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# NBP, a zebrafish homolog of human Kank3, is a novel Numb interactor essential for epidermal integrity and neurulation

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ABSTRACT

Numb is an adaptor protein implicated in diverse basic cellular processes. Using the yeast-two hybrid system we isolated a novel Numb interactor in zebrafish called NBP which is an ortholog of human renal tumor suppressor Kank. NBP interacts with the PTB domain of Numb through a region well conserved among vertebrate Kanks containing the NGGY sequence. Similar *NBP* and *Numb* morphant phenotype such as impaired convergence and extension movements during gastrulation, neurulation and epidermis defects and enhanced phenotypic aberrations in double morphants suggest that the genes interact genetically. We demonstrate that the expression of *NBP* undergoes quantitative and qualitative changes during embryogenesis and that the protein accumulates at the cell periphery to sites of cell–cell contact during gastrulation and later in development it concentrates at the basal poles of differentiated cells. These findings imply a possible role of NBP in establishing and maintaining cell adhesion and tissue integrity.



## Introduction

*Numb* was originally identified as a gene required for normal development of the peripheral nervous system in *Drosophila* (Uemura et al., 1989). Later on, *Numb*, encoding several splicing isoforms, and the related *Numblike*, were isolated in vertebrates (Yan, 2009 for review). In the last years, plenty of studies in *Drosophila* and mammals have demonstrated the importance of Numbs in different fields such as developmental neurobiology, cancer biology and neurodegenerative diseases and at cellular level, their implication in regulating cell fate determination, dendritic spine development, endocytosis, protein recycling, cell cycle and proteosomal protein degradation (reviewed by Gulino et al., 2009). Despite the fact that the precise biochemical role of Numbs in diverse cellular processes is mostly waiting to be uncovered, it becomes evident that they may act as adaptors facilitating the formation of multi-protein complexes. The majority of interacting partners isolated to date act either like Numb-associated E3 ligases participating in endocytosis or they undergo endocytosis and recycling mediated by Numbs (Gulino et al., 2009). No Numb-interacting partner has been identified yet in lower vertebrates. In the current study, we isolate in zebrafish a new interactor of both Numb splicing variants, NBP (Numb-Binding Protein) which is an ortholog of human Kank3 (known also as ankyrin repeat domain 47, ANKRD47). Kank3, together with Kank1 (ANKRD15), Kank2 (ANKRD25) and Kank4 (AMKRD38), belongs to the Kank (Kidney ankyrin repeat-containing protein) family defined recently (Kakinuma et al., 2009; Zhu et al., 2008). There is limited information about the function of Kanks yet and the only studies on vertebrates Kanks are coming from cell cultures. The human Kank1 was originally found as a tumor suppressor of renal cell carcinoma (Sarkar et al., 2002). Its deletion is connected also with familiar cerebral palsy (Lerer et al., 2005). Findings that Kank1 in mammalian cells may regulate the actin cytoskeleton by interacting with both IRSp53 and 14-3-3 proteins (Kakinuma et al., 2008; Roy et al., 2009) outline the importance of Kank proteins in various cellular pathways. The human Kank2 (called also SIP) binds steroid receptor coactivators and by their sequestering in the cytoplasm participates in transcription regulation (Zhang et al., 2007). Overexpression of the Kank2 mouse homolog in chicken embryo fibroblasts and human osteosarcoma cells increases the cell proliferation rate and induces their

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oncogenic phenotype (Harada et al., 2005). VAB-19, the ortholog of Kank proteins in *Caenorhabditis elegans*, is involved in epidermis cell elongation also through the actin cytoskeleton (Ding et al., 2003). CG10249, the single ortholog of Kank proteins in *Drosophila*, has been identified in the screening for genes required for normal neuronal cells development (Sepp et al., 2008). There is no information on the function of Kank3 and Kank4 in vertebrates and no Kank protein has been characterized in lower vertebrates. In the present study, we reveal that zebrafish NBP interacts with Numbs through a novel motif well conserved in all vertebrates Kanks. We demonstrate that NBP is a protein with nonredundant function during zebrafish embryogenesis as its deficiency causes serious embryo defects and lethality within 48 h. Cell biology and genetic approaches reveal a putative role of NBP in establishing and/or maintaining cell adhesion and polarity.

## Material and methods

### *Fish strains and staging*

Zebrafish embryos were obtained from spontaneous spawning and staged according to Kimmel et al. (1995). Adult fish were kept at 28.5 °C on a 14 h light/10 h dark cycle.

### *Yeast two-hybrid assay, cDNA library and 5′-RACE*

The HybridZAP® 2.1 two-hybrid system (Stratagene) was used to screen a zebrafish cDNA library with either the PTBL domain of zebrafish Numb1 (AY583653.1) or the PTBS domain of Numb3 (AY583654.1) as baits. For details on cDNA library construction, plasmids preparation and primers sequences see Supplemental Material and Methods. Twenty and twelve million colonies, respectively, were tested in $His^+$ and X-Gal assays and not-self-activating clones were sequenced. For further studies, we selected the gene which we named *NBP*. The missing 5′-end of the cDNA was amplified by 5′-RACE System (Life Technologies). For quantitative analysis of protein binding and for identification of interaction domains we used the yeast two-hybrid assay and fluorometry method (for detailed procedure, plasmids construction and primers sequences see Supplemental Material and Methods).

### *Phylogeny, domain and protein structure analysis*

The NBP amino acid sequence predicted from the cDNA obtained in the present study was used as a query for searching against the non redundant protein NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Full-length protein sequences with structures resembling NBP were aligned by ClustalW XXL (http://www.ch.embnet.org/software/ClustalW-XXL.html) and phylogram was displayed by TreeView X program. Alignment of *Danio*-NBP and representative sequences from the ANKRD47 subfamily (Homo, Mus and Taeniopygia) and alignment of putative Numb-binding regions were performed by ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). For programs used for searching conserved domains, motives and localization predictions see Supplemental Material and Methods.

### *In vitro binding procedures and Western blotting*

The fusion proteins were expressed in *Escherichia coli* BL21 (for vectors preparation see Supplemental Material and Methods) and isolated with PBS (pH 8.1) buffer supplemented with 1 mM PMSF, 1 mM DTT and 0.1% Triton X-100. His-fusion proteins were purified using TALON metal affinity resin (Clontech), GST-fusion proteins with Glutathione Sepharose 4B beads (GE Healthcare). Supernatants of His- and GST-fusion proteins (10 μg each) were mixed with 1 μg of either GST-fusion protein coupled to Glutathione Sepharose 4B beads or His-fusion protein coupled to TALON metal affinity resin, respectively. Beads and resin were washed with PBS buffer, resuspended in SDS-Laemmli sample buffer and used for Western blotting. Proteins from 24 hpf wild-type and morphants were isolated by lysis buffer (50 mM Tris–HCl pH 7.6, 1% Nonidet P-40, 1 mM PMSF, 2 mM DTT, 10% glycerol and 0,5%, Protease Inhibitor Cocktail, Sigma P 8340) and used for Western blotting (antibodies and procedure are described in Supplemental Material and Methods).

### *Creation of knockdown embryos and EGTA treatment*

NBP and Numbs knockdown embryo lines were generated using the antisense morpholino oligonucleotides (MOs, Gene Tools) technology. The following MOs were used: *NBP* MO (5′-TGCACAGATTGGGTCATTTTATGTA-3′), the NBP mismatch MO (*NBP* MOC, 5′-TGCAGACATTGCGTGATTTTATCTA-3′), *Numb* MO (5′-TCAGACGAACTGCTCGTATCCACAC-3′) and the Numb mismatch MO (*Numb* MOC, 5′-TCACACCAACTCCTCCTATCGACAC-3′). *NBP* MO and *Numb* MO were injected in 5 nl Danieau's buffer (Nasevicius and Ekker, 2000) at 100–600 μM concentration into one-four-cell stage embryos to create single or, in combination, double knockdown morphants. For mRNA rescue experiments, Danieau's buffer containing 200 ng/μl NBP mRNA (see Supplemental Material and Methods) was injected along with 600 μM *NBP* MO. Control experiments were performed with *NBP* MOC and *Numb* MOC at 600 μM. For EGTA treatment, embryos were kept with the chorion and briefly washed with calcium–magnesium-free medium followed by incubation for 1 h in 20 mM EGTA.

### *Bimolecular fluorescence complementation analyses (BiFC), GFP technology and immunohystochemistry*

Capped mRNAs for BiFC (Hu et al., 2002) and GFP technologies were amplified in vitro using the mMessage mMachine kit (Ambion) from the Numbs-HA-YC and NBP-cMyc-YN plasmids, and from the pCS2 + EGFP-NBP plasmid, respectively (for plasmid construction see Supplemental Material and Methods). 1 ng of RNAs in 5 nl aliquots were injected into one-four-cell stage wild-type embryos. For immunohistochemistry, 24 hpf embryos used in the BiFC assay were fixed with 4% formaldehyde in MTSB buffer, dehydrated, embedded in Steedman wax and sectioned (Vitha et al., 2001). Sections were probed with primary antibodies for 1 h, washed and treated for 1 h with appropriate secondary antibodies (antibodies are listed in Supplemental Material and Methods).

### *RT-PCR, in situ hybridization and TUNEL assay*

Total RNA was extracted with Trizol (Invitrogen). First-strand DNA synthesis was performed with the Invitrogen Superscript first-strand synthesis system. RT-PCR (30 cycles) was carried out using either NBP1 and NBP2 primers generating a 1730 bp fragment of NBP or β actin1 and β actin2 primers producing a β actin fragment as a control (primer sequences are in Supplemental Table S1). Whole-mount in situ hybridizations were performed as described (Thisse et al., 1993). RNA probes and primer sequences are present in Supplemental Material and Methods. For detailed histological studies, probed embryos were fixed by 4% formaldehyde and embedded in Araldite (Serva) and sectioned. For TUNEL assay, 24 hpf embryos injected with either 1 mM *NBP* MO or *Numbs* MO were fixed with 4% formaldehyde for 4 h, permeabilized with acetone at −20 °C, washed twice for 10 min with PBS (pH 7.2) supplemented with 0.001% Triton X-100 and 0.1% sodium citrate and labeled for apoptotic cells by In situ Cell Death Detection Kit (Roche).