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Characterisation of expression patterns and functional role of Cactin in early zebrafish development

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ABSTRACT

The immune system of teleost zebrafish (*Danio rerio*) shows high similarity to mammalian counterparts sharing many innate immune components including Toll-Like Receptors (TLRs), cytokines, chemokines and complement molecules. As in mammals, zebrafish also contains the transcription factor NF-κB that plays dualist roles in innate immunity and early development. Indeed NF-κB members are expressed in different temporal patterns during the early stages of zebrafish embryogenesis indicating that each molecule is involved in specific developmental events. In the present study we employ zebrafish as a model to characterise the expression pattern and role of a novel NF-κB regulator, termed Cactin, in early development. Cactin was first characterised in *Drosophila* as a new member of the Rel pathway that could affect the generation of dorsal–ventral polarity. To explore the potential developmental role of Cactin in zebrafish, we initially investigated its expression pattern and functional role during early embryonic developmental stages. We detect Cactin expression at all stages of early development and knockdown of Cactin by specific morpholino antisense oligonucleotides causes developmental abnormalities manifested by an overall dysmorphic cellular organisation. These results indicate that Cactin has been highly conserved during evolution and plays a key role in early embryonic development.

1. Results and discussion

The transcription factor NF-κB plays a key role in regulating the innate and adaptive arms of the immune system (Hayden et al., 2006; Vallabhapurapu and Karin, 2009). However members of the NF-κB family have also been demonstrated to act as important regulators of early *Drosophila* development (Govind, 1999). Such dualist roles for NF-κB in immunity and development have led to intense investigation on the signalling pathways that regulate its activation. Inhibitory roles have been described for I-κB proteins that bind directly to NF-κB subunits and block activation of the latter by sequestering them in the cytoplasm and inhibiting their DNA-binding capacity (Moynagh, 2005). Various immune and developmental stimuli activate NF-κB by triggering signalling cascades that culminate in phosphorylation of I-κB proteins leading to their ubiquitination and proteasome-mediated degradation. The proteolysis of I-κBs allows for nuclear translocation of NF-κB and its activation of transcription of a plethora of genes including ones that promote the generation of dorso-ventral polarity in early development. Given the highly conserved nature of the NF-κB/I-κB families *Drosophila* has served as a highly valuable model organism in defining the early developmental role of NF-κB (Govind, 1999; Minakhina and Steward, 2006). Thus *dorsal*, the NF-κB homologue in *Drosophila*, is regulated by the I-κB family member Cactus and the activation of dorsal drives dorso-ventral polarity formation in developing *Drosophila*. Whilst many of the regulators of NF-κB have been well studied there undoubtedly remains less well characterised modulators of this important developmental pathway. One such molecule is Cactin. The latter was initially identified in *Drosophila* as a novel Cactus-interacting protein by using Cactus as bait in a yeast-two hybrid screen (Lin et al., 2000). *Drosophila* Cactin was shown to be maternally inherited and its over-expression furthered the Cactus mutant phenotype in *Drosophila* by enhancing embryonic lethality and ventralisation (Lin et al., 2000). The latter study implicates Cactin in the dorsal–ventral pathway and suggests that it positively regulates dorsal function. However no studies have since characterised the developmental role of Cactin and there is no existing data on its function in higher organisms. In the present study we employ zebrafish as a model organism to define for the first time the role of Cactin in vertebrate development. Zebrafish is an especially suitable model for evaluating the importance of Cactin as a novel regulator of NF-κB in development given that NF-κB/I-κB members have been previously characterised in zebrafish with inhibition of NF-κB interfering with notochord differentiation and

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generating no tail-like embryos (Correa et al., 2004). Given this important role for NF-κB in zebrafish development and the previous characterisation of Cactin as a protein binding partner for the I-κB homologue Cactus we characterised the expression patterns and functional role of zebrafish Cactin in early development.

1.1. Cloning and bioinformatic analysis of Cactin in zebrafish

Zebrafish is a genetically tractable model organism, since the entire genome has been sequenced and assembled and this facilitates the assignment of biological gene function (Meeker and Trede, 2008). The known human Cactin sequence was used to search zebrafish genomic sequence data using the BLAST program in the Ensembl Genome database. This search uncovered a zebrafish Cactin orthologue on chromosome 2. The zCactin gene spans about 13 kb (kilobases), is composed of 10 exons and is predicted to encode a protein of 771 amino acid (Archive Ensembl GenBank Accession Number LOC567213; see http://oct2007.archive.ensembl.org/Danio_rerio/transview?transcript=ENSDART00000083710&db=core). In order to examine the evolutionary relationships between Cactin orthologues across an extended taxonomic range, a multiple sequence alignment was constructed (see Supplementary figure). Cactin is highly conserved across species (Fig. 1A). From the multiple alignment it is clear that the C-terminal region of the protein has undergone selective pressure and is highly conserved across the species (Supplementary figure). With the exception of the N-terminal region there is a remarkable overall degree of conservation among the diverse organisms examined indicative of a key role(s) for the protein. A phylogenetic alignment of Cactin orthologues was also generated using ClustalW database (Fig. 1B). Cactin is found throughout eukaryotes in different multicellular animals. Despite the genome-amplification events at the base of vertebrate evolution only a single copy of the Cactin gene is found in the zebrafish genome.

1.2. Expression of Cactin in early embryonic developmental stages

The expression patterns of zCactin were next investigated with RT-PCR analysis being performed to characterise expression levels of fully spliced mRNA at various stages of development (Fig. 2A). zCactin mRNA transcript was observed at all stages of early development. Maternal zCactin transcript is also present, since expression is already detected at the 8-cell stage, which is prior to the onset of zygotic gene expression (van der Sar et al., 2006). Interestingly this correlates with the maternal expression of upstream regulators of NF-κB such as Toll-like receptors and associated adaptor proteins (van der Sar et al., 2006). To determine the spatial and temporal expression of zCactin mRNA in embryonic developmental stages whole mount *in situ* hybridisation was carried out (Fig. 2B). Embryos were probed with DIG-labelled antisense and sense zCactin RNA. zCactin shows extensive expression from the 8-cell stage to the 48 hpf. zCactin expression is not spatially restricted at the 8-

A

Sequence 1: Human	758 aa
Sequence 2: Mouse	772 aa
Sequence 3: Xenopus	604 aa
Sequence 4: Zebrafish	771 aa
Sequence 5: Drosophila	762 aa
Sequence 6: Caenorhabditis	667 aa
Sequences (1:2) Aligned	Identity: 87%
Sequences (1:3) Aligned	Identity: 80%
Sequences (1:4) Aligned	Identity: 81%
Sequences (1:5) Aligned	Identity: 50%
Sequences (1:6) Aligned	Identity: 38%
Sequences (2:3) Aligned	Identity: 79%
Sequences (2:4) Aligned	Identity: 80%
Sequences (2:5) Aligned	Identity: 50%
Sequences (2:6) Aligned	Identity: 38%
Sequences (3:4) Aligned	Identity: 76%
Sequences (3:5) Aligned	Identity: 52%
Sequences (3:6) Aligned	Identity: 67%
Sequences (4:5) Aligned	Identity: 52%
Sequences (4:6) Aligned	Identity: 38%
Sequences (5:6) Aligned	Identity: 36%

B

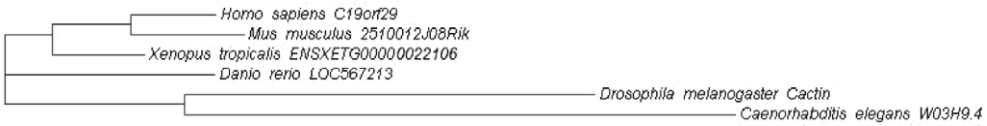

Fig. 1. Sequence and phylogeny analysis of Cactin. (A) The percentage identities for Cactin sequences between species pairs are indicated. Sequences were compared using BLASTP. (B) The phylogenetic alignment of protein sequences from Cactin orthologues was generated using ClustalW. The branch length is proportional to the amount of inferred evolutionary change.