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RESEARCH ARTICLE

Nusap1 is essential for neural crest cell migration in zebrafish

Jing Nie[1,3], Hua Wang[2], Fuchu He[1,3,4] ✉, Huizhe Huang[1,2] ✉

[1] Department of Biology Sciences and Biotechnology, Tsinghua University, Beijing 100084, China
[2] School of Life Sciences, Xiamen University, Xiamen 361005, China
[3] State Key Laboratory of Proteomics, Beijing Proteome Research Center, Beijing Institute of Radiation Medicine, Beijing 100850, China
[4] Institutes of Biomedical Sciences, Fudan University, Shanghai 200032, China
✉ Correspondence: huangh@xmu.edu.cn (Huang H.), hefc@nic.bmi.ac.cn (He F.)
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ABSTRACT

Microtubules play important roles in mitotic spindle assembly and chromosome segregation to maintain normal cell cycle progression. A number of microtubule-associated proteins have been identified in epithelial and neural cell cultures; however, their physiological significance is not well characterized due to the lack of appropriate *in vivo* animal models. Nucleolar spindle-associated protein (NuSAP) is a microtubule-binding protein and is reported to be involved in mitosis by cell culture studies. In this report, we identified the zebrafish homologue of human NuSAP and investigated its expression profile and functions. Using *in situ* hybridization, we demonstrated that transcripts of zebrafish *nusap1* are specifically expressed in the retina, forebrain, hindbrain and neural crest. When the *in vivo* expression of *nusap1* was knocked down through antisense oligonucleotide morpholino technology, the morphants of *nusap1* showed impaired morphogenesis in the trunk and yolk extension, implying the involvement of Nusap1 in cell migration. Mechanistic studies revealed that *nusap1* morphants have an altered expression pattern of neural crest markers *crestin* and *sox9b*, but normal expression of blood vessel and notochord markers *gata1* and *shh*. In addition, *nusap1* mRNA injection caused serious apoptosis in retina and hindbrain tissue, and these phenotypes can be rescued by co-injection of morpholino against *nusap1*. These observations not only suggest a role for Nusap1 in connecting apoptosis with cell migration, but also provide strong evidences that Nusap1 is potentially involved in morphogenesis in vertebrates.

KEYWORDS Nusap1, zebrafish, microtubule, apoptosis, migration, neural crest

INTRODUCTION

Cell cycle control has significant roles in multiple physiological processes, such as stem cell pluripotency, cell proliferation and differentiation, and embryonic morphogenesis (Vousden and Lu, 2002; Ornitz, 2005). Mitotic spindle assembly and chromosome segregation are the pivotal events for regulating the cell cycle, especially for mitosis phase progression. A number of scaffold proteins have been identified that associate with the mitotic spindle and play important roles in spindle assembly and cell cycle control. Examples of these proteins include γ-tubulin, NuMA, TPX2, dynactin and nucleolar spindle-associated protein (NuSAP) (Merdes et al., 1996; Hetzer et al., 2000). NuSAP is a well-known microtubule-associated protein. The homologues of human NuSAP have been reported in orangutan, dog, mouse and frog. The N-terminal box and C-terminal KEN box share the most protein sequence identity between amphibians and primates. Carmeliet's group found that NuSAP has altered expression during cell cycle progression at both mRNA and protein level (Raemaekers et al., 2003). Recent reports showed that NuSAP can be degraded by anaphase promoting complex/cyclosome formation (APC/C) via the ubiquitin/proteasome system (Li et al., 2007). The KEN box is recognized by APC/C-cdh1 or APC/C-cdc20, which controls

the protein level of NuSAP during cell cycle progression. However, the biological significance of the degradation or knockdown of NuSAP still remains unclear.

The GTPase Ran is essential for regulating nucleocytoplasmic transport in interphase cells and for organizing the spindle apparatus during mitosis (Yudin and Fainzilber, 2009). NuSAP is targeted by Ran GTPase to stabilize microtubule (Ribbeck et al., 2006). During mitosis, dissociated NuSAP is immobilized on chromatin and produces a high concentration of microtubules around chromatin (Ribbeck et al., 2007). Moreover, overexpression or knockdown of NuSAP results in mitotic spindle deficiency (Raemaekers et al., 2003). In spite of that these *in vitro* studies which support the involvement of NuSAP in linking microtubule with mitotic chromosomes during cell cycle regulation, the *in vivo* roles of NuSAP on cell fate and cell behaviors are largely unknown.

Here, we identified a novel homologue of NuSAP in zebrafish, which shares 36% amino acid sequence identity with human NuSAP. The transcripts were restrictively expressed in the brain, neural crest and blood island as shown by *in situ* hybridization. We used antisense oligonucleotide morpholino to knockdown *nusap1* mRNA in zebrafish and found that the *nusap1* morphants display impaired morphogenesis and defective cell migration in the trunk and yolk extension. These phenotypes can be rescued by co-injection of *nusap1* mRNA. Interestingly, injection of *nusap1* mRNA alone causes apoptosis in the eyes and hindbrain. These original phenotypes confirm the previous *in vitro* observations about NuSAP in regulating microtubule stabilization and mitosis, and further suggest the *in vivo* significance of Nusap1 in cell migration and cell cycle progression.

RESULTS

Cloning and identification of zebrafish *Nusap1* gene

By screening FGF-responsive genes in a zebrafish cDNA microarray, we classified 32 candidate ORF fragments into strong-, moderate- and weak-response groups to Fgf8 (Huang et al., 2007). Although *in situ* hybridization data suggested that Fgf8 increased *nusap1* transcription only slightly (Data not shown), we still focused on this gene for the interest in its functional relationship with human NuSAP. Sequencing of full length *nusap1* showed that its product was the same as that from a locus in zebrafish contig 13 (XP_695834.1) with the open reading frame (ORF) between position 121 and 1461. To determine the homology of NuSAP, protein sequences from different species were aligned using ClustalW software, and the evolutionary tree indicated that the primary sequence of Nusap1 is highly conserved among vertebrates. The identity of NuSAP between zebrafish and human is 36% (Fig. 1A), and zebrafish Nusap1 has highest homology to its frog orthologue (Fig. 1B).

Although phylogenetic analysis reveals that zebrafish Nusap1 branching is basal to other species and the mammalian Nusap1s are clustered into a separate group, several conserved markers surrounding zebrafish and human orthologues are arranged in the same orientation, including *Ndufaf1* (NADH dehydrogenase-ubiquinone 1 alpha subcomplex, assembly factor 1) and *Rtf1* (Paf1/RNA polymerase II complex component). A detailed scheme of gene positions surrounding Nusap1 is shown in Fig. 2, indicating the authentic orthologous relationship of zebrafish Nusap1 on chromosome 13 with human NuSAP on chromosome 15.

Expression profile of *nusap1*

To confirm that Nusap1 is expressed in zebrafish, its expression pattern was probed by whole mount *in situ* hybridization. As shown in Fig. 3, *nusap1* was present in single-cell embryos, suggesting that it is a maternal gene. The zygotic expression was ubiquitous detected from the 1024 cell stage to mid-gastrulation stage (Fig. 3B–D and 3D'), and the pattern in a salt and pepper manner implied its functions in cell cycle progression and proliferation by several experiences from large scale *in situ* screening work in other institutes (Herzog et al., 2003). After the somite formation, *nusap1* transcripts were detected in forebrain, hindbrain, neural crest, blood island and proliferative cells (Fig. 3E–F and 3F'). The same stages of embryo development were analyzed by a sense-control probe in parallel, and analysis of a 30% epiboly embryo is shown in panel H. *nusap1* expression gradually weakened after 24 hpf, and cannot be detected by the same method after 3 dpf (Fig. 3G). Consistently, RT-PCR also detected the expression of *nusap1* transcripts post fertilization in a gradually weakened manner (Fig. 3I).

Knockdown of *nusap1* causes cell migration defect in neural crest

Although *in vitro* evidence supports the roles of NuSAP in mitosis and microtubule stabilization (Ribbeck et al., 2006), the *in vivo* significance of Nusap1 function is still largely unknown. The unique temporal and spatial expression pattern of *nusap1* in zebrafish suggests that Nusap1 may have important roles *in vivo*. The cellular and genetic function of Nusap1 was further tested by morpholino knockdown. To specifically knockdown *nusap1* at the mRNA level, two ATG morpholinos were designed against its 5'-UTR and start codon regions respectively. As shown in Fig. 4A–C, microinjection of plasmid with N-terminal Nusap1 and GFP fusion resulted in highly mosaic GFP expression, while co-injection of Nusap1-GFP plasmid with *nusap1* MO1 efficiently inhibited the transient GFP expression. Consistently, semi-quantitative Western blot showed reduced GFP expression in *nusap1*

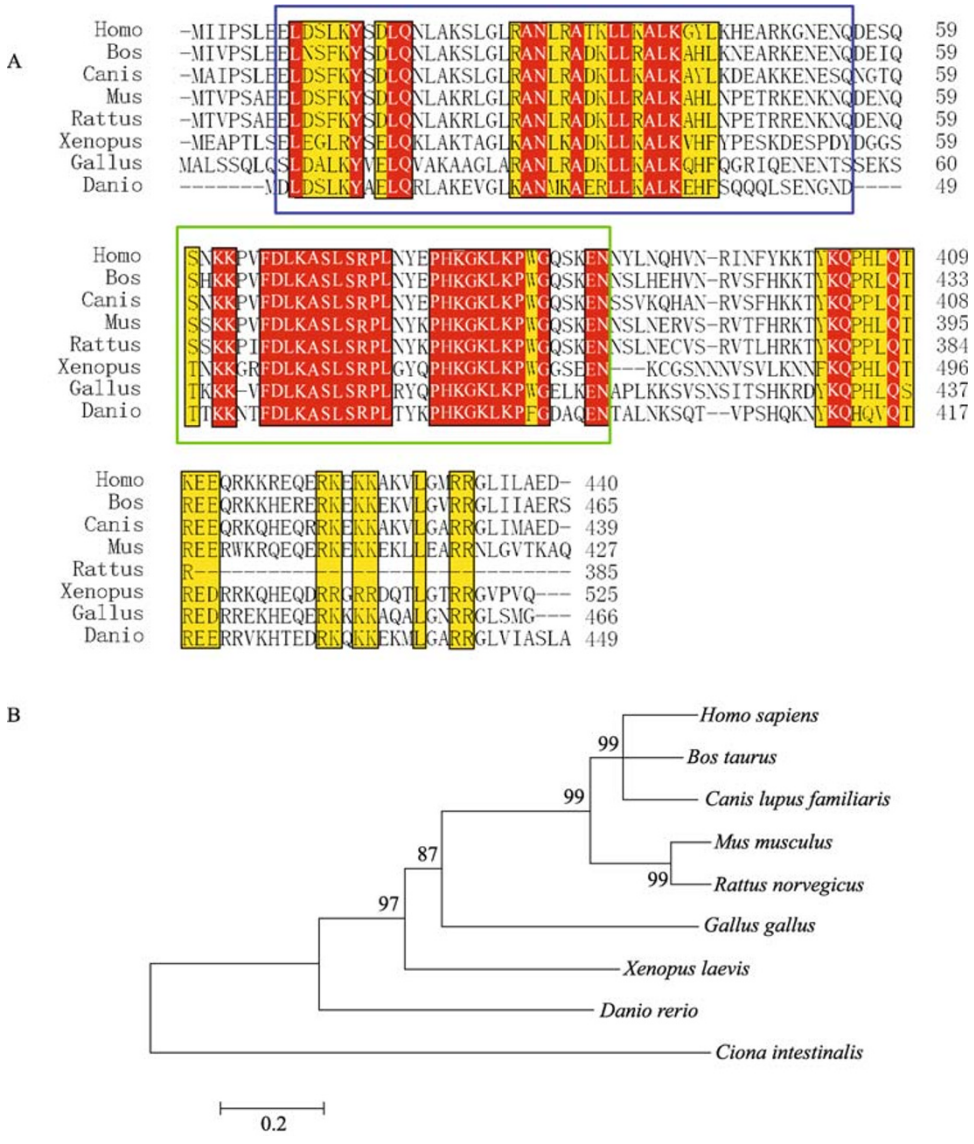

Figure 1. Bioinformatic and genetic relationship of Nusap1 proteins. (A) Alignment of Nusap1 amino acid sequences from fish to mammals. The N-terminal SAP box is indicated by the blue frame, and the C-terminal conserved region containing the KEN box is indicated by the green frame. (B) Nusap1 protein phylogenetic tree constructed from the following species: *Homo sapiens, Bos taurus, Canis lupus familiaris, Mus musculus, Rattus norvegicus, Gallus gallus, Xenopus laevis, Danio rerio* and *Ciona intestinalis*. The values of each node are confidence values derived from 1000 bootstraps resembling of the alignment data. The branch length in the tree is proportional to the number of amino acid substitutions, and the scale bar indicates 0.2 amino acid substitution per position in the sequences.

morphants (Fig. 4D). These results confirmed that *nusap1* MO1 specifically inhibited Nusap1 gene translation in a dose-dependent manner.

In comparison to the ctrMO injected embryos, the trunk of *nusap1* morphants were rougher and shorter, with a smaller and curving tail (Fig. 5A and 5B). TUNEL assay did not detect apparent apoptosis, and angiogenesis and myogenesis appeared normal in *nusap1* morphants (Supplemental Fig. 1). Consistent with the above phenotypes, *in situ* hybridization revealed that *nusap1* morphants have impaired expression patterns of *crestin* and *sox9b*, which are two markers for neural crest (Fig. 5C–F, 5C' and 5D'). Staining of neural crest cells and precursor of pigment cells with these two markers indicated a reduced distance of cell migration. However, the *nusap1* morphants and control embryos showed a similar mRNA expression level of these markers in whole embryos as measured by quantitative real-time PCR (Fig. 5G and 5H). These results implicate that Nusap1 is an

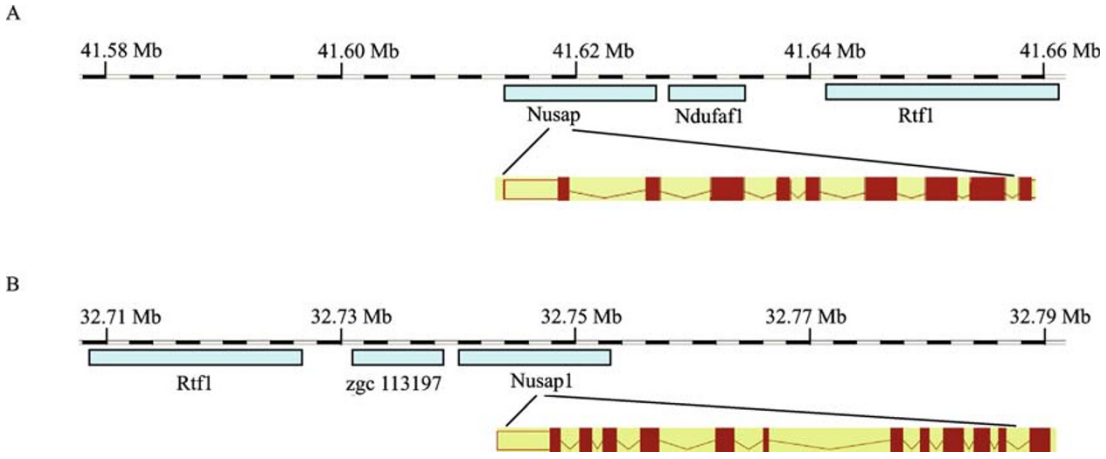

Figure 2. The chromotin orientation of genes around Nusap1. (A) The gene cluster in human chromosome 15 displayed by the Ensembl online program (version GRCh27). (B) The gene cluster in zebrafish chromosome 13 displayed by Ensembl online program (version Zv8). Zgc113197 indicates Ndufaf1 in zebrafish.

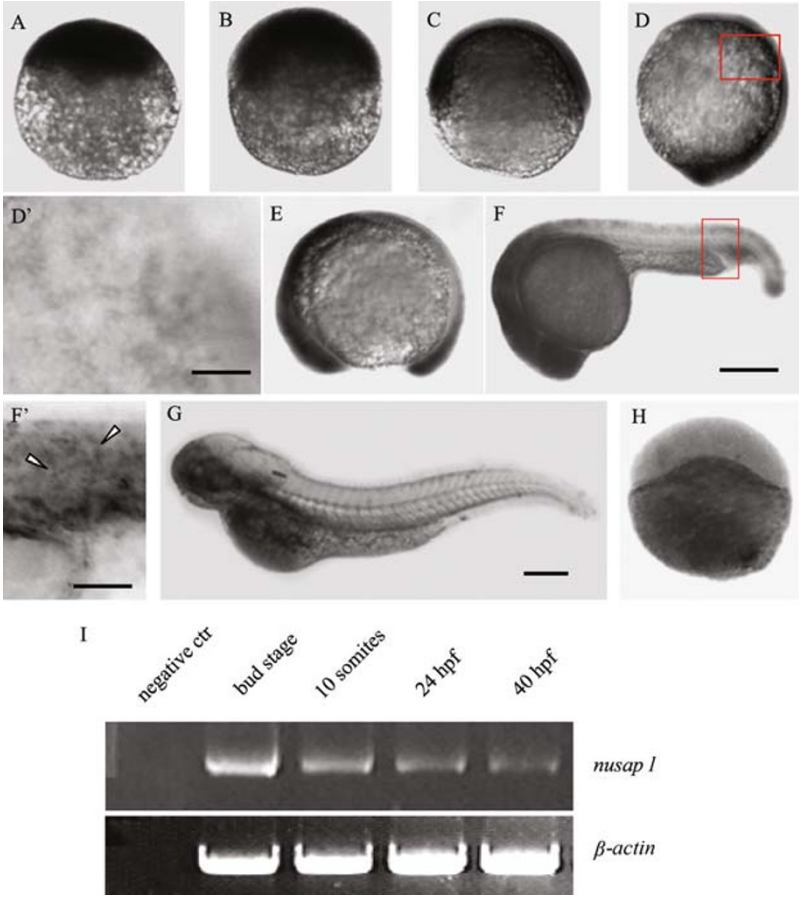

Figure 3. Expression profile of *nusap1* in zebrafish. *nusap1* transcripts were probed using *in situ* hybridization at the following stages: (A) One cell stage; (B) 30% epiboly stage; (C) 50% epiboly stage; (D) Bud stage; (E) 10 somites stage; (F) 24 hpf (scale bar indicates 0.5 mm in panels A–D, E, F and H); (G) 3 dpf (scale bar indicates 0.5 mm). Panels D' and F' were inserts from D and F, respectively. Scale bars indicated 100 μm. (H) Control assay using sense-RNA probe in parallel, and 30% epiboly embryo was listed as example. Embryos in panels E–G display a lateral view with anterior to left orientation; the others display a lateral view with animal pole to upward orientation.

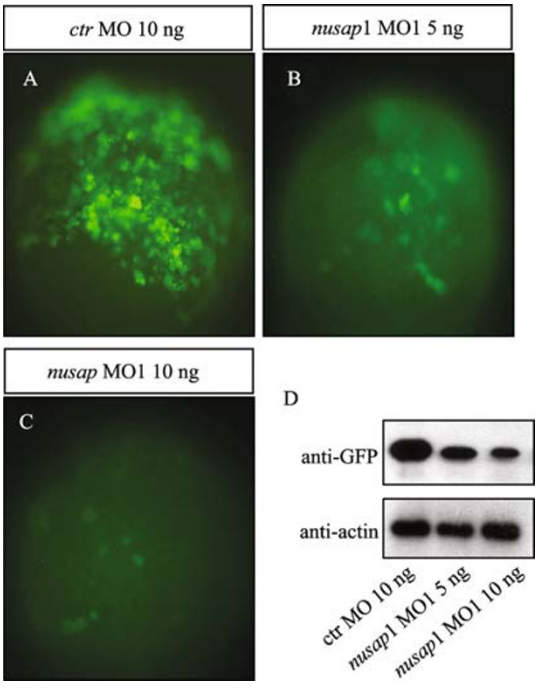

Figure 4. Specificity and efficiency test of morpholino against zebrafish *nusap1*. Fluorescent views of the embryos injected with Nusap1-GFP fusing plasmid. (A) Embryo co-injected with 10 ng ctrMO; (B) Embryo co-injected with 5 ng *nusap1* MO1; (C) Embryo co-injected with 10 ng *nusap1* MO1 (D) Semi-quantitative assay of embryo lysates Western blot. One hundred embryos were lysed using SDS buffer with Coomassie brilliant blue, and 20 μL of lysate was analyzed with an anti-GFP antibody, the half value of a loading control was detected by anti-β-actin antibody.

important regulator in neural crest development, and the differences in the *crestin* and *sox9b* expression patterns are possibly due to abnormal cell behaviors, such as cell migration, rather than the altered cell fate.

Overexpression of *nusap1* results in embryonic apoptosis

To further test the specificity and efficiency of MO, mRNA encoding the *nusap1* full length open reading frame was co-injected with MO (Fig. 6A–C). This overexpression can abrogate *nusap1* morphants, and interestingly, mRNA of *nusap1* alone caused drastic apoptosis (Fig. 6A). A whole mount TUNEL assay showed that apoptosis was concentrated in the retina and hindbrain (Fig. 6E and 6F). Statistics data revealed that the small tail caused by *nusap1* MO injection was also rescued by its overexpression (Fig. 6D). These observations imply that *nusap1* MO1 is able to rescue the apoptosis phenotype caused by *nusap1* over-expression.

DISCUSSION

Nusap1 is a novel microtubule-associated protein identified several years ago, and both its expression and distribution are regulated by cell cycle progression. The human NuSAP expression profile shows that it is mainly expressed in immune organs, including the thymus, bone marrow and fetal liver. Sequence analysis indicates that Nusap1 is conserved among vertebrates. So far, all characterized Nusap1s have been identified in vertebrates, from human to puffer fish. Only a similar member was found in *Ciona intestinalis* (XP_002127601). Thus, whether invertebrates contain Nusap1 or not is still unknown. Our on-going progress includes characterizing the expression of Nusap1 in sea squirts and amphioxuses, as well as, investigating its significance in CNS development and the evolution of Nusap1.

In this report, we used zebrafish as an animal model to investigate the expression and biological functions of Nusap1. As revealed by *in situ* hybridization analysis, zebrafish *nusap1* is a maternal transcript, and its zygotic expression can be detected in the salt and pepper pattern and decreases after two days, which is similar to the expression of human NuSAP. The temporal and spatial expression profile of *nusap1* suggests its potential roles in neural crest development and apoptosis regulation.

When the *nusap1* expression was up- or down-regulated by microinjection of mRNA or morpholino, respectively, development of zebrafish embryos was disrupted. This is consistent with the functions of human NuSAP—deletion or overexpression of which destroys the normal cell cycle progression (Raemaekers et al., 2003). Microtubule-associated proteins control the assembly of the mitotic spindle and chromosome segregation, and thus, their expressions are strictly and precisely regulated. The TUNEL assay we showed here had moderate signal treated by sub-lethal dose of UV activity. Recent paper investigated the relationship between cell cycle arrest and cell death by different environmental stress and different p53 phosphorylation mechanism (Li et al., 2009). It is possible that Nusap1 is involved in this mechanism.

The *nusap1* overexpression induced apoptosis, which often involves p53 activation. However co-injection of morpholino against zebrafish *p53* with *nusap1* mRNA failed to rescue the above phenotype (data not shown), suggesting that the apoptosis phenotype may be not caused by p53 activation (Robu et al., 2007). Nevertheless, due to the pivotal roles of p53 and its complicated post-translation modifications, we could not exclude that the functions of Nusap1 are independent of p53 signaling. Studies from clinical cases indicate that methionine stress induces increased toxicity in NuSAP and p53 double minus condition (Kokkinakis et al., 2005), implying the potential relationship between NuSAP with p53 and suggesting a further direction of Nusap1 as a tumor suppressor.

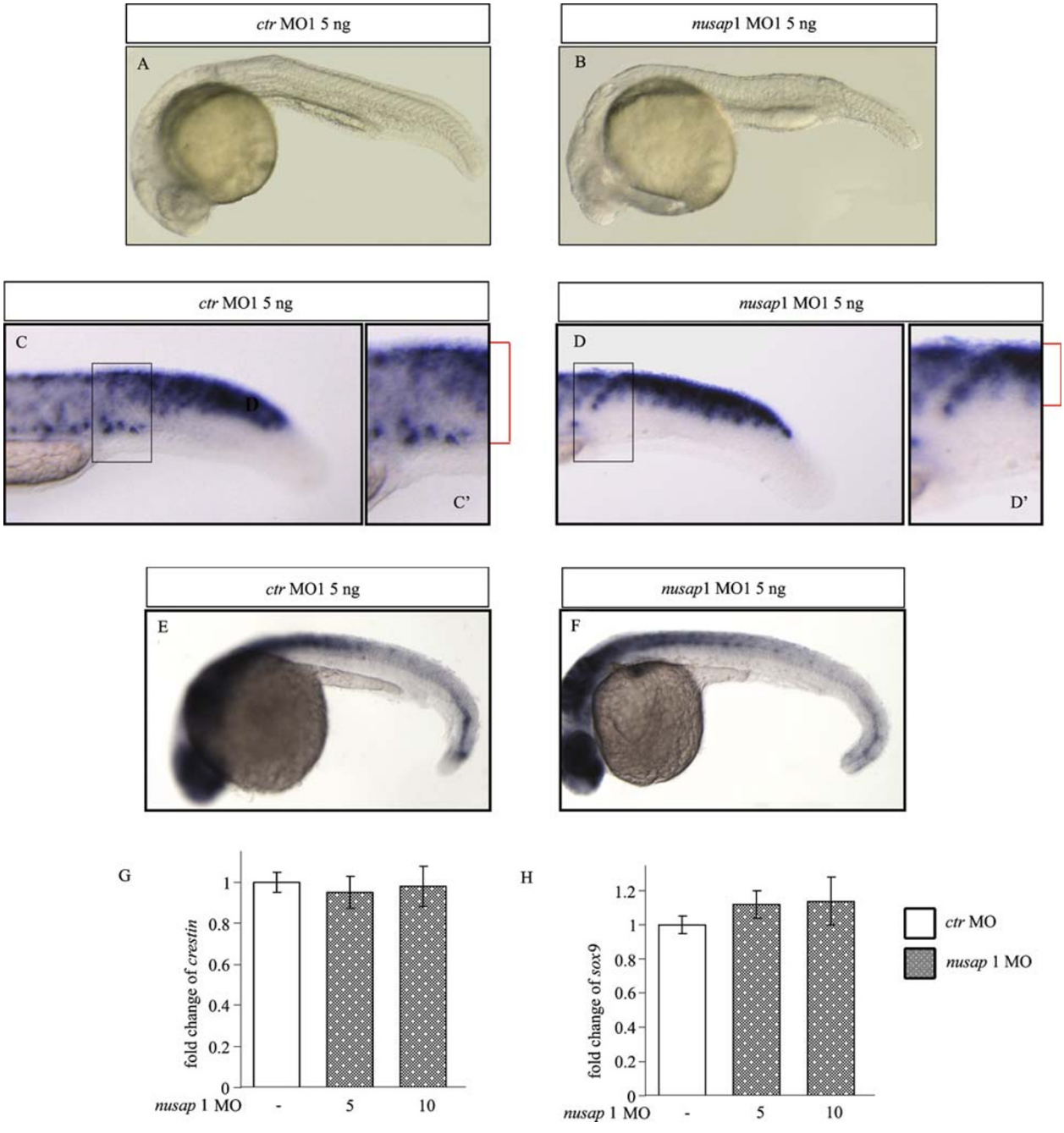

Figure 5. Impaired cell migration in *nusap1* morphants. (A) Live photo of embryo injected with 5 ng control MO; (B) Live photo of embryo injected with 5 ng *nusap1* MO1. (C–F) Expression of *crestin* (C, D) and *sox9b* (E, F) detected by *in situ* hybridization using anti-sense probes. Insertions in C' and D' emphasized a deficient neural crest in *nusap1* morphants. The red frames indicated the level of neural crest migration. All embryos were detected at 24 hpf. (G) The expression level of *crestin* in the indicated conditions was measured by qRT-PCR. (H) The expression level of *sox9b* in the indicated conditions was also measured by qRT-PCR.

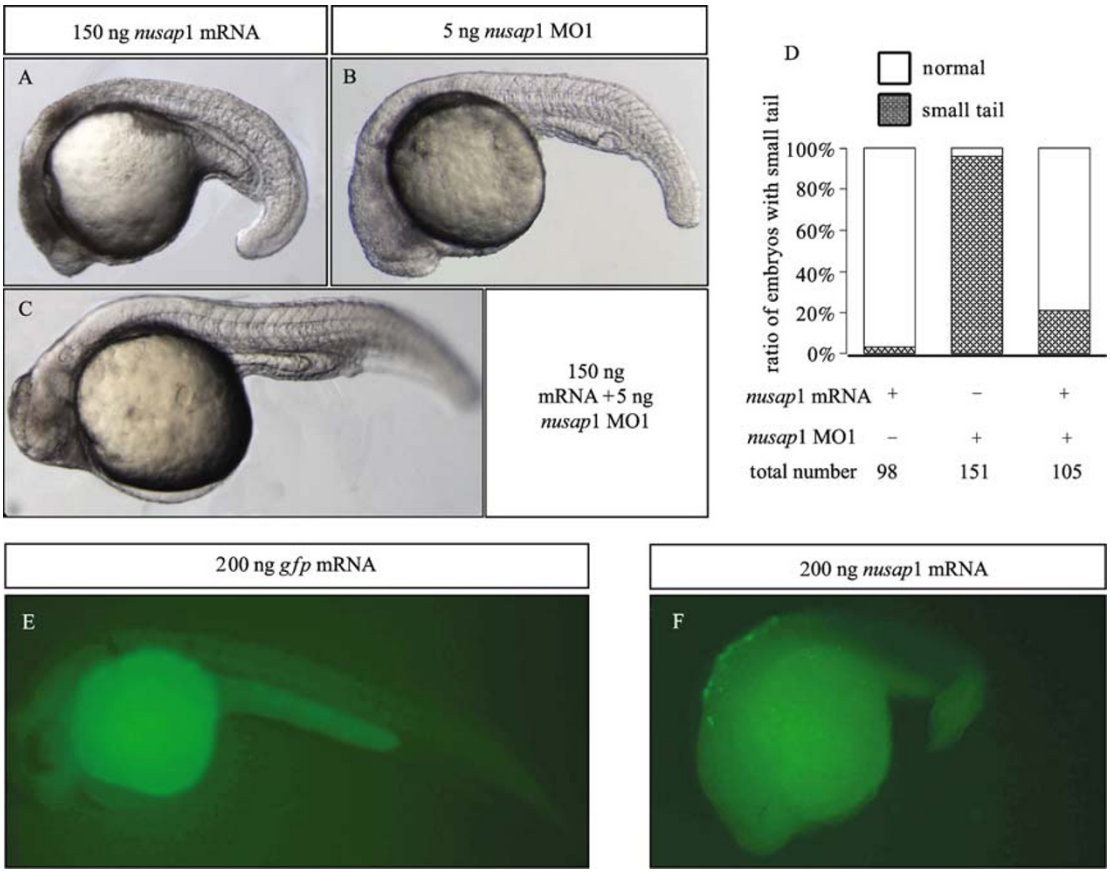

Figure 6. Apoptosis induced by overexpression of *nusap1*. (A) The apoptotic phenotype caused by injection with 150 ng of *nusap1* mRNA. The grey and dark tissue around hindbrain implied classic cell death by apoptosis. (B) The small tail phenotype caused by cell migration defect. (C) MO and mRNA co-injection abrogated both phenotypes on apoptosis and cell migration. All embryos are shown at 24 hpf, and statistics of each phenotypes is listed at panel (D). Apoptosis in GFP (E) and *nusap1* mRNA (F) injected embryos were determined by the fluorescent TUNEL assay and analyzed by confocal microscope. And last two panels were merged by bright and fluorescent channels using PHOTOSHOP (Adobe Company).

MATERIALS AND METHODS

Zebrafish maintenance and plasmid construction

The AB zebrafish strain was raised and kept under standard conditions at ~28.5°C. Embryos were staged according to Zebrafish Book. The coding sequence of zebrafish *nusap1* was amplified with a pair of specific primers as zNSF (5'-CCGCTCGAGATAACATGGATTTAGACTC-3') and zNSR (5'-CGCGGATCCGGTCAGAATAGTTTTTCTTC-3') from a 24 hpf zebrafish cDNA, and cloned into pBLUE-scriptKS$^+$ and pXT7 expression vector that contains the UTR from *Xenopus β-globin*. All PCR products were verified by sequencing.

Synthesis of *nusap1* mRNA

Capped mRNA was synthesized *in vitro* from linearized plasmid DNA using T7 or SP6 RNA polymerase (Cap-Scribe, Roche 11581066001). After removal of DNA by treatment with RNase-free DNase, the mRNA was purified with the RNeasy Mini Kit (QIAGEN) and dissolved in RNsae-free water.

Morpholinos design and microinjection

Two morpholinos were designed to knockdown endogenous *nusap1* with a standard control: 5'-TTGAAGAGGCACAAGTA-TAATA-3' (*nusap1*-MO1, −48 to −25 from ATG), 5'-TATTGCCTATAAAATCGACGTAAG-3' (*nusap1*-MO2, −26 to −3 from ATG) and 5'-CCTCTTACCTCAGTTACAATTTATA-3' (ctrMO). To verify the knockdown efficiency of the morpholinos, the a Nusap1-GFP construct was generated by subcloning a 1016-bp fragment of *nusap1* that contains a 95-bp 5'-UTR and its adjacent coding sequence for the first 307 amino acids in-frame into pEGFP-N1 vector, and Reverse Transcription PCR for testing endogenous *nusap1* mRNA level in Fig. 3 was performed using same pair of primers as RT-F (5'-ACAAACCCAAACTAACCGATC-3') and RT-R (5'-ACGAGCTGGAGTCTTCACCAG-3'). The total embryo lysates were probed by Western blot using an anti-GFP and anti-β-actin antibody. The capped mRNA was diluted to 300 ng/μL concentration before microinjection. The mRNA or morpholino was microinjected into the yolk or cytoplasm between one- and two-cell stages as previously described (Meng et al., 1999).

Whole-mount *in situ* hybridization and real-time PCR

Digoxigenin-UTP-labeled antisense RNA probe was generated by *in vitro* transcription and used for *in situ* hybridization. Whole-mount *in situ* hybridization followed standard procedure with minor modifications (Thisse et al. 2001). Quantitative RT-PCR was performed as previously described (Xiong et al., 2006). Specific pairs of primers were referred to followings: 5'-AGACTGCTGTAAGGAGTGTCCTC-3' (chd forward), 5'-CCATGAAGTCCTCTATGCATTCCG-3' (chd reverse), 5'-CAGAGCTCACTTAGGGAAAGGCTC-3' (bmp2 forward), 5'-CCAATAGTCTAGTGATGGGCTCCTG-3' (bmp2 reverse), 5'-CCGGTCTGCTCAGTCCAGACC-3' (gata1 forward), 5'-GGAAAGGGCTACTGGACCAGAC-3' (gata1 reverse), 5'-GGACAGCCTCCTCCCTAAGGC-3' (ctn forward), 5'-CAGTTCTAGCTGGTTGATGCGG-3' (ctn reverse), 5'- ATGGATGATGAAATTGCCGCAC-3' (β-actin forward), 5'-ACCATCACCAGAGTCCATCACG-3' (β-actin reverse), 5'-TCAGACGAGAAGACGGAACA-3' (myod forward), 5'-CACGATGCTGGACAGACAAT-3' (myod reverse), 5'-GGGACCATTGTGGTCGACAG-3' (shha forward), 5'-GCTTGAGTTTACTGACATCCC-3' (shha reverse), 5'-CCCGCGCGGAGCCGCCGCTGC-3' (sox9b forward) and 5'-GCAGGTGCGGGTACTGGTCCGC-3' (sox9b reverse) (Maves et al., 2007; Warga et al., 2009).

TUNEL assay

Embryos at 24 hpf were fixed with 4% paraformaldehyde overnight and subjected to the TUNEL assay using the fluorescein cell death detection kit (Roche 11684795910) as previously described (Chen et al., 2009).

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ABBREVIATIONS

dpf, days post fertilization; hpf, hours post fertilization; MO, morpholino; NuSAP, nucleolar spindle-associated protein; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling;

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