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Cloning of a functional 25-hydroxyvitamin D-1α-hydroxylase in zebrafish (*Danio rerio*)

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Abstract

Activation of precursor 25-hydroxyvitamin D_3 (25D) to hormonal 1,25-dihydroxyvitamin D_3 (1,25D) is a pivotal step in vitamin D physiology, catalyzed by the enzyme 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase). To establish new models for assessing the physiological importance of the 1α-hydroxylase-25D-axis, we used *Danio rerio* (zebrafish) to characterize expression and biological activity of the gene for 1α-hydroxylase (*cyp27b1*). Treatment of day 5 zebrafish larvae with inactive 25D (5-150 nM) or active 1,25D (0.1-10 nM) induced dose responsive expression (15-95 fold) of the vitamin D-target gene *cyp24a1* relative to larvae treated with vehicle, suggesting the presence of Cyp27b1 activity. A full-length zebrafish *cyp27b1* cDNA was then generated using RACE and RT-PCR methods. Sequencing of the resulting clone revealed an open reading frame encoding a protein of 505 amino acids with 54% identity to human CYP27B1. Transfection of a *cyp27b1* expression vector into HKC-8, a human kidney proximal tubular epithelial cell line, enhanced intracrine metabolism of 25D to 1,25D resulting in greater than 2-fold induction of *CYP24A1* mRNA expression and a 25-fold increase in 1,25D production compared to empty vector. These data indicate that we have cloned a functional zebrafish *CYP27B1*, representing a phylogenetically distant branch from mammals of this key enzyme in vitamin D metabolism. Further analysis of *cyp27b1* expression and activity in zebrafish may provide new perspectives on the biological importance of 25D metabolism.

Keywords

CYP27B1; vitamin D; cytochrome P450; CYP24A1; metabolism

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Conflict of Interest
The authors have declared that there is no conflict of interest.

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Introduction

Optimal vitamin D status has long been recognized as a key factor in the prevention of bone diseases but in the last decade it has become increasingly clear that facets of extra-skeletal physiology may also be influenced by vitamin D [6,13]. Cholecalciferol (vitamin D_3) is produced by UVB photoconversion of 7-dehydrocholesterol in skin or consumed in vitamin D_3 containing foods or oral supplements. Vitamin D_3 undergoes a hydroxylation step in the liver yielding prohormone 25-hydroxyvitamin D_3 (25D) followed by a subsequent hydroxylation (in renal and extra-renal sites) yielding the active 1,25-dihydroxyvitamin D_3 (1,25D) [30]. 1,25D produced by the kidney acts in an endocrine manner to regulate calcium metabolism and is vital to bone health while the actions of 1,25D in extra-skeletal physiology appear to be dependent on tissue-specific conversion of prohormone 25-hydroxyvitamin D_3 (25D) to active 1,25-dihydroxyvitamin D_3 (1,25D) [26,28], with target cells expressing the enzyme 25D-1α-hydroxylase (CYP27B1) that catalyzes this reaction [26,27,28]. Coincident expression of the nuclear receptor for 1,25D (vitamin D receptor, VDR) by these cells suggests that many effects of vitamin D are mediated via an intracrine, rather than an endocrine mechanism. In this setting, given that 25D is the major circulating form of vitamin D, it is likely that impaired vitamin D status will compromise local synthesis of 1,25D and associated physiological responses [5,25]. A role for vitamin D in non-classical, extra-skeletal physiology is supported by data from various studies using mouse models. These include mice with knockout of the murine genes for *Cyp27b1* [35], *Vdr* [21,31,60], and mice with dietary deficiency of vitamin D [32].

The actions of vitamin D in zebrafish specifically, and fish in general, remains unclear. Vitamin D metabolism has been demonstrated in fish [44,46], and it has been assumed that vitamin D contributes to the skeletal homeostasis of these animals in much the same way as other vertebrates. Although no specific requirements have been set, standard diets for zebrafish routinely include vitamin D [1,2]. The zebrafish genome project has identified putative genes for various components of the vitamin D system such as: *vdr* (*vdra*, and *vdrb*), *cyp2r1, cyp27a1* (*cyp27a1.2*, and *cyp27a1.4*), *cyp27b1, cyp24a1* and *gc*. Despite this abundance of genome project data for zebrafish, only a handful of reports have documented functional responses to vitamin D in these animals. To date, these studies have focused exclusively on the effects of active 1,25D upon calcium handling, *vdr* expression and transcriptome analysis [16,17,18,34]. By contrast, actions of precursor 25D in zebrafish are much less well understood. In the current study, we have sought to expand this by cloning the zebrafish gene for *CYP27B1*, which may be important for both the classical (endocrine) and non-classical (intracrine) effects of vitamin D in this organism.

As a model system, zebrafish have the advantage of being relatively inexpensive, small, fast growing organisms with a larval state that can be easily visualized and genetically manipulated [7]. As such, zebrafish are a potentially attractive model system to study vitamin D's role in bone development and immune function. In regards to immune response to infection in particular, zebrafish can be infected with, *Mycobacterium marinum* [47,49,56], a mycobacterial pathogen with similarities to the *Mycobacterium tuberculosis* pathogen that causes tuberculosis in humans. Thus, *Danio rerio* (zebrafish) may provide a

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useful alternative animal model for studies of vitamin D and its potential role in infection and immunity.

Material and methods

Animal care

Fish were raised on a 14/10 hour light/dark cycle at 28.5°C. Embryos were maintained in a 28.5°C incubator. Experiments were performed at larval stages when male and female zebrafish cannot be distinguished. The Chancellor's Animal Research Care Committee at the University of California, Los Angeles, approved all experiments.

qRT-PCR analysis of mRNA expression in zebrafish

Zebrafish larvae (day 5) were incubated with 25D (0 - 150 nM) or 1,25D (0 - 10 nM) for 6 hours. In the inhibitor studies, itraconazole (0, 0.1, and 1.0 μM) was added one hour prior to the six hour incubation with vehicle, 5 nM 25D or 0.1 nM 1,25D. RNA from zebrafish larvae was extracted by Trizol (Life Technologies, Carlsbad, CA) and cDNA generated by Super Script III Reverse Transcriptase (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. After cDNA synthesis, samples were diluted three-fold with RNAse free water, and 2 μl aliquots were used in qPCR reactions. A master mix of SYBR-qPCR enzyme mix (Agilent, Santa Clara, CA) and 50 nM primer pairs (either reported or designed on Primer3 [53]) was prepared and added to templates to a final volume of 25 μl. qPCR analysis was performed on MX3005P instrument (Agilent, Santa Clara, CA) using the following amplification program: 10 min 95°C (1×), 30 sec 95°C, 1 min 55°C, 1 min 72°C (40×). Amplification program was followed by a dissociation program: 1 min 95°C, 30 sec 55°C, 0.2C°/sec ramp up until 95°C. Ct values were determined by instrument software. Ct values for the gene of interest, zebrafish 24-vitamin D hydroxylase (*cyp24a1*; Genbank NM_001089458; forward 5′ AAAAGTCAACGGC AAAATGG 3′; reverse 5′ GTGTGGTCCTTCCACGTCTT 3′) were subtracted by the Ct values or the calibrator gene, zebrafish elongation factor 1-alpha [39] (*elfa*; Genbank AY422992; forward 5′ CTTCTCAGGCTGACTGTGC 3′; reverse 5′ CCGCTAGCATTACCCTCC 3′) to yield Ct values. Data were expressed as either Ct or fold change (fold = 2^{Ct}) relative to vehicle treated sample. Error bars were displayed as standard deviation (SD) unless indicated otherwise. Equal variance two-tailed Student's t-test (Microsoft Excel) was used for statistical analysis and results with $p < 0.05$ were deemed significant.

Cloning of zebrafish cyp27b1

First Choice RLM-RACE Kit (Ambion, Austin, TX) was utilized to clone 5′ and 3′ ends. Following the manufacturer protocol, 5′ and 3′ RACE cDNA was generated from five-day old zebrafish RNA. The predicted *cyp27b1* cDNA sequence (ENSDARP00000066177) was entered nto Primer3 software [53] to design primers that were synthesized (Life Technologies, Carlsbad, CA) for usage in RACE cDNA synthesis. Primers used are listed in Table 1. The cDNAs were then PCR-amplified with Ambion supplied RACE outer primers and *cyp27b1* specific outer primers, followed by nested PCR with Ambion supplied RACE inner primers and *cyp27b1* specific inner primers. *Taq* polymerase (Life Technologies, Carlsbad, CA) was used for nested PCR reactions and middle fragment PCR. To clone the

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overlapping middle fragment, cDNA rom 0.5 μg of RNA was synthesized with Super Script III Reverse Transcriptase (Life Technologies, Carlsbad, CA) followed by PCR with *cyp27b1* specific primers. Reaction conditions for the PCR were: 94°C for 3 min followed by 35 cycles of 94°C for 45 sec, 60°C for 30 sec and 72°C for 2 min with a final 10 min extension at 72°C. Fragments were separated by agarose gel electrophoresis, and isolated with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The fragments were then ligated into the pCR-TOPO vector (Life Technologies, Carlsbad, CA) and sequenced by the UCLA Sequencing and Genotyping Core. The 5′ RACE and 3′ RACE fragments were combined with the middle fragment through splicing by overlapping extension (SOE) PCR. *Pfx* polymerase (Life Technologies, Carlsbad, CA) was used for subsequent template generation. Reaction conditions for *Pfx* were: 94°C for 2 min followed by 35 cycles of 94°C for 15 sec, 61°C for 30 sec and 68°C for 2 min. The 5′ SOE and 3′ SOE fragments were combined by ligation at a common *Kpn I* site, yielding a full-length cDNA, which was cloned into the TOPO TA pcDNA3.1 vector (Life Technologies, Carlsbad, CA). The cDNA sequence has been deposited to Genbank (KM262796).

Zebrafish tissue analysis for cyp27b1 by qPCR

Two independent preparations of RNA by Trizol extraction were obtained from organs (kidney, heart and spleen) of two or three 18-month old male zebrafish. cDNA was synthesized as described above for qPCR analysis of ELFA for normalization purposes. To facilitate detection of the low expression of *cyp27b1*, a separate batch of cDNA was synthesized using *cyp27b1* gene specific primer (5′ TGGTTTCCTCCGCTGTGTTT 3′) located near the 3′ end. qPCR analysis was conducted with *cyp27b1* probe/primer set near the 5′ end: forward 5′ GTTCGCTAAAGGACACATTGAC 3′; reverse 5′ CCTGAGACAGAAAGTACGTGAG 3′; and probe 5′ CAGCAGGAGAAGCAGAAGCTGGAG 3′ modified with 5′ FAM, ZEN internal Quencher, and 3′ Iowa Black FQ (Integrated DNA Technologies, Coralville, Iowa). qPCR analysis was performed on MX3005P instrument (Agilent, Santa Clara, CA). Ct = Ct(*cyp27b1*) – Ct(*elfa*) was calculated for each sample and fold change relative to the lowest expressing organ was determined.

Transfection of HKC-8 for qPCR and HPLC analysis

Human proximal kidney tubule cells (HKC-8 cells) were detached by trypsin, diluted with Opti-MEM (Life Technologies, Carlsbad, CA) and 2.5×10^5 cells were seeded into each well of 12-well plates. 8 hours later, 0, 0.5 or 1.0 μg pcDNA3.1-z*cyp27b1* were normalized to a total of 1.0 μg with pcDNA3.1 and diluted with 100 μl Opti-MEM for each well to be transfected. 4 μl of Lipofectamine 2000 (LF2000, Life Technologies, Carlsbad, CA) was diluted with 100 μl of Opti-MEM for each well to be transfected. The diluted plasmid DNA was combined with the diluted LF2000, mixed vigorously, incubated for 20 minutes to allow complex formation, and then seeded onto the plated cells. 18 hours later, LF2000/DNA containing media was removed by aspiration and fresh Opti-MEM media was added. 0 or 100 nM 25D dissolved in ethanol was added to each well at a 0.1% volume. Six hours later, media were removed, and 1 ml of Trizol (Life Technologies, Carlsbad, CA) was added to the cells. RNA extraction and cDNA synthesis was according to manufacturer's protocol. CYP24A1 (Hs00167999_m1) and 18S rRNA (4310893E) probe/primers were used in

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TaqMan qPCR analysis (Life Technologies, Grand Island, NY). For HPLC analysis, LF2000 transfection of pcDNA3.1 and pcDNA3.1-*zcyp27b1* was scaled up according to product protocol for 24 μg plasmid per 10cm dish. 48-hours after transfection, cells were detached with trypsin and one million cells were incubated with 300,000 cpm ^{3}H-25-hydroxyvitamin D_3 (^{3}H-25D, 155 Ci/mmol; Perkin Elmer, Waltham, MA) per glass tube for two hours. Metabolites were extracted, purified and analyzed by HPLC as previously described [8]. Protein concentration of cellular material used in metabolism experiment was determined by Bradford assay (Bio-Rad, Hercules, CA) to normalize samples with results expressed as fmol/hr/mg.

Phylogenetic Analysis of DNA sequences

The evolutionary history of CYP27B1 protein sequences were inferred by using the Maximum Likelihood method as implemented in MEGA 5 [48]. CYP27B1 sequences analyzed were NCBI reference sequences from human (NP_000776.1), mouse (NP_034139.2), rat (NP_446215.1), cow (NP_001179213.1), pig (NP_999160.1), rhesus (NP_001181642.1), cat (XP_003989015.1), dog (XP_538254.3), chimpanzee (XP_509175.2), xenopus (NP_001006907.1), and horse (NP_001157429.1), and Ensembl sequences from zebrafish (ENSDARP00000066177), and anole (ENSACAP00000012941). NCBI reference sequences of CYP27A1 from chicken (XP_422056.3) and zebrafish (XP_001923080.3), and CYP27C1 from chicken (XP_422077.2) and zebrafish (NP_001106808.2) were also included in the analysis.

Results and Discussion

Effects of 25D and 1,25D on whole zebrafish gene expression

Day 5 zebrafish larvae were treated with varying doses of inactive precursor 25D (5-150 nM), active metabolite 1,25D (0.1-10 nM), or vehicle (0.1% ethanol) for six hours. Total RNA from the zebrafish was then used to quantify expression of mRNA for zebrafish equivalent of the 1,25D-inducible catabolic enzyme vitamin D-24-hydroxylase (*cyp24a1*). Data in Figure 1A and 1B indicate that both 25D (36 to 95-fold) and 1,25D (15 to 69-fold) induced *cyp24a1* expression relative to ethanol-treated control larvae. The ability of pro-hormone 25D to induce this change in gene expression suggested that zebrafish express a CYP27B1 gene that is associated with synthesis of 1,25D in mammals. To confirm the presence of a functional 1α-hydroxylase activity in zebrafish, day 5 larvae were treated with vehicle (0.1% DMSO) or with the 1α-hydroxylase inhibitor itraconzole (ITRA; 0.1 or 1.0 μM) for one hour prior to initiation of six hour incubations with either 25D (5 nM) or 1,25D (0.1 nM). Subsequent analysis of mRNA expression in these larvae showed that ITRA pre-treatment decreased 25D-induction of *cyp24a1* by 50%, but did not have any effect on 1,25D-induced *cyp24a1* (Figure 1C). These data suggest that the effects of 25D on gene expression in zebrafish larvae involve a functional 1α-hydroxylase enzyme. Further studies were therefore carried out to clone the gene associated with this enzyme activity in zebrafish.

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Cloning of zebrafish cyp27b1

Rapid amplification of cDNA ends (RACE) and sequence data from the zebrafish genome database were used to clone a cDNA for zebrafish *cyp27b1*. The full-length cDNA sequence (Genbank accession number KM262796) cloned contains an open reading frame (ORF) yielding a predicted 505-amino acid protein. The cloned sequence (Genbank accession number KM262796) shares 99% identity at the nucleotide level and 98% identity at the protein level with the predicted sequence in the zebrafish genome database (Genbank accession number XP_003199448.2). Amino acid sequence comparison analysis between zebrafish and human 1α-hydroxylases showed 54% identity between zebrafish 1α-hydroxylase and its human homolog (Figure 2). Functionally important regions of the human 1α-hydroxylase protein such as the heme-binding domain (bold) [45], ferredoxin binding domain (underlined) [40], and residues believed to be vital for enzymatic function (Q66 and T408; bold and asterisk) [59] were found in the zebrafish sequence with high identity.

Zebrafish cyp27b1 is expressed in kidney, spleen and heart

Expression of CYP27B1 was found first and at high levels in kidney [33,61]; however, it has also been found expressed in extra-renal sites [3,24]. To determine relative tissue expression levels in zebrafish, kidney, spleen and heart tissues were obtained from adult male fish and qPCR analysis conducted. In Figure 3, the heart exhibited the lowest expression relative to the other organs analyzed while the kidney, as expected, had the highest (17 and 65 fold greater than heart) and the spleen had intermediate levels (4 to 7 fold higher compared to heart). These findings are consistent with reports from other animals that revealed low but detectable expression of CYP27B1 in spleen [11,42] and heart [11,12].

Zebrafish cyp27b1 is biologically active

To determine whether the *cyp27b1* identified in Figure 2 is biological active, a cDNA expression construct for *cyp27b1* was transfected into HKC-8, human proximal tubule kidney cells, which are known to support a modest level of conversion of 25D to 1,25D [9,14]. Following transfection with either empty vector, 0.5 or 1 μg of pcDNA3.1-z*cyp27b1*, HKC-8 cells were incubated with vehicle (0.1% ethanol) or 25D (100 nM) for six hours. Subsequent RT-PCR analysis of mRNA from these cells showed that 25D-induced expression of mRNA for human *CYP24A1* increased in a dose-dependent fashion in HKC-8 cells that received *cyp27b1* cDNA relative to cells that received empty vector (Figure 4A). To further confirm the functionality of the cloned *cyp27b1*, HPLC analysis of 1,25D production was conducted. HKC-8 cells were transfected with pcDNA3.1 or pcDNA3.1-*zcyp27b1* and 48-hours post-transfection, cells were detached and incubated for two hours with ^{3}H-25D. Vitamin D metabolites were then extracted, purified and analyzed by HPLC (Figure 4B-4D) and results expressed as fmol/hr/mg protein. As shown in Figure 4B, HKC-8 transfected with pcDNA3.1-*zcyp27b1* produced 25-fold more 1,25D relative to the empty vector transfected cells (815.3 vs. 32.6 fmol/hr/mg). These data confirm that the cloned zebrafish *cyp27b1* encoded a functional 25-hydroxyvitamin D-1α-hydroxylase.

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Phylogenetic analysis of zebrafish cyp27b1

Based on sequence analysis, cytochrome P450 genes have been grouped into 11 clans [41] of which CYP27B1 belongs to the mitochondrial clan. The cladogram in Figure 5 shows a phylogenetic analysis of CYP27B1 protein sequences retrieved from thirteen vertebrates, along with two CYP27A1 and two CYP27C1 sequences as outgroups. The phylogenetic analysis indicates that the zebrafish CYP27B1 is derived from an ancient bifurcation from the lineage eading to mammals. As such, the most similar sequences to zebrafish 1α-hydroxylase are amphibian (xenopus) and reptile (anole) 1α-hydroxylases. Predictably, within the mammalian CYP27B1, sequences grouped as expected with most similarity within rodent and primate species with less confident clustering of the other mammalian species. Though zebrafish 1α-hydroxylase/*cyp27b1* was distinct from the mammalian cluster, it remains firmly within the CYP27B1 family, clearly separated from the CYP27A1 and CYP27C1 lineages.

In view of the fundamental importance of vitamin D for skeletal homeostasis, it seems likely that all genes such as VDR and CYP27B1 would be common to all species in the subphylum vertebrata. However, an unexpected result of our analysis was finding an absence of any avian members of the CYP27B1 sequence family within the genome database. Some of the early work on vitamin D metabolism utilized chickens [22,33] and they are known to synthesize 1,25D [4,23,57], so the absence of CYP27B1 in avian genome databases is surprising. Investigators in this field recognized that there is 5-10% of the chicken genome where sequence data remains absent or of poor quality [20,58] thus, perhaps, explaining the current absence of CYP27B1 in the chicken genome database. On the other hand, numerous avian species have CYP27A1 and CYP27C1 represented in the databases, thus it is unlikely that CYP27B1 has simply been missed by avian sequencing projects. This suggests the avian lineage uses an alternate, as yet unidentified, enzyme to fulfill the function of a vitamin D 1α-hydroxylase, possibly another member of the very large P450 superfamily. For example, although CYP2R1 is understood to be the major contributor to 25D production, it is not the only CYP with vitamin D-25-hydroxylase activity [62]. By contrast, *Cyp27b1* knockout mice [19,43], and natural CYP27B1 mutations in humans [37] strongly suggest that this gene is solely responsible for production of 1,25D in these species. The conspicuous absence of CYP27B1 in avian genome databases is an area of active investigation (Wes Warren, personal communication) and firm conclusions about the presence or absence of CYP27B1 awaits further research.

Zebrafish as a potential model system for vitamin D and TB

In mammals, 25D is the major circulating form of vitamin D and the principal marker of vitamin D status [29]. In recent years the importance of 25D in vitamin D physiology has been elevated with the recognition that many effects of vitamin D may be mediated via local intracrine, conversion of 25D to 1,25D, with resulting tissue-specific actions of 1,25D bound to the VDR [6,13,15]. As a consequence there has been renewed interest in the expression and activity of the *CYP27B1* gene product, 1α-hydroxylase, which catalyzes conversion of 25D to 1,25D [24,52]. The site-specific impact of 1α-hydroxylase/CYP27B1 on vitamin D physiology has been studied extensively in murine models [35,36,42,55], but has yet to be assessed in zebrafish.

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Cloning of a functional *cyp27b1* provides a useful complement to the previous characterization of zebrafish *vdr* [16] adding additional evidence for the existence of the vitamin D system in this organism. Future studies aimed at manipulating expression of these two components of the vitamin D system through morpholino knockdown or zinc-finger nuclease mutation will help clarify the relative activities of these gene products in diverse facets of vitamin D physiology. The functional 1α-hydroxylase enzyme activity demonstrated in this report further underlines the versatility of zebrafish as a model system. Of particular interest is the role of vitamin D in immune responses to infection [27]. Vitamin D-deficiency has been closely linked to the mycobacterial infectious disease tuberculosis (TB) [38,54], a major global health problem. However, current animal models for TB are poor and greatly limit the scope of studies aimed at defining the potential effect of molecules such as vitamin D as treatment for or prevention of this disease. By contrast zebrafish are a highly informative model for infection with *Mycobacterium marinum*, a mycobacterium related to the TB pathogen, *Mycobacterium tuberculosis* [10,49,50,51]. Thus, in future studies, zebrafish may be a useful model for assessing the role of vitamin D metabolism and signaling in the pathophysiology and treatment of diseases such as TB.

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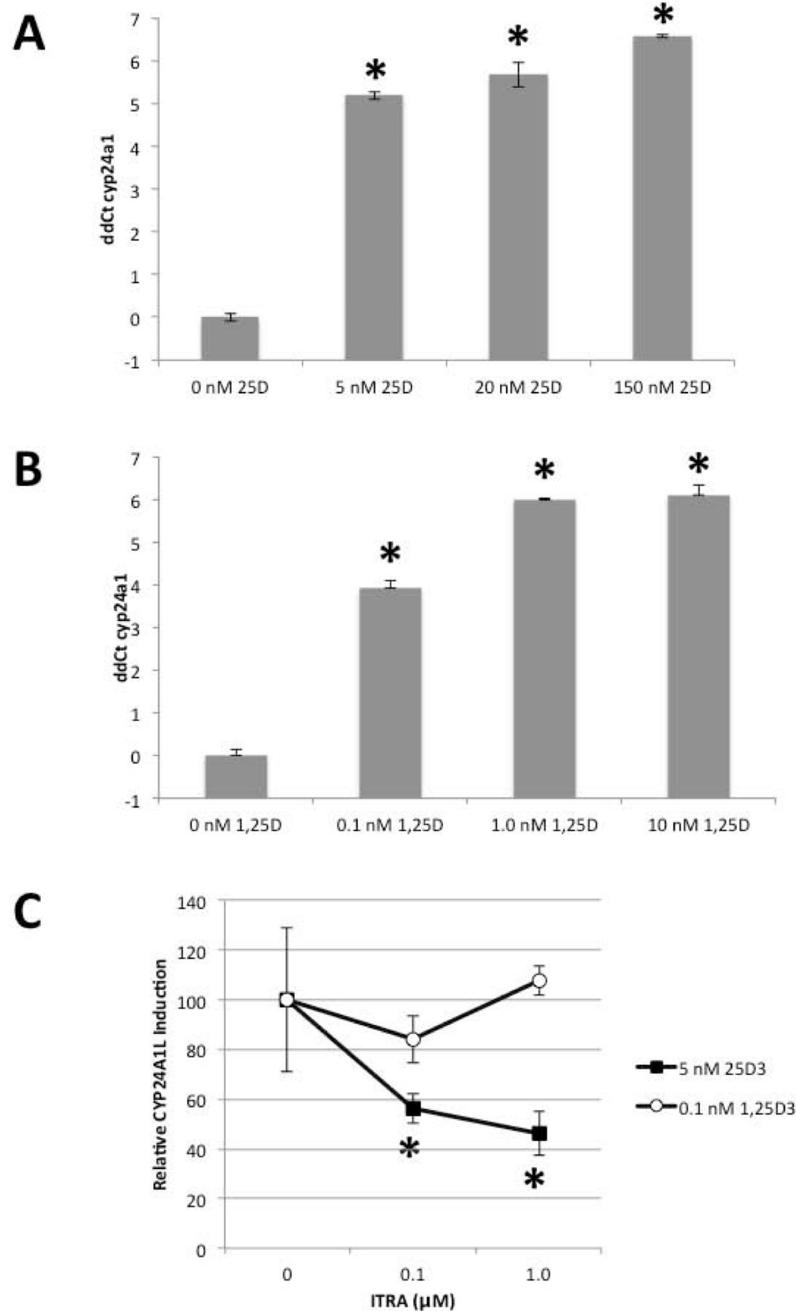

Figure 1. Induction of *cyp24a1* mRNA expression by 25D and 1,25D identifies a functional 25-hydroxyvitamin D-1-hydroxylase in zebrafish

Zebrafish larvae (day 5) were treated with increasing doses of: A) 25D (5-150 nM), B) 1,25D (0.1-10 nM) or vehicle (0.1% ethanol) for six hours, and expression of *cyp24a1* mRNA determined by qRT-PCR. Data are expressed as Ct = Ct (*cyp24a1*) - Ct(*elfa*). C) Zebrafish larvae (day 5) were treated with increasing doses of itraconazole (ITRA, 0, 0.1, and 1.0 µM) one hour prior to six hour incubation with vehicle, 5 nM 25D or 0.1 nM 1,25D. Expression of *cyp24a1* was then determined by qRT-PCR and expressed relative to vehicle ITRA set to 100%. Fold = 2^{Ct}. Data are shown as n=3 replicates ± SD. * $P < 0.05$.

```
>>Seq1 = zfish (505 aa); Seq2  = human (508 aa)
 s-w opt: 1741  Z-score: 2126.9  bits: 403.1 E(): 1.2e-116
Smith-Waterman score: 1741;  53.992% identity (55.269% ungapped)
in 476 aa overlap (39-505:35-508)
               10        20        30        40        50        60
Seq1    LKVTGRSALPLLRFAERWADVIRAPPTPQVKTLEQMPGPSPARFIRDLFMKRGFSRLHQL
                                      ..: ..::::   :. .:: : :.::::.:
Seq2    LKYASRVFHRVRWAPELGASLGYREYHSARRSLADIPGPSTPSFLAELFCKGGLSRLHEL
                  10        20        30        40        50        60

               70        80        90       100       110       120
Seq1    QLEGRQKYGPMWKASFGPILTVHVAEPELIQQVLRQEGQHPVRSELSSWKDYRALRGEGY
        :..:  ..::.: :::: . ::.:: : :....:::: .: :  .: : ..:  : ..
Seq2    QVQGAAHFGPVWLASFGTVRTVYVAAPALVEELLRQEGPRPERCSFSPWTEHRRCRQRAC
        *   70        80        90       100       110       120

              130       140       150       160       170       180
Seq1    GLLTAEGEEWQCVRSLLSKHMLRPQAVEAYDGALNAVVSDLLQKLKLRSQESSS--RIVS
        ::::::::::: .:::::.  .::::::.  : :.:: :: ::...:. .  ....   .:
Seq2    GLLTAEGEEWQRLRSLLAPLLLRPQAAARYAGTLNNVVCDLVRRLRRQRGRGTGPPALVR
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               190       200       210       220       230       240
Seq1    DISAEFYRFGLEGISSVLFESRIGCLDAVVPVETERFIQSINTMFVMTLLTMAMPQWLHR
        :...:::.::::::..::. ::.:::.: :: .:: ::.......:: :::::::::.::..
Seq2    DVAGEFYKFGLEGIAAVLLGSRLGCLEAQVPPDTETFIRAVGSVFVSTLLTMAMPHWLRH
                190       200       210       220       230       240

               250       260       270       280            290       300
Seq1    LLPKPWDTFCRCWDVMFEFAKGHIDQRLQEEKQKLECGEQLE-----GRYLTYFLSQAGL
        :.: ::  .:: :: :: ::. :...:  : .   .. : : :      : .::.:: .  :
Seq2    LVPGPWGRLCRDWDQMFAFAQRHVERR--EAEAAMRNGGQPEKDLESGAHLTHFLFREEL
                250       260       270            280       290       300

                  310       320       330       340       350
Seq1    PLTSVYSNVTELLLAGVDTISSTLSWSLYELSRHPDVQTALRDEVLSVMKDRS--VPQAS
        :  :. .::::::::::::.:.::::.::::::::.:::::..:. ....  :   :.:.
Seq2    PAQSILGNVTELLLAGVDTVSNTLSWALYELSRHPEVQTALHSEITAALSPGSSAYPSAT
                  310       320       330       340       350       360

             360       370       380       390       400       410
Seq1    DVAAMPLLKAVVKEILRLYPVIPANARVINKDIEVGGYVIPKNTLITLCHYATSRDPQQF
         .. .::::::::.:::::.:.:.:: .:::.:: :.:::::::.:::::::::: ::
Seq2    VLSQLPLLKAVVKEVLRLYPVVPGNSRVPDKDIHVGDYIIPKNTLVTLCHYATSRDPAQF
                  370       380       390       400     * 410       420

             420       430       440       450       460       470
Seq1    RDPDSFRPQRWGDRSDRSHPYATVPFGVGKRSCIGRRIAELEVYLALSRILMHFTMEPVR
         .:.:::: ::  ..   ::.:..::: ::::.:::.::::. .::..:: :: ..:
Seq2    PEPNSFRPARWLGEGPTPHPFASLPFGFGKRSCMGRRLAELELQMALAQILTHFEVQPEP
                  430       440       450       460       470       480

        480       490       500
Seq1    ENDTVHPMTRTLLVPERQIDLRFTER
            :.: :::.::::.:.:.: .:
Seq2    GAAPVRPKTRTVLVPERSINLQFLDR
                  490       500
```

Figure 2. Amino acid sequence comparison between zebrafish Cyp27b1 and human CYP27B1

The amino acid sequence for Cyp27b1 was compared with human CYP27B1 using NCBI BLAST. Heme binding domain was marked in bold. Ferredoxin binding domain was marked with underline. Q66 and T408 (bold and asterik) have been identified as residues found mutated in human type I rickets suggesting functionally significant amino acids. The cDNA sequence has been deposited to Genbank (KM262796).

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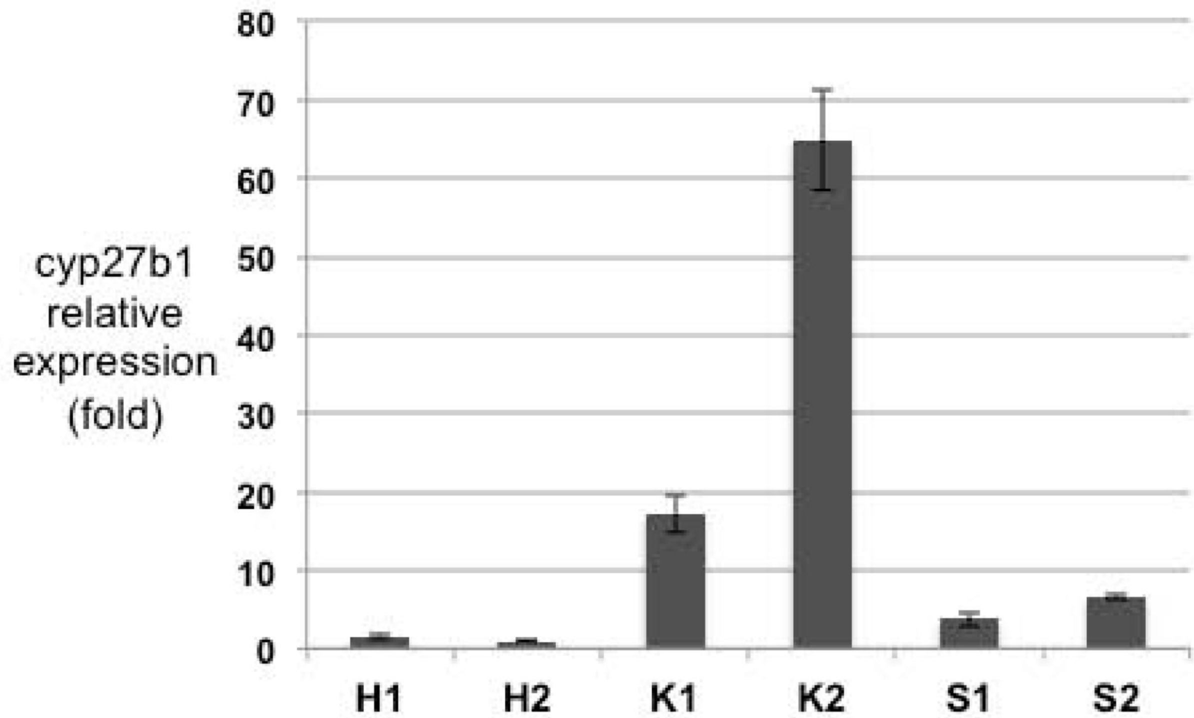

Figure 3. Expression of zebrafish *cyp27b1* in kidney, spleen and heart

Quantitative real-time PCR was conducted on cDNA prepared from heart (H1, H2) spleen (S1, S2) and kidney (K1, K2). The graphs display the data from triplicate qPCR assessments of two independent preparations of the respective organs. The data is presented as relative fold expression with H2 (lowest expression sample) set to one-fold and error bars ± SD.

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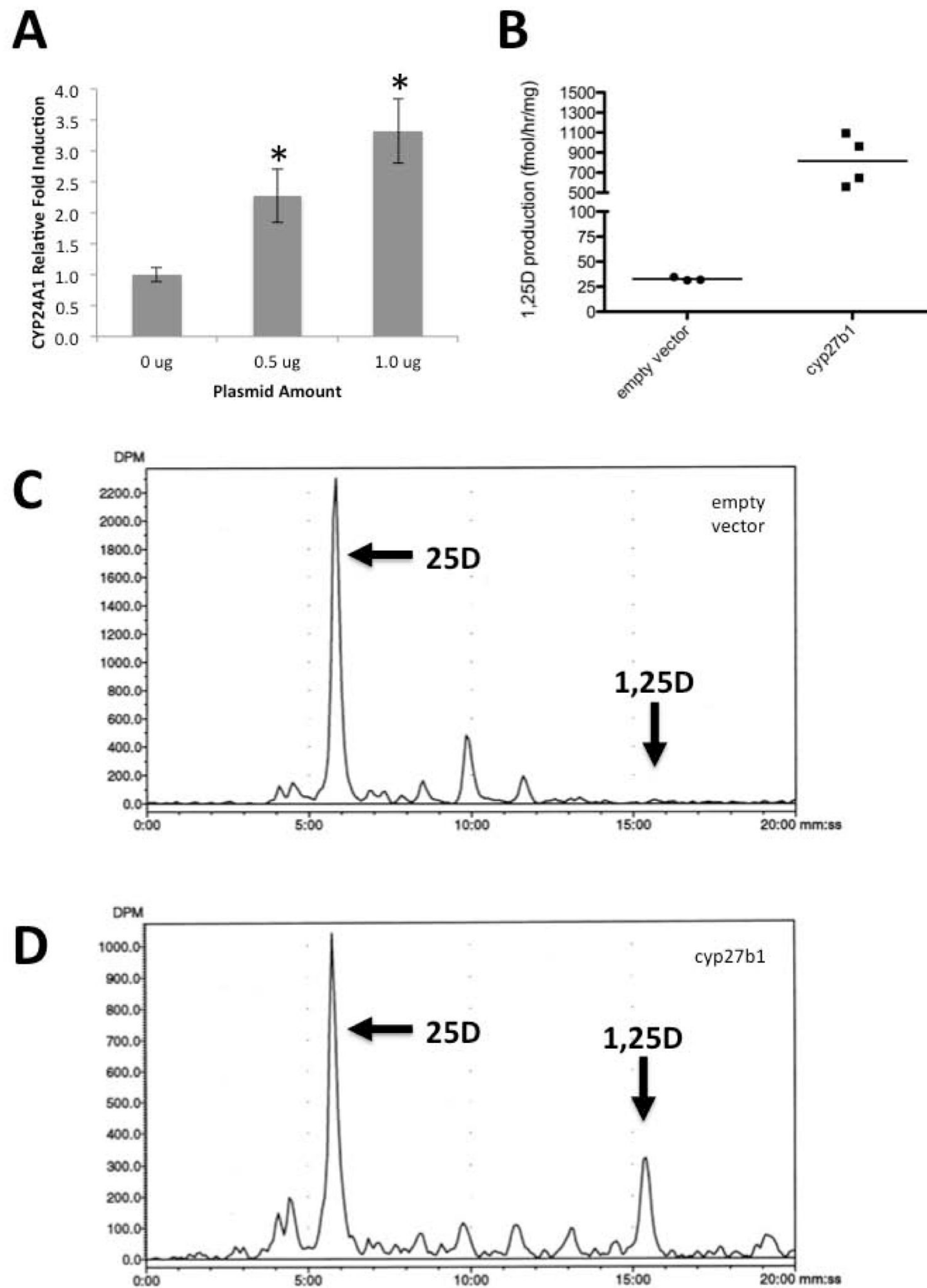

Figure 4. Zebrafish *cyp27b1* cDNA encodes a functional 25-hydroxyvitamin D-1α-hydroxylase activity

A) HKC-8, a human proximal tubule kidney cell line, was transfected with indicated amounts of pcDNA3.1-z*cyp27b1* expression plasmid or empty vector. The resulting cells were incubated with vehicle (0.1% ethanol) or 100 nM 25D for six hours. qRT-PCR analysis was used to quantify expression of mRNA for the 1,25D-VDR-induced catabolic enzyme 24-hydroxylase (*CYP24A1*) in human HKC-8 cells. Data are expressed as fold-induction of *CYP24A1* expression relative to 100 nM 25D incubated empty vector control cells set to a value of one. Data are shown as mean ± SE for n=3 replicates; * $P < 0.05$. B) HKC-8

transfected with plasmid expressing zebrafish *cyp27b1* or empty vector was analyzed by HPLC for $1,25D_3$ synthesis after two-hour incubation with 300,000 cpm ^3H-$25D_3$. ^3H-$1,25D_3$ production was calculated and normalized to mg protein per sample and expressed as fmol/mg/hr. The graph displays the average of N=3 (empty vector) and N=4 (cyp27b1) HPLC runs. Representative traces are shown of analysis of HKC-8 transfected with C) pcDNA3.1 empty vector or D) pcDNA3.1-*zcyp27b1*.

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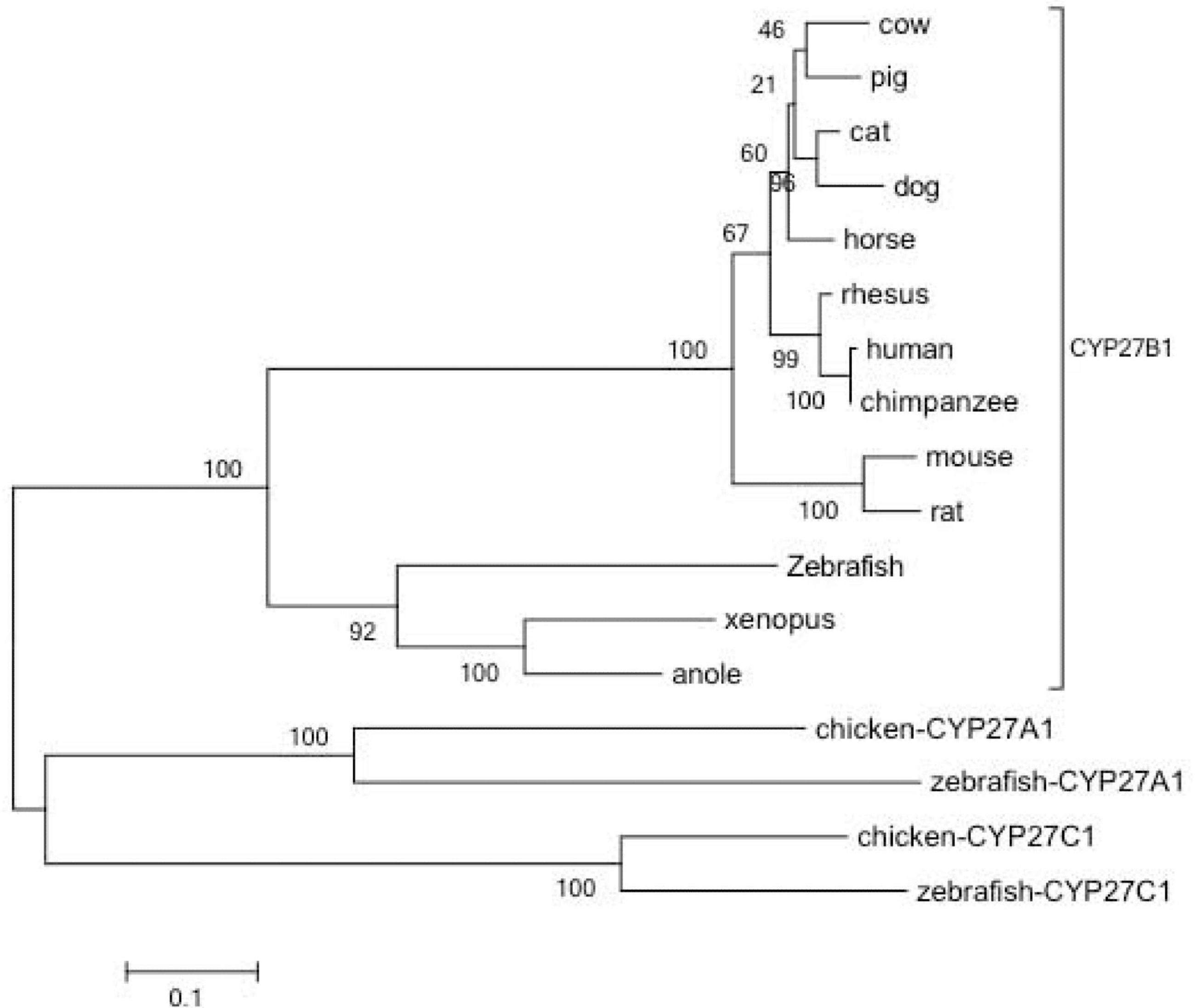

Figure. 5. Phylogenetic analysis of CYP27B1 found in various species

CYP27B1 evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood (-8036.2717) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 17 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 487 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. Zebrafish Cyp27a1 and Cyp27c1 were included as

outgroups. CYP27A1 and CYP27C1 from chicken were included since no CYP27B1 was found for that species.

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Table 1

Oligonucleotide primer sequences used for cloning

Cloning Step	Oligo name and sequence
Middle fragment PCR	(1F) 5′-CATGTGGAAGGCCAGTTTCG-3′ (4R) 5′-TACACCTCCAGCTCAGCGAT-3′
Nested 5′ RACE PCR Reaction 1	Forward 5′ RACE outer from Ambion 5′-GCTGATGGCGATGAATGAACACTG-3′ (2R) 5′-GGTCAGCAGAGTCATCACGA-3′
Nested 5′ RACE PCR Reaction 2	Forward 5′ RACE inner from Ambion 5′-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3′ (1R) 5′-CGAAACTGGCCTTCCACATG-3′
Nested 3′ RACE PCR Reaction 1	(3F) 5′-CACGATCTCCAGCACACTGT-3′ Reverse 3′ RACE outer from Ambion 5′-GCGAGCACAGAATTAATACGACT-3′
Nested 3′ RACE PCR Reaction 2	(4F) 5′-ATCGCTGAGCTGGAGGTGTA-3′ Reverse 3′ RACE inner from Ambion 5′-CGCGGATCCGAATTAATACGACTCACTATAGG-3′
Splice overlap Extension PCR 5′ RACE + Middle fragment as template	Forward 5′ RACE inner from Ambion 5′-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3′ (4R) 5′-TACACCTCCAGCTCAGCGAT-3′
Splice overlap Extension PCR Middle fragment + 3′ RACE as template	(1F) 5′-CATGTGGAAGGCCAGTTTCG-3′ Reverse 3′ RACE inner from Ambion 5′-CGCGGATCCGAATTAATACGACTCACTATAGG-3′