bp downstream of the first ATG. Though both ATG have Kozaks sequence (AGCATGT and GGTATGG), the consensus (A/GNNATGG) sequence associated with the second ATG is strong. Therefore, it seems that the second ATG is functional. Sequence comparison in several mammals including human and other fishes with \textit{H. fossilis} indicates the initiation codon is the second ATG of \textit{H. fossilis} of WDR13. The polyadenylation signal with a sequence of ‘AGTAAA’ is present 468bp downstream of the stop codon TAA. The polyadenylation signal detected in \textit{H. fossilis} is different from the canonical sequence of ‘AATAAA’ (Beaudoing \textit{et al.}, 2000; Nag \textit{et al.}, 2006) found in the case of human, mouse or in zebrafish. The 3'UTR has several stretches of di-nucleotide repeats (GT and GA) called microsatellites. Microsatellites or simple sequence repeats (SSR) are important molecular markers for development of genetic linkage maps (Li \textit{et al.}, 2004). They can be present in exons, introns and UTRs. Among these the UTRs harbors more microsatellites than the coding regions (Li \textit{et al.}, 2004). The UTRs have more of di-nucleotide repeats as compared to coding regions which have tri-nucleotide repeats (Li \textit{et al.}, 2004). Data presented of WDR13 in \textit{H. fossilis} is also consistent with the report that the 3'UTR in the channel catfish has biasness towards AC/GT repeats (Liu \textit{et al.}, 2001) as in WDR13 UTR. Studies in channel cat fish have shown that the cDNA’s which are expressed in brain have more of di-nucleotide repeats.
in their 3′UTRs. This also is in agreement with the present study since WDR13 is highly expressed in brain. The 3′UTRs of zebrafish, tetradon, medaka, fugu and Gasteracetalus are also analyzed for the presence of such di-nucleotide repeats. All these fishes have “GT” repeats but the lengths are small with 3 repeats spread in stretches. The 3′UTR of zebrafish, tetradon and medaka in addition to the GT repeats have ‘GA’ and ‘CA’ di-nucleotide repeats. The 3′UTR of mouse and human when analyzed for such repeats, showed the presence of “AT” repeats but not the “GT” repeats which are present in the fish species. Genes having similar function can have different di-nucleotide repeats which essentially serve the same function because these di-nucleotide repeats might replace each other during evolution of UTRs of orthologous genes (Riley and Krieger, 2004; Riley et al., 2007; Riley and Krieger, 2009). Di-nucleotide repeats regulate genes by enhancing the transcription of the genes when present in their UTR (Hamada et al., 1984). Tri-nucleotide repeats and their expansion are mostly associated with neurological disorders in humans (Ashley and Warren, 1995). No such function is attributed to di-nucleotide repeats but these repeats are mostly present in genes present in developmentally important genes, particularly genes involved in brain development and modulation (Nithianantharajah and Hannan, 2007; Fondon et al., 2008). Thus the presence of the di-nucleotide repeats in the 3′UTR of WDR13 gene and its expression predominantly in brain suggests that this gene may have function in brain development and modulation.

**In silico analysis of WDR13 protein**

The WDR13 of *H. fossilis* has a CDS of 1446bp with ATG at 169bp with the Kozak’s consensus sequence “GGTATGG” (Kozak, 1986a, b). When the CDS is translated *in silico* forms a protein of 482amino acids with a molecular mass of 53kDa and pI of 9.01. Compared to human and mouse protein (which have 6 WD repeats), zebrafish and *H. fossilis* protein have only 4 WD repeats. WDR proteins posses a common structure (β propeller) but perform diverse functions and can be grouped into at least thirty different functional families (Yu et al., 2000) which include signal transduction, RNA synthesis and processing, chromatin assembly, regulation of vesicular trafficking, cytoskeletal assembly, cell cycle regulation, programmed cell death etc (Li and Roberts, 2001).
The WDR13 protein of mouse is localized in the nucleus (Suresh et al., 2005). Analysis of the *H. fossilis* WDR13 protein *in silico* for the presence of NLS using the program pSORT II did not detect any such signal but it showed the protein to be nuclear in localization using Reinhardt's method for nuclear and cytoplasmic discrimination (Reinhardt and Hubbard, 1998). SMART program has identified three regions of low compositional complexity in this protein. The low complexity regions are abundant in multi-domain polypeptides which are important in morphogenesis, embryonic development, RNA processing, signal transduction, intracellular and extra cellular structure and integrity (Wootton and Federhen, 1993; Wootton, 1994). These sequences are also important in molecular interaction and biological roles of the protein (Brocchieri, 2001; Xue and Forsdyke, 2003). The presence of low complexity regions in the WDR13 protein indicates that it may be involved in any of the functions mentioned above. PROSCAN program has identified several potential sites for post-translational modifications (PTM) which included glycosylation, phosphorylation, and myristoylation in this protein. WDR13 protein has three N-glycosylation sites. This type of PTM plays a role in protein folding and thus confers stability. It also targets the proteins to subcellular and extra-cellular sites as well as cell matrix. Besides it is of high importance because of its role in development and homeostasis and is needed for viability in eukaryotes. Glycosylation also plays an important role in cell-cell adhesion and cell cycle progression (Kukuruzinska and Lennon, 1998; Roth, 2002). The WDR13 protein has N-myristoylation sites which could play a role in stabilizing the 3D conformation. It also has a role in membrane binding and membrane targeting of the protein (Resh, 1999). The protein also has various phosphorylation sites; one site of cAMP-and cGMP-dependant protein kinase, twelve sites for protein kinase C and five sites for casein kinase II enzymes. All three kinases are Ser/Thr phosphotransferase. Protein kinase C (PKC) is involved in receptor desensitization, modulating membrane structure, transcription regulation, mediating immune response, regulating cell growth, proliferation and differentiation, learning and memory among many other functions. PKCs are activated by pathways which involve the production of diacylglycerol. It is mostly involved in signal transduction involved in cell growth, proliferation and differentiation (Allende and Allende, 1995; Newton, 1995, 2001). Similarly casein kinase II (CK II) phosphorylation is also involved in cell growth, proliferation and differentiation (Krek et al., 1992; Bossemeyer, 1995). Presence of these phosphorylation sites in WDR13 protein show that the function of WDR13 proteins can be modulated by...
the action of these protein kinases. The presence of PKC and CK II shows that these proteins might play a role in cell growth, proliferation and differentiation.

**Genomic organization of WDR13 gene in *H. fossilis***

To know the structure of the WDR13 gene in *H. fossilis*, instead of screening a genomic library, PCR method was adopted. Clustal analysis with human, mouse and zebrafish sequences with *H. fossilis* cDNA allowed marking of the exon and intron boundaries which are assumed to be conserved in all these species. Primers were then designed to amplify the entire gene. The total gene is amplified in 5 pieces and the sequence assembled. The gene is 11023bp in size and is larger in size than human gene (7.3Kb) (Singh *et al.*, 2003) and mouse gene (8Kb) (Suresh *et al.*, 2005). The WDR13 of *H. fossilis* has 10 exons and 9 introns. The WDR13 gene of human, mouse and chimpanzee have 9 exons and 8 introns where even the position of the introns is conserved. Though *H. fossilis* and zebrafish have 10 exons and 9 introns but the length of exons from exon 3 to exon 10 are very similar to human indicating their conserved nature. Since position of the introns in orthologous genes are conserved (Moore, 1983), we have analyzed the number of introns and exons of WDR13 gene in other fish species. Fugu and tetradon have 9 exons and 8 introns similar to human and mouse gene whereas the *Gasterosteus* and *Oryzias* have 10 exons and 9 introns similar to *H. fossilis* and zebrafish. The difference in number of introns present in a gene between the species is thought to be due to differential selection pressure causing intron loss or intron gain (Stoltzfus *et al.*, 1997; Patthy, 1999; Jeffares *et al.*, 2006). Evolutilional studies show that multicellular organisms have evolved from intron rich ancestors (Roy, 2006; Roy and Gilbert, 2006) which eventually lost introns over a period of time (Roy and Gilbert, 2005). On the other hand it is also shown that gain of introns in some genes during evolution is not uncommon (Roy, 2004; Jeffares *et al.*, 2006). The presence of intron one in *H. fossilis* can be explained as an ancestral intron which has been lost in humans during evolution or a new intron which is formed in *H. fossilis* due to transposition. The rate of intron loss is 20 fold more than the intron gain (Jeffares *et al.*, 2006). Though intron loss prevails, it is not possible to believe that intron one is an ancestral intron and it is lost in humans during evolution because the intron loss is more towards the 3’ end of the gene (Roy and Gilbert, 2005; Jeffares *et al.*, 2006) and the intron one is in the 5’ region of the gene which have greater selective importance due to greater concentration
of regulatory elements in that region (Fedorova and Fedorov, 2003). Thus the intron one in fishes may be a new intron which is formed due to the activity of transposons (Roy, 2004) and also intron gain also favors 5' end of the gene (Roy and Gilbert, 2005). The WDR13 gene varies in length from species to species even among fishes. It is 11kb in *H. fossilis*, 17Kb in zebrafish, 5.2Kb in fugu, 6.97Kb in Gasterosteus, 4.6Kb in tetradox and 6.3Kb in medaka. The difference in the length of the gene is due to variation in the lengths of the introns (Stoltzfus et al., 1997; Yandell et al., 2006). It is shown that the length of introns of fugu is eight times small as compared to that of humans, consistent with the sizes of their genomes (Vinogradov, 1999; McLysaght et al., 2000). This shows that the size of the gene is dependent on the length of the introns which is in proportionate with the size of the genome. Thus *H. fossilis* and zebrafish the gene is big owing to the presence of long introns and the other species have small introns owing to the genome size of the species (Ogata et al., 1996; Petrov and Hartl, 2000). Introns act as a good tool for studying the evolutionary history of a gene (Fedorova and Fedorov, 2003) because the other reason for difference in size of the introns of the same gene among species is due to accumulation of small deletions during course of evolution in certain species (Ogata et al., 1996). Genes which are expressed in fewer tissues and which have low expression levels have longer introns compared to genes with higher expression and those expressed in all the tissues (Jeffares et al., 2008). Highly expressed genes have stronger selection pressure to shorten their introns to maintain economy, because transcribing long stretches of DNA continuously is a time and energy consuming process (Pozzoli et al., 2007). In human and mouse WDR13 gene is expressed in all the tissues (Singh et al., 2003; Suresh et al., 2005) which is similar to *H. fossilis*, the gene is also expressed in tissue specific manner which explains the small size of the gene. The CDS of the WDR13 gene of *H. fossilis* has 60% G+C whereas the entire transcript has G+C of 52%. This shows that this gene can be present in a GC rich isochore. Isochores are the regions in the genome where there is difference in gene concentration and relatively higher GC content (Bernardi, 1993; Eyre-Walker and Hurst, 2001). One more point to support its position in the GC isochores is that the genes present in these regions are small (Duret et al., 1995). Housekeeping genes or the widely expressed genes are present in the regions of GC isochores (Lercher et al., 2003; Versteeg et al., 2003) which have open chromatin which are easily accessible for transcription factors (Cremer and Cremer, 2001; Vinogradov, 2003a). Tissue specific genes are also present in GC rich regions but the house keeping genes are slightly richer as compared to tissue specific genes.
The GC content of WDR13 gene supports the ubiquitous nature of the gene which needs continuous transcription, and also the tissue specificity where the alternate splice forms of the gene are tissue specific which will be discussed later.

The SSRs and satellite repeats in the non-coding regions play an important role in regulating the transcription of the gene, proper splicing of the mRNA or its export to cytoplasm (Li et al., 2004). SSRs also play a major role in genome packing by acting as punctuation marks. They often have roles in long range interactions mediated by proteins which bind to these repeats (Kumar et al., 2010). Satellite repeats can influence chromosome organization, transcription as well as post transcriptional aspects of gene regulation. The SSRs and the satellites present in the introns of WDR13 gene might be playing a role in transcriptional control of either of the splice forms or all the splice forms.

Alternative splicing of WDR13 gene in *H. fossilis*

Alternative splicing is predominant among vertebrates and 40%-60% of human genes are alternatively spliced (Modrek and Lee, 2002; Xu et al., 2002). Human and mouse WDR13 is alternatively spliced (Singh et al., 2003; Suresh et al., 2005). Human have two alternate transcripts; one expressing in all the tissues and the other expressing in testis and ovary. In addition splice forms having different length of 3'UTRs and retention of intron 1, intron 1 and 2 and a portion of Intron 5 have also been reported. In *H. fossilis* variation in the UTR size in the transcripts of WDR13 is not detected in any tissues. While amplifying the CDS of WDR13 gene by RT-PCR, three amplicons were detected. To eliminate non-specific amplifications, high stringent conditions of PCR were used. These amplicons were cloned and sequenced. The 1.5Kb transcript named WDR13a has all the ten exons and represents the constitutively spliced form of the gene. The other transcript is 1.3Kb in size (WDR13b) has an in-frame deletion of exon 3. This type of alternative splicing is known as a cassette exon type of alternative splicing (Smith et al., 1989; Black, 2003). In the third amplicon in addition to having a cassette exon 3, it also has retention of 45bp of intron 5 (WDR13c). This transcript is having both cassette exon as well intron retention type of alternative splicing (Smith et al., 1989; Black, 2003). All these alternatively spliced WDR13 mRNAs have open reading frame without any premature stop codons. WDR13a which is the constitutively spliced form codes for a protein of 53kDa with a pI value of 9.01.
WDR13b and WDR13c code proteins of 43kDa and 44kDa respectively with pi of 6.83 and 8.46. This shows that the exon 3 which is spliced out is basic in nature which has brought down the pi of the protein to 6.83 in the case of WDR13b. Neither the splicing out of the cassette exon nor the retention of intron 5 in the alternate splice forms change the number of WD repeats in the proteins when translated in silico.

**Implications of Alternative splice forms**

The alternative splice forms of WDR13 with the retention of intron in human and mouse are expressed in a tissue specific manner (Singh et al., 2003; Suresh et al., 2005). The retained introns might introduce stop codons resulting in the premature stopping of the translation producing truncated or inactive proteins in the tissues where this form of variant is present (Smith et al., 1989). This is one type of control where protein is not required. The best studied case is *Drosophila P* element, a transposon expressed only in the germ line where all the introns are spliced out forming an active protein. In somatic cells the intron 3 is retained thus altering the reading frame and making the protein inactive (Kaufman and Rio, 1991; Rio, 1991). In other cases the intron when retained in frame would code for an extra peptide which might change the property of the protein, including localization or binding properties (Rogers et al., 1987). Intron retention may result in the introduction of a premature stop codon, thereby resulting in translation of a truncated protein downstream of the retained introns as shown in human WDR13 (Singh et al., 2003; Suresh et al., 2005). In comparison with human and mouse the retained stretch of intron in WDR13 of *H. fossilis* does not insert any stop codons and the reading frame is maintained. This points out that the retained intron may confer an additional function as shown in the case of fibronectin gene (Rogers et al., 1987; Schwarzbaucer et al., 1987). In human and mouse other alternative splice forms of WDR13 include variation in the length of the 3'UTR present in different tissues. The UTR harbours certain AU rich elements (Zubiaga et al., 1995) which play a role in degrading the RNA as in the case of colony stimulating factor-1 gene which has the same coding region but different length in their 3'UTRs. One of the UTRs has an AU rich regions which turns out its RNA rapidly (Ladner et al., 1987). Thus the gene is regulated at the mRNA level. The same would be the case in the human and the mouse WDR13 gene which has different length of UTRs in different tissues. As the WDR13 gene of *H. fossilis* does not have variation in length of UTRs the regulation of this gene...
at this level may be ruled out. This shows that the alternative splice forms of *H. fossilis* is different from human and mouse and therefore may have an entirely different function.

Cassette type of alternative splicing seen in the WDR13 of *H. fossilis* can change the function of the protein. Due to the loss of a stretch of amino acids of the protein the localization signal may change as in the case of NCAM's which are membrane bound or cytoplasmic depending upon the use of cassette exons (Cunningham *et al.*, 1987). Glial cell line-derived neurotrophic factor is a potent therapeutic target for neurodegenerative disorders. It has a cassette exon 2. When this exon is included in the mRNA it forms a secreted protein and exclusion of this exon results in the formation of a protein which is retained within the cell (Grimm *et al.*, 1998). Fibronectin is a cell adhesive molecule and can exist in both soluble and insoluble forms which are present in the plasma membrane or the extra cellular matrix. The property of the protein depends upon the use of cassette exons and retention of introns (Schwarzbauer *et al.*, 1983; Rogers *et al.*, 1987; Schwarzbauer *et al.*, 1987). The transcripts may also be cell specific as in the case of N-type calcium channel α1B which is localized to synapses throughout the nervous system and couples excitation to release of neurotransmitters. This gene has two splice forms mα1B-b and mα1B-d which are reciprocally expressed in brain and sympathetic ganglion. These splice forms differ at two regions where one has four amino acids and the other has two amino acid stretch. The difference in function of the splice forms is the insertion of the two amino acids in the isoform (Lin *et al.*, 1999), altering the property of the protein. Similar type of splicing is seen in the WDR13 gene of *H. fossilis*, where splicing out of exon 3 might result in loss of a peptide in the protein formed, or the retention of the 45bp of intron 5 would result in the addition of an extra peptide which may cause differential localization of the protein.

Modulation of the protein function may occur in the case of WDR13 in *H. fossilis* as the splicing out of exon 3 results in the loss of a peptide which may have some functional sites or binding sites required for interaction with other proteins. The retention of the intron 5 would add an extra peptide which may impart an additional function or a binding site as seen in the case of calcitonin gene. The calcitonin gene produces both calcitonin (CT) and calcitonin gene-related peptide (CGRP) by alternative splicing and poly (A) addition site usage. Splicing out of exon 3 forms CGRP and splicing out of exon 4 forms CT (Rosenfeld *et al.*, 1984).

Sub cellular localization studies of WDR13a of *H. fossilis* in COS and HeLa cell lines showed the protein is localized in the nucleus which is in consistent with the
mouse data (Suresh et al., 2005). The alternate splice forms WDR13b and WDR13c are localized in the cytoplasm. Taking cellular localization data it can be speculated that the exon 3 may have nuclear localization signal or posses binding sites which form a complex with other proteins which can localize the proteins to the nucleus. The loss of the exon 3 in the alternate splice form may lose such signal or the binding site, due to which the proteins are unable to enter the nucleus. The other splice form, WDR13c has retained intron 5, may have an entirely different function.

The three splice forms of the WDR13 gene are expressed in brain and muscle of *H. fossilis*. Immuno staining on *H. fossilis* brain sections were carried out using WDR13 antibody raised in our laboratory (will be discussed in the next section). In consistent with cellular localization data (COS and HeLa cells), localization is seen in the nucleus and cytoplasmic compartments. The protein formed by the constitutively spliced form is localized predominantly in the nucleus of cerebellum and hypothalamus and certain other cells which are spread sparsely in the brain. The proteins from the alternatively spliced forms are localized in the cytoplasm in groups of cells distributed throughout the brain. Cell having the WDR13 protein in both the nucleus and cytoplasmic is not seen showing that the splice forms are not expressed in the same cell. From this study it can be concluded that the nuclear form is the WDR13a and cytoplasmic form is either WDR13b or WDR13c *in vivo*. Distinguishing the later two forms was not possible as both are in cytoplasm and the antibody detects both the forms. This indicates that WDR13 is not only present in different compartments of the cell, but also present in different cell types, and may function differently in their respective compartments and cells.

### Identification of putative nuclear localization signal in WDR13 protein

The localization of the WDR13a (constitutively spliced) in the nucleus and the other splice forms WDR13b and c in the cytoplasm, points that exon 3 might have a role in localizing the protein to the nucleus. The exon 3 may possess a novel NLS which is not detected by pSORT II program. Manual analysis of this region detected a stretch of amino acids which has high content of basic amino acids. Clustal analysis of this region with the WDR13 protein available in the data base indicated that this region is conserved from *Nematostella vectensis* (cnidaria) to human. NLS are of two types: mono partite where four amino acids in a stretch of six amino acids can act as an NLS (Boulikas,
1994); the bipartite NLS in which two clusters of basic amino acids are separated by a stretch of 10 amino acids (Dingwall and Laskey, 1991; Boulikas, 1993). Analysis of the amino acid stretch obtained in the manual search of exon 3 showed the features of a bipartite NLS. It has 3 basic amino acid residues, and a spacer of 16 amino acids followed by 2 more basic amino acids. Though it is defined that the spacer length in the bipartite NLS should be 10 amino acids, later NLSs were identified which have spacer length of more than 10 amino acids. In the case of adenovirus DNA binding protein the spacer length is 37 amino acids (Morin et al., 1989). In the influenza virus polymerase basic protein has an NLS of 16 amino acids similar to the WDR13 proteins and in most of these NLS, reduction in length of the spacer does not affect the localization of the protein (Nath and Nayak, 1990). In the bipartite NLS the first stretch has two basic amino acids and following the spacer has another three basic amino acids. In the case of WDR13 protein this orientation is in reverse, where it has three basic amino acids followed by a spacer and than two basic amino acids. Because of the presence of basic amino acids in reverse order, it might represent a novel type of NLS in WDR13. Since some NLS are formed during protein folding where the spacer is looped out and all the basic amino acids come together and form a continuous stretch of amino acid like the mono partite NLS which can bind to the importin. Some proteins have atypical NLS where it has three or two amino acids within a hexa-peptide can act as a NLS. In WDR13 the last three basic amino acids can act as atypical NLS. Phosphorylation of the flanking sequences of NLS also enhance nuclear uptake of a protein as seen in the case of SV40 T protein, where the protein has casein kinase II phosphorylation site which enhance the nuclear uptake and mutation in the same region affect nuclear uptake (McVey et al., 1989). WDR13 also has casein kinase II phosphorylation sites in this basic amino acid stretch which may play a role in its nuclear transport.

**WDR13 gene expression is tissue specific in *H. fossilis***

Alternative splice forms of mRNA may be tissue specific. The constitutive splice form may express in one tissue and alternatively spliced forms may be present in another tissue (Smith et al., 1989). Besides some splice forms may be present in different cell types (Lopez, 1998) in the same tissue or may be developmentally regulated. The alternate splice forms of WDR13 are differentially expressed. The constitutively spliced form is expressed in all the tissues studied (brain, fat, heart, intestine, kidney, liver,
muscle, ovary, pituitary, testis). The alternate splice forms though present in small amount in all tissues but predominantly expressed in brain, pituitary and skeletal muscle. This observation shows that the constitutively spliced form of the gene may have a similar function in all the tissues and the alternatively spliced forms may have an additional function in brain, pituitary and skeletal muscle.

**WDR13 gene is maternally deposited in *H. fossilis***

WDR13 gene in *H. fossilis* is maternally deposited as seen by RT-PCR. Early development of animal is controlled/supported by the information stored in the egg as maternal RNA (Crippa and Gross, 1969). These maternal transcripts are expressed spatially and temporally and are deposited in the fertilized egg in an inactive state complexed with the proteins mRNP3, mRMP4, FRHY2 or other unidentified proteins (Ranjan *et al.*, 1993; Hake and Richter, 1997). Maternal mRNA carries various functions like the anterior-posterior axis formation and dorso-ventral patterning of the organism. WDR13a mRNA is present in the unfertilized eggs, single cell stage and throughout the life of the individual indicating it is both maternally and zygotically important gene. The alternative splice forms of the gene, WDR13b and WDR13c are present in small amount in the unfertilized and single cell embryos but are more pronounced in embryos at 24hpf onward. During this time brain and muscle development starts in the embryo. Previously it is shown that WDR13 splice forms are present significantly in the brain and muscle. Such a correlation indicates the role of WDR13 splice forms in developmental regulation and maintenance of brain and muscle.

**Splicing regulatory elements present in the introns around the regulated exon**

Constitutive splicing requires 5’GT, 3’AG, branch point, and poly pyrimidine tract as cis factors whereas snRNPs, U2AF and other proteins as trans factors. In addition to these, alternative splicing requires additional cis and trans factors. The cis factors include ESEs (exonic splicing enhancers), ESSs (exonic splicing suppressors), ISEs (intronic splicing enhancers) and ISSs (intronic splicing suppressors). These cis factors act in cohort with the trans factors in bringing alternate splicing. WDR13 gene in *H. fossilis* has cassette exon 3 and 45bp retention of intron 5. There are many ESEs present all through the WDR13 cDNA which mark both constitutively and alternatively spliced
exons (Blencowe, 2000; Fairbrother et al., 2002). SR proteins bind to ESEs and mark them and bring about the inclusion of the exons (Hertel et al., 1997; Blencowe, 2000). Exon 3 has one ESS, these are bound by hnRNP proteins (Krecic and Swanson, 1999). The best example of the role of ESE and ESS in bringing about alternative splicing is seen in tat 3 exon in HIV. WDR13 exon 3 in *H. fossilis* also has both ESEs and ESSs. The ESEs might play a role in the inclusion of the exon 3 in constitutively spliced from which is expressed ubiquitously. The alternative spliced form which is predominantly expressed in brain and skeletal muscle might require the action of the ESS present in the exon 3 along with other protein factors which might counteract the SR proteins to exclude the exon from the transcript.

Introns harbour ISEs and ISSs having defined functions in alternate splicing (Black, 2003). Many diseases are associated with mutations that affect these signals in the introns (Pozzoli and Sironi, 2005). In most of the cases the introns surrounding the tissue regulated exons are conserved since they harbor regulatory elements for alternate splicing (Sorek and Ast, 2003; Sugnet et al., 2006). Analysis of the upstream and the downstream intron of the WDR13 exon 3 have identified ISEs and ISS. The upstream intron has the sequence “UGCAUG” which can act as splicing enhancer, but this ISE may not be that significant because it can acts an ISE when present in the downstream of the regulated exons. The downstream intron also has the similar sequence of ISE. This sequence is known to play a role in tissue specific splicing of exons in many neuronal genes like c-src, exon EIIIb of the rat fibronectin gene and non muscle myosin heavy chains. This is a candidate sequence for most of the exons which are regulated in brain and to a some extent in muscle (Brudno et al., 2001). This sequence in most cases is duplicated which increases the inclusion of N1 exon in the neuronal cells (Modafferi and Black, 1997; Brudno et al., 2001). This sequence alone could not bring about the tissue specific splicing in WDR13 gene in *H. fossilis* as it is not duplicated.

The intron downstream of exon 3 in WDR13 has enhancer elements “GGGGG” “UGCAUG” and several repeats of “CUCUCU”, a silencer element. The intronic enhancers often have both positive and negative elements assembling into large RNP complexes which is seen in splicing of N1 exon in c-src and is very similar to WDR13 ISEs and ISS (Black, 1992; Chan and Black, 1997; Modafferi and Black, 1997). The introns have binding sites for hnRNP B, hnRNP C, hnRNP F and hnRNP H. hnRNP B and C in the upstream and downstream of the introns are negative regulators of splicing which play a role in modulation of splice site selection. *In vitro* studies carried
out between competing 5′ splice sites have shown that the presence of this group of proteins results in use of the distal 5′ splice site (Mayeda et al., 1994). In K-SAM exon of rat fibronectin gene this protein factor in association with ESS brings about shift in 3′ splice site choice there by resulting exon skipping (Del Gatto et al., 1996). Same kind of regulation can be seen in the partial retention of intron 5 in WDR13 where an hnRNP binding site may shift the distal 3′ splice site. hnRNP F and H proteins (belong to the “H” group) can act as both splicing activators or repressors. In c-src gene this protein binds to intronic sequence and brings about the inclusion of the N1 exon in the neuronal cells (Chou et al., 1999). The same protein in the case of β-tropomyosin binds to exonic sequence and causes the exclusion of exon 7 in non muscle cells (Chen and Helfman, 1999; Chen et al., 1999). Thus the function of this protein depends on the place where it binds; activating when bound in intron region and suppressor when bound in exon region. The introns present in the upstream and downstream of WDR13 gene also have binding sites for SR group of proteins SRp40 and SRp55. SR proteins act as silencer when bound in the intronic regions unlike when bound in exons. The introns on either side of the exon 3 have various binding sites for poly pyrimidine tract binding protein (PTB). This protein has an affinity to bind to poly pyrimidine tract of the intron and makes that site inaccessible for U2AF and other splicing factors, which ultimately leads to repression of splicing (Chou et al., 2000; Wagner and Garcia-Blanco, 2001). In the tissue regulated c-src exon N1 PTB brings on introns on either side of the N1 exon forms a larger complex with brings about the exclusion of the regulated exon in the non neuronal cells by forming a larger complex with hnRNP F and H to loop out the exon, which is not present in the transcript (Chou et al., 2000). In the non neuronal cells the PTB protein is replaced by nPTB and the PTB binding to the introns is destabilized by an ATP dependant activity. In such conditions the downstream regulatory sequence “UGCAUG” acts to enhance the inclusion of the exon in the non neuronal cells. Splicing out of the WDR13 exon 3 in the brain and muscle might involve the complex interplay of ESS, or the combination of ISEs and ISS present as seen in c-src exon which is neuron specific.

In addition to these factors the downstream intron has binding sites for highly tissue specific splicing regulator Nova protein. This protein is exclusively expressed in neurons of the central nervous system (Buckanovich et al., 1993; Yang et al., 1998). Nova protein binding sites are also present adjacent to the regulated 3A exon of glycine receptor α2 and brings about the inclusion of this exon in neurons (Polydorides et al.,
2000). This can be seen as one of the ways in which the exon 3 of WDR13 is regulated in brain but since this protein is exclusively neuronal in origin it cannot bring about splicing in muscle. We have also identified binding sites for muscle specific splicing factor Fox1 and Fox2 sites in WDR13 gene. These proteins can act as both splicing repressor and an activator. Its acts in muscle specific skipping of exon 9 in ATP synthetase γ and activates the inclusion of skeletal muscle specific exon in α-actinin, possibly by antagonizing the PTB (Pyrimidine Tract Binding) protein (Jin et al., 2003).

Thus alternate splicing in WDR13 gene in H. fossilis might be a complex process where there can be interplay of ESS and combined action of ISE and ISS causing the splicing out of exon 3 or retention of intron 5. Tissue specific splicing factors can also have a possible role in the brain or muscle specific splicing of the WDR13 gene in H. fossilis.

**Expression and purification of WDR13 protein**

The three splice forms of the WDR13 gene are cloned individually in pET28b vector and expressed in bacteria. Induction of 53KDa protein is seen with WDR13a clone whereas 44Kda protein is induced with both WDR13b and WDR13c splice forms. Purification of the 53kDa protein is taken up as this is the constitutively spliced form and expressed in all the tissues studied. Over-expression of the protein in the bacteria formed inclusion bodies (Fahnert et al., 2004) which are biologically inactive. Solubilizing the protein in vivo by changing temperatures (22 °C, 18 °C and 15 °C) and composition of the media failed. Therefore the protein was purified under denaturing conditions. The protein was purified from the inclusion bodies and solubilized in 8M urea. The solubilized protein was further purified using a Talon column resulting in pure protein of 53kDa which was used for raising antibodies.

**Raising of antisera against WDR13 protein.**

Several attempts to raise antibodies against the human WDR13 in mouse, rabbits and guinea pigs at CCMB failed. Singh et al (2003) used the 43kDa, the alternatively spliced form of the gene to raise antibodies. The human and mouse WDR13 protein is very similar, differing only in four amino acids. It was then hypothesized (Singh et al., 2003) that due to the conserved nature of this protein the immune system is not recognizing this protein as an antigen and thus no antibodies were produced. Besides, the protein used for raising the antibody lacks the first ninety two stretch of amino acids.
which are hydrophilic and can be antigenic (Singh et al., 2003). While raising the antibodies against *H. fossilis* WDR13 protein, the constitutively spliced form is used. This protein has 85% similarity with the mouse protein. When tested the antiserum and the ascetic fluid both reacted with the purified WDR13 proteins (53kDa, 43kDa and 44kDa) indicating that the antibodies cross react with all the three splice forms of WDR13 protein of *H. fossilis*. It is thus presumed that we were able to raise antibodies since the antigenic stretch that is the first ninety two amino acids is included and also there is a significant difference between the proteins from both the species. The antibody also showed cross reactivity with the *H. fossilis* tissue lysate showing the 53kDa and 44kDa proteins. Alternatively spliced forms could not be detected as individual bands probably because the proteins could not be resolved on the SDS-PAGE as both the forms differ only by 1.2kDa. *H. fossilis* proteins though differ by 6 amino acids with zebrafish protein, showed cross reactivity of the antibody with that of the zebrafish tissue lysate.

**Cloning and characterization of WDR13 promoter**

Inverse PCR or adapter PCR approaches used to amplify the promoter of WDR13 were unsuccessful. Finally using Universal genome walker kit a 3.4Kb segment upstream of WDR13 gene was cloned. Sequence analysis of this clone did not identify a typical TATA box at -25 to -35 (Smale and Kadonaga, 2003) upstream of transcription start site (+1 Figure 27) indicating this may be a TATA-less promoter (Muller et al., 2007). The sequence is also analyzed using TF search and SIGNALSCAN for transcription binding factors. It identified binding sites for SRY, SP-1, SP-2, AP-1, 2, 3, CdxA, Brn-2, CRE-BP, Pbx-1, IK-2, c/EBP, CBF(1), CDF, HiNF-A, HNF-3 and IK-2,3,4 transcription factor. It is now known that not all promoters have a TATA box (Sandelin et al., 2007). The TATA dependant promoters are single peak promoters; with a well defined transcription start site whereas the TATA-less promoters are broad peak promoters, which have randomly distributed transcription start sites over 100bp window. Promoters possessing TATA box constitute only 10% of the genes and are tightly regulated tissue specific promoters whereas TATA-less promoters are associated with the ubiquitously expressed genes (FitzGerald et al., 2004) which is also in accordance with the ubiquitous nature of WDR13 gene.
Promoter analysis of WDR13

The TATA-less promoter are activated by GC rich regions known as CpG islands where specific class of transcription factors called SP factors (SP1, SP2 and SP3) bind. SP1 has a pivotal role in transcription initiation of these promoters (Crawford et al., 1999; Segal et al., 1999). Analysis of the promoter regions of WDR13 identified several SP 1 and other regulatory protein binding sites. To study the minimal length of the promoter, deletion analysis from 5' region was carried out. Maximum promoter activity is seen in the regions -359/+322. This spans the 5'UTR, exon 1 and intron 1. The deletion of -359/+116 region resulted in no change in promoter activity which spans the region of exon 1 and intron 1. The upstream of this region is the 5'UTR and the upstream promoter region (-359/+116) has three binding sites for SP1 and AP 1 transcription factors. Deletion from +116 to +1 decreases promoter activity showing that the SP1 binding site in this region is important for promoter activity. Further deletion of the upstream region (-359/-200) resulted in the loss of promoter activity showing that the SP1 binding sites in this region are also important for the promoter activity of the gene. When both these regions were deleted (-200/+56) the promoter activity reduced to minimal level indicating the requirement of multiple SP1 binding sites for the promoter activity. Phospho fructo kinase (PFKP) promoter has two SP1 binding sites, mutation of either result in the decrease of promoter activity showing that the multiple SP1 binding sites are required for promoter activity (Hannemann et al., 2005). In other clones studied there is an increase of promoter activity in the region from -2288/-2797. Analysis of the sequence in this region shows binding sites for transcription factors GATA-1 and2 and 3. These are zinc finger DNA binding domains which are lineage specific, playing an important role in tissue specific gene expression and differentiation. GATA factors are involved in hematopoietic cell specification (Simon, 1995; Patient and McGhee, 2002). In addition to their role in erythroid cell lineage specificity, GATA-2 and 3 factors are detected in developing brain suggest its role in the development of nervous system (Nardelli et al., 1999). GATA-3 gene is also expressed during human kidney embryogenesis (Labastie et al., 1995). Targeted disruption of GATA-3 gene resulted in severe abnormalities in the nervous system and the animals die in utero. The other abnormalities of these animals include uneven spinal cord, hypo pigmentation of retina, reduction in thickness of neuroepithelium, and collapse of brain ventricles (Pandolfi et
The presence of GATA 1, 2, 3 in the promoter of WDR13 gene and its expression in brain indicate the role of WDR13 gene in the development of brain.

**In vivo and in vitro analysis of promoter**

In zebrafish, the zygotic transcription starts at 4hpf during the mid-blastula transition period (Kane and Kimmel, 1993). To see the time point at which the zygotic transcription of WDR13 gene starts, WDR13Pro-EGFP was injected into the single cell embryo of zebra fish. Zygotic transcription of WDR13 starts at around 10hpf. In zebra fish at 10hpf the development of notochord begins which plays a major role in support as well as patterning of the body.

It is also established that WDR13 gene is maternally expressed and zygotically active from 10hpf. To determine in which tissue this gene is expressed, injection of WDR13Pro-EGFP construct into the single cell zebrafish embryos is carried out and the GFP expression is checked after 24 hours. GFP expression is seen in notochord, muscle and head regions. The beginning of the zygotic transcription of WDR13 at 10hpf, and the appearance of the GFP in the notochord and muscles strongly support its role in the development of the notochord which in turn play a role in formation of body axis and muscles. Maternal deposition of the gene indicates that the protein might play a role very early in the development. Expression of all the three splice forms in brain and muscle and its expression during development also support their role in development. In mouse it is shown that the levels of WDR13 gene are up regulated in the injured brains along with other genes like astrotactin and calineurin gene which have a role in repairing the damaged brain (Price et al., 2003). It is hypothesized that WDR13 might be having some role in signaling in response to neuro-degeneration and reactive synaptogenesis in the brain as suggested by Price et al. Considering this it can be speculated that WDR13 of *H. fossilis* may also have role in signaling during the development of notochord, muscle and brain, besides may be playing the same role in repairing the brain when it is injured.

**Functional analysis of WDR13 gene using morpholinos**

To determine the function of the WDR13 gene, the loss of gene function approach was adopted. This can be achieved by gene mutation, targeted gene knock-out or knock-down of WDR13. There is no well-characterized mutant available for the WDR13 gene hence the phenotype analysis of WDR13 through loss of functions is not possible. In *H. fossilis* it is not possible to make complete gene knock-out due the non availability of stem cells. Thus gene knock-down using morpholino based antisense
oligomers (Summerton et al., 1997; Summerton and Weller, 1997; Summerton, 1999), a well established approach in zebrafish and *Xenopus* (Nasevicius and Ekker, 2000; Sumanas and Larson, 2002) has been undertaken to analyze the function of WDR13 gene. Morpholinos are phosphorodiamidate oligonucleotides. In these oligos the ribose sugar in the backbone is replaced by morpholine moiety. Phosphodiester linkage is replaced by phosphorodiamidate non ionic linkage which makes the morpholinos highly stable and resistant to wide range of nucleases and proteases (Summerton and Weller, 1997). In the present study two morpholinos are used. One oligo is antisense (AS) morpholino, designed against the ATG start codon. This binds to the leader sequence and sterically blocks the translation of the protein, thus knocking down the function of the gene (Heasman, 2002). Since AS construct prevent proteins translation it is expected to block both the maternal and the zygotic transcripts of the WDR13 gene. The other morpholino, the splice block (SB) binds to splice sites and sterically blocks the splicing of the pre-mRNA, causing altered splicing of mRNA resulting in a truncated protein or there would no formation of mature mRNA (Partridge et al., 1996; Draper et al., 2001). As the SB morpholino acts at the level of mRNA formation, it will specifically block the zygotic transcripts of WDR13 in the embryo, but it cannot act on the maternally deposited transcript in the eggs. Hence it is expected that these two morpholinos designed for knocking down the WDR13 gene, could efficiently distinguish the maternal WDR13 mRNA from the newly formed mRNA by zygotic transcription. For each oligo (AS, SB, Std) 200 embryos are injected and similar number of uninjected embryos were taken as WT controls. The mortality rate in the WT is 3.5% whereas in Std it is 7.5%, and the development of the embryos in both the groups are normal without any visible morphological defects. Both the test oligo groups show high mortality and defects in the development of the embryo. The mortality rate of AS oligo is high in first 24 hours as compared to the SB oligo, since it blocks the translation of both the maternal transcript and the zygotic transcript (Scholpp and Brand, 2001). The SB blocks only the zygotic transcript, the individual survives till the maternal transcript is available resulting in a lower mortality rate as compared to the AS at 24hpf. The survival rate is similar in both the test oligos after 24hpf up to 7 days of development showing that once maternal deposit is exhausted both the oligos behave in similar a manner in knocking down the WDR13 mRNA. Besides the individuals surviving up to the seventh day have mild phenotypic defects possibly the morpholino concentration may be diluted. This makes the WDR13 gene transcription to outnumber the morpholinos at some time point as
development proceeds making the WDR13 translation to carry on, though the animal survives with some defects.

Two types of phenotype: moderate and severe were observed after morpholino injection. The knock down due to morpholinos is dose dependant (Draper et al., 2001). This is also evident from the western blot analysis where there is clear difference in the amount of WDR13 protein knock down, which is noticed between the severe and moderate phenotypes in the present study.

The morphants with moderate phenotype have less pigmentation whereas in the severe phenotype complete absence of pigmentation is seen. In both the phenotypes defects in craniofacial skeleton and jaw arches are noticed. Pigment cells are derived from neural crest (Eisen and Weston, 1993). These cells after migration and colonization form highly patterned structures including much of craniofacial skeleton, peripheral nervous system and pigment patterns. The cells which migrate between the somites and the skin, form chromatophores which are essential for the development of pigments of zebrafish and the cells which migrate between the neural tube and somites form the structures listed above (Raible and Eisen, 1994). Pigmentation in zebrafish starts around 24hpf and is visible first in retinal epithelium (Malicki et al., 1996). Mutations in zebrafish have been identified which affect the pigment formation including chromatophore patterning, pigment cell proliferation, chromatophore survival, chromatophore differentiation and those which block melanin synthesis (Driever et al., 1996; Kelsh et al., 1996). The pigmentation in the WDR13 severe morphants is completely absent. The colourless (cls) mutant of zebrafish has complete absence of pigment cells whereas the retinal pigmentation is unaffected but in the case of WDR13 morphants complete absence of pigmentation in the retina is seen. White tail (wit) mutant has severe pigmentation defect along with improper development of craniofacial skeleton similar to the WDR13 morphant phenotype. This shows that there might be some defect at the specification of chromatophores by the neural crest as seen in these mutants. In both the mutants there is pigmentation in the retina but it is absent in the WDR13 morphants. This may be due to melanophore degeneration as seen in the mutants like fade out (fad), fading vision (fdv), Quasimodo (qam) where the retinal pigment also becomes paler associated with retinal degeneration. Similar phenotype is seen in WDR13 morphants. The mutations involving the blockage in melanin synthesis is seen in albino (alb), sandy (sdy), golden (gol), nickel (nkl) where complete absence of pigmentation is seen in the body as well as the retina. Such a function might not be likely
for the WDR13 gene since there is pigmentation in the moderate phenotype, though there is complete absence of pigmentation in the retina.

The neural crest cells are the precursors for pigment patterning and the craniofacial skeleton. We can speculate that WDR13 might be having some role in specification of neural crest cells. In neural crest mutations, defects are also seen in other structures like ear and eye (Malicki et al., 1996; Odenthal et al., 1996; Whitfield et al., 1996). The eye of the morphants in the severe phenotype showed severe degeneration including disorganized retina. In the moderate phenotype there is formation of retina but it does not have pigmentation. The neurons of the retina are developed from an uniform sheet of pseudo-stratified neuroepithelium (Schmitt and Dowling, 1994). Various mutants are available which affect the retinal development. The mutant oko meduzy (ome), glass onion (glo), and nagie oko (nok), the eye pigmentation is patchy or absent with disorganized retinal neurons, abnormal brain and curved body (Malicki et al., 1996) which is similar to the phenotype of the WDR13 morphants. The glo mutants also have defects in the entire neural tube, brain and tail development. The vertebrate brain and retina consists of various neurons arranged in distinct laminae which have a common mode of development. Mutations in mice, reeler (rl) and weaver (wv) have been attributed their function to neuroepithelium development which supports the above said hypothesis (Caviness and Rakic, 1978). Such a correlation has to be proved in zebrafish. Mutants like glo can be a candidate in the development of neuroepithelium. All the splice forms of WDR13 is expressed in the brain and knock down using morpholino oligos against WDR13 resulted in the abnormal development of brain and severe degeneration of the retina similar to the retinal mutants. This suggests the role of WDR13 in neuronal patterning and development of neuroepithelium. In most cases defects in pigmentation are associated with the defects in the development of ear, eye and other neural cell derivatives (Haffter et al., 1996; Malicki et al., 1996; Whitfield et al., 1996).

The otic cavity appears in early somitogenesis stage forming semicircular otic vesicles by thickening and cavitation. The otoliths which are dense deposits of calcium carbonate are formed over the maculae which play a role in swimming and balance of the animal (Haddon and Lewis, 1996; Haddon et al., 1998). In moderate phenotype of WDR13 morphants the otic cavity is small and round as compared to the semicircular cavity as seen in the control. The anterior otolith is small and the posterior otolith appears degenerate. The otoliths appear close to each other and are displaced compared to the control. Comparison of the phenotype obtained after WDR13 morpholino injection
was done with the mutations which affect the phenotype of the inner ear. The mutants which affect the ear can be classified into 1) those which affect specifically otoliths, with no other ear defect and 2) those which affect the epithelial morphology. The **dog-eared** (**dog**) mutant affects both the phenotype of ear morphology and otoliths. Semicircular canals though formed are disorganized. Development of jaw is also affected in these mutants. Some mutants affecting ear development also show defects in jaw like **van vogh** (**vgo**), **boxer** (**box**) and **dackel** (**dak**) (Whitfield et al., 1996). The **vgo** mutant show defects in otic vesicles and jaw similar to WDR13 morphants but possess well developed otoliths. Some ear mutations have association with pigmentation defects as well (Kelsh et al., 1996; Whitfield et al., 1996). The **cls** mutant discussed earlier has pigment defects also has defects in the ear development where it has a small otic vesicle like the **vgo** mutant and the otoliths are also small (Kelsh et al., 1996) as seen in the WDR13 morphants. In mutants like **vgo**, **dog**, **mtc** in addition to the defects in the ear, defects are observed in the neural crest derivative lineages, either in the pigment cells or the cartilaginous elements of jaw (Kelsh et al., 1996; Odenthal et al., 1996). Studies in chick have shown that the neuronal cells contribute to the cartilaginous otic vesicle (Noden, 1988). From the defects seen in the ear, pigmentation and craniofacial morphology in the morphants of WDR13 it can be hypothesized that this gene may play a role in development of these organs. It should be noted that all these organs have a common origin from the neural crest cells. Similar to these mutants WDR13 may have a role in development of the ectodermal neurogenic populations or formation of cartilaginous structures of jaw and otic vesicle. Some ear mutants are also associated with defects in the development of brain as seen in the **snakehead**, **otter**, **fullbrain**, and **acerebellar** (Jiang et al., 1996; Whitfield et al., 1996). Most of the mutants which have a hind brain defect has a defective ear as seen in mice, showing that inductive signals from the hindbrain play a role in the development of inner ear (Steel and Brown, 1994). On a holistic view the pigment cells, ear and eye all are related to the sensory system which is finally connected to the brain. Defect in pigment cell and ear can be attributed to the neural crest cell defect and the defects in eye and ear can be attributed to the neuroepithelium. Thus it can be hypothesize that WDR13 may act as a signaling molecule during the development of these organs. In the WDR13 morphants with severe phenotype there is no otic vesicle or the otoliths, ear is represented by a small dent, or there is complete absence of otic vesicle at 24hpf. So far mutants without ears could not be identified in mutant screen for ear defects (Whitfield et al., 1996). It is hypothesized
that, either there is no master gene regulating ear development or there may be multiple genes governing the formation of ear or the genes responsible for the development of ear might have function in the early development of the embryo and the mutation in those genes might be so drastic that ear phenotype would be masked by early lethality. Genes which have function in the early development are maternally deposited which can compensate for the lack of zygotic expression in the mutants (Whitfield et al., 1996). This may be true in the case of WDR13 as it is maternally deposited which shows some function in the early development of the animal, as in the ‘severe’ phenotype not only the ear but eye and the craniofacial skeleton are affected and the individuals did not survive past 36hpf. Knocking down both the maternal transcript and zygotic transcript of WDR13 resulted in the severe phenotype. All these results suggest that WDR13 may have a role in the early development of the animal.

In addition to these defects the morphants also have defects in notochord and somite patterning. During gastrulation the dorsal organizer forms the chordamesoderm, which forms the notochord. During development the central cells of the notochord differentiate and acquire vacuoles which gives shape and rigidity to the notochord (Schulte-Merker et al., 1992). Notochord plays a major role in patterning of the adjacent tissues, neuroectoderm, paraxial mesoderm and forms the floor plate and can independently signal the formation of motorneurons. It plays role in the formation of somites which forms the sclerotome and dermamyotome (Stemple, 2005). Mutations in development of notochord in zebrafish can be divided into two groups, one which affects the formation of notochord and the second group which affects the development of the notochord (Odenthal et al., 1996; Stemple et al., 1996). The mutants no tail (ntl), floating head (flh), momo (mom) and doc (Odenthal et al., 1996) belong to the first group which affect the formation of notochord. In these mutants the notochord is completely absent or partially present either in the trunk region or the tail region. The mutations which affect the development of notochord are further divided into groups depending upon the stage at which they affect. The mutants which are classified having differentiation defects are further divided into three groups the one which affects only notochord, those which affect notochord and brain and the other which affects notochord and later the entire embryo. The mutants sleepy (sly), grumpy (gup), bashful (bal) does not have a well differentiated and vacuolated notochord, have brain defects and does not from the “V” shaped somites with defects in the eye and abnormal body axis, which is similar to phenotype seen in the WDR13 morphants. This shows that WDR13 gene
might be playing a role in the differentiation of the notochord. In the mutants *dopey* (*dop*), *sneezy* (*sny*) and *mirky* (*mik*) in addition to a lack of differentiated notochords the animals degenerate and become necrotic. In the present study the severe morphants also appear necrotic. The mutants *snow white* (*snw*), *mind bomb* (*mib*), *changeling* (*chg*), *maggot* (*mgt*) are grouped in the mutations which are involved in maintenance of notochord. These animals have a vacuolated notochord but the vacuoles take a spherical shape instead of a scalloped shape. Besides these mutants have an abnormal head shape, under developed brachial arches and malformed ear similar to the phenotype of the WDR13 morphants. The tails of these mutants are also bent similar to the WDR13 morphants. This shows that WDR13 in addition to playing a role in the differentiation of the notochord might also be playing a role in maintaining the shape.

As discussed earlier notochord plays a role in patterning of the somites, the myotome, sclerotome and the floor plate (Stickney *et al.*, 2000). Sonic hedge hog signaling is very important in patterning the somites and also the specification of slow muscle formation in zebrafish (Blagden *et al.*, 1997). This shows that the interaction between the notochord and paraxial mesoderm are necessary for proper somite patterning and myogenic fates in the somites. In the mutants whose notochords are affected, the somites are also affected. The mutations which affect the somitogenesis as reported in *fused somites* (*fss*), *beamter* (*bea*) affect all the somites, whereas *deadly seven* (*des*), *after eight* (*aei*) and *white tail* (*wtl*), affect the more posterior somites. In the *you*-type genes mutations, the somites are “U” shaped (van Eeden *et al.*, 1996). In all these mutants there are no defects in the notochord but only in the somites. This shows that the defects in the somites seen in the WDR13 morphants is due to the defect in the differentiation of the notochord which is failing in its signaling duties due to which the somites are affected, as discussed earlier (Stemple *et al.*, 1996; Stemple, 2005). The somites in the morphants have abnormal morphology with the loss of the “V” shape and are asymmetric in the body axis with less of epaxial mesoderm which forms the muscles of neck and trunk and more of hypaxial mesoderm which forms the thoracic and anterior abdominal muscles. This asymmetry is also due to the notochord which is unable to deliver its function in playing a role in patterning of paraxial mesoderm into the epaxial and hypaxial mesoderm (Stickney *et al.*, 2000; Stemple, 2005). Taking into account all the phenotypic traits, it can be hypothesized that the defects in the formation of notochord is due to differentiation and maintenance of the notochord. The malformed notochord is compromised in its function of signaling which resulted in the malformed
somites, and asymmetric body axis. There is also no proper development of the neural tube which resulted in the malformed brain. The abnormalities seen in the ear, eye and the pigment defects can be attributed to a defect in the defective specification of the neural crest cells which is affecting the formation of the neuroepithelium. The necrotic appearance seen all through the animal in case of the severe phenotype in WDR13 morphants shows that the gene might have a possible role in cell signaling during early development and differentiation of notochord which has pleiotrophic effects on the development of other organs. The maternal deposit shows that the gene might be having a role early in the development, and this maternal deposit suffices the embryo until the zygotic transcription starts. The zygotic transcription of WDR13 starts at 10hpf shows that gene is required all through the development of the animals. The gene is also ubiquitously expressed in all the adult tissues studied, points that the gene is also needed during the entire life span of the animal. The gene is regulated by a TATA-less promoter and is activated by the presence of SP1 transcription factors show, that it housekeeping gene. The alternate splice forms might be having a different function compared to the constitutively spliced form since the localization of the splice forms are different. In the present study it was not possible to pin point the phenotype, whether it is due to the constitutively spliced form or the alternatively spliced forms since the morpholinos used in the study target all the three splice forms.

Besides isolation and characterization of the WDR13 gene, the present study also extends to find out the function of the gene. In this process it is shown that WDR13 gene is alternatively spliced and all three splice forms are expressed in brain and skeletal muscle and the gene is maternal deposited indicating a function in the early development. It has a TATA-less promoter with specific binding sites for SP1 and SP2 transcription factors. The zygotic expression of the gene starts at 10hpf and the gene being expressed in head, muscles and notochord at 24hpf indicating that the function of the gene might be important in the development of brain, muscles and notochords. Knock down studies have shown defects in craniofacial structures, pigmentation, development of head structures like eye, ear and jaw arches. The body is severely deformed with malformed somites, notochords and bent tails. Some of the morphants also appeared necrotic. From the above observations it is hypothesized that WDR13 gene is a developmentally important gene which might be playing a role in signaling during development and may carry out the same/similar functions in different tissues in the adult fish.