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Pescadillo is required for *Xenopus laevis* eye development and neural crest migration

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Abstract

Pescadillo is a multifunctional, nuclear protein involved in rRNA precursor processing, ribosomal assembly, and transcriptional regulation. Pescadillo has been assigned important functions in embryonic development and tumor formation. We previously identified pescadillo as a potential downstream target of non-canonical Wnt-4 signaling. Here we have investigated for the first time the function of the *Xenopus laevis* homolog of pescadillo during early embryogenesis on a molecular level. Loss of function analysis indicates that pescadillo is required for eye development and neural crest migration. BrdU incorporation and TUNEL assays indicate that a loss of pescadillo function affects proliferation and triggers apoptosis through a p53-mediated mechanism. Furthermore, pescadillo affects the expression of early eye-specific marker genes, likely independent of its function in regulating proliferation and apoptosis, and in addition migration of cranial neural crest cells. Our data indicate that pescadillo has multiple important functions during *X. laevis* development and that its function is highly conserved among different species.

Keywords: *Xenopus*; Eye development; Neural crest; Cartilage; Pescadillo; p53; Wnt

Introduction

Embryonic development of multicellular organisms is governed by different processes including cellular differentiation, proliferation and migration. These processes depend on each other and need to be tightly regulated with respect to each other to ensure proper development. These different aspects are regulated by different growth factors functioning in the early embryo. Wnt proteins, for example, are extracellular glycoproteins that can activate different intracellular signaling pathways during development. The canonical Wnt/β-catenin pathway involves stabilization of cytoplasmic β-catenin upon ligand/receptor interaction which then can interact with transcription factors of the TCF/LEF family to activate *Wnt* target genes. Canonical Wnt signaling is involved in dorsal–ventral axis formation, posteriorization of neural tissue and neural crest induction as well as other processes (Logan and Nusse, 2004). In contrast, non-canonical Wnt pathways are independent of β-catenin and involve certain kinases like jun-N-terminal kinase (JNK), protein kinase C (PKC), or calcium-calmodulin-dependent kinase II (CamKII) (Veeman et al., 2003). Non-canonical Wnt signaling has been linked to different embryological processes including regulation of gastrulation movements (Heisenberg et al., 2000), eye development (Cavodeassi et al., 2005; Lee et al., 2006; Maurus et al., 2005; Rasmussen et al., 2001), or neural crest cell migration (De Calisto et al., 2005; Garriock and Krieg, 2007). Recently we aimed to identify potential non-canonical *Wnt-4* target genes in a neural context (Maurus et al., 2005). For this purpose we neuralized *Xenopus laevis* animal caps through treatment with noggin and FGF and in addition overexpressed Wnt-4 in part of the caps. Through a subsequent subtractive cDNA cloning approach we identified several genes that are potential downstream factors of Wnt-4, including the *X. laevis* homolog of pescadillo.

Pescadillo was originally identified in the zebrafish, *Danio rerio*, in an insertional mutagenesis screen (Allende et al., 1996). During zebrafish development, organs with high pescadillo

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expression are the eye, the optic tectum, the fin buds, the liver primordium, the gut and the branchial arches. In zebrafish, loss of pescadillo results in reduced eyes, a smaller brain as well as defects in the visceral skeleton, which derives from the branchial arches and therefore from the neural crest cells. Fish lacking pescadillo die at embryonic day 6 whereas pescadillo-deficient mice die before implantation (Lerch-Gaggl et al., 2002). These earlier data on pescadillo function indicate an important role of pescadillo during embryonic development. The molecular mechanism through which pescadillo acts during early development remained unsolved.

Data from yeast and eukaryotic cell culture systems implicate a role for pescadillo in ribosome biogenesis, cell proliferation control, DNA replication as well as regulation of gene transcription (Adams et al., 2002; Du and Stillman, 2002; Grimm et al., 2006; Lapik et al., 2004; Lerch-Gaggl et al., 2002; Oeffinger et al., 2002; Sikorski et al., 2006). Misregulation of pescadillo function has been linked to cancer and chromosomal instability (Killian et al., 2004; Kinoshita et al., 2001; Maiorana et al., 2004; Prisco et al., 2004; Zhang et al., 2005). Pescadillo encodes a nuclear protein harboring a nuclear localization signal (Haque et al., 2000; Kinoshita et al., 2001), a BRCT protein–protein interaction domain as well as a potential sumoylation site. Most data suggest a role for pescadillo in ribosome biogenesis. Pescadillo physically interacts with Bop1 and WDR12 and is required for pre-rRNA processing during assembly of 60S ribosomal subunits (Holzel et al., 2005; Lapik et al., 2004). Misregulation of ribosomal assembly can lead to developmental defects through activation of p53, an important regulator of apoptosis as well as embryonic differentiation (Cordenonsi et al., 2003; Takebayashi-Suzuki et al., 2003). Normally, p53 concentration in the cell is kept low due to the action of Mdm2, an E3 ubiquitin ligase that targets p53 for proteasomal degradation. Failure in ribosome biogenesis leads to free ribosomal proteins L5, L11, and L23 that can interact with Mdm2, thereby activating p53 (Dai et al., 2004; Pestov et al., 2001; Rubbi and Milner, 2003). Furthermore, pescadillo has recently been shown to interact with the insulin receptor substrate-1 (IRS-1) which is an important mediator of insulin and insulin-like growth factor signaling (Maiorana et al., 2004). Signaling through IRS-1 has been implicated in different processes like cellular differentiation and migration. Furthermore, pescadillo has been shown to directly bind DNA and to regulate gene expression. Recently, pescadillo has been shown to interact with Mtap1b, the microtubule-associated protein 1B (Lerch-Gaggl et al., 2007). These observations open the possibility that pescadillo may have different functions during early embryogenesis.

Here we provide a first characterization of pescadillo function in *X. laevis* embryogenesis. Expression of pescadillo was dependent on the function of Wnt-4 as well as Frizzled-3 confirming that pescadillo is a Wnt-4 downstream factor. Knock-down of pescadillo function results in an eye and neural crest cell/cartilage phenotype. Despite the fact that pescadillo is involved in regulating apoptosis and proliferation, the pescadillo loss of function phenotype was mainly due to defects in cellular differentiation and migration. These data therefore provide a first hint to uncover novel functions of pescadillo during early development and suggest that pescadillo is a multifunctional protein. Furthermore, *X. laevis* will be a suitable organism to analyze these functions.

Methods

Embryo cultures

X. laevis embryos were generated and cultured by standard methods (Sive et al., 2000) and staged according to Nieuwkoop and Faber (1975).

Cloning and mutagenesis

The pescadillo construct used was obtained by the RZPD (Deutsches Ressourcenzentrum für Genomforschung) [RZPD clone ID: IRAKp961C1697Q2, Acc. No.: BC043950]. The original pescadillo construct was in pCMV-SPORT6 and for further experiments subcloned into pCS2+ (Rupp and Weintraub, 1991). To generate a construct missing the morpholino oligonucleotide binding site, the QuickChange Site-Directed Mutagenesis Kit (Stratagene) was used. For designing optimal mutagenic primers a special software program (Stratagene) was used (http://labtools.stratagene.com/QC). Mutagenesis was done in two steps. First, three silent mutations within the open reading frame were realized (pescadillo sequence: **ATG** GGT GGT CTG; pescadillo_3Mut sequence: ATG GGT GGC TTA, nucleotides exchanged are underlined). Second step, 6 bp in front of the ATG codon was mutated (pescadillo sequence: GCG GAG ATG; pescadillo_Mut: AGA TCT **ATG**). As a consequence of the mutagenesis procedure, the mutated sequence was: pescadillo MO binding site: GCG GAG **ATG** GGT GGT CTG GAG AA; pescadillo_Mut MO binding site: AGA TCT ATG GGT GGC TTA GAG AA. This mutated binding site was not recognized by the pescadillo MO (see below) and was used for rescue experiments. Deletion constructs of pescadillo were created by inverse PCR with proof reading Pfu-polymerase (Promega) to remove the BRCT-domain, SUMO-domain or NLS-domain, respectively, starting from the pescadillo_Mut construct. Primer sequences were: pescadillo_ΔBRCT_r: 5′-ATG TTT TTC CTC CTC CTG-3′; pescadillo_ΔBRCT_l: 5′-CCT GGG GTG CTT CTG CCC-3′; pescadillo_ΔSUMO_r: 5′-AAC CTT GCC TGC TGT TAC-3′; pescadillo_ΔSUMO_l: 5′-CGT CTA GCA ATT ATG ATG-3′; pescadillo_ΔNLS_r: 5′-CAT CAT CAT AAT TGC TAG-3′; pescadillo_ΔNLS_l: 5′-TAA TCT AGA ACT ATA GTG-3′. All primers for inverse PCR were 5′-phosphorylated. Removal of the corresponding domain was verified by sequencing.

Morpholino oligonucleotides (MOs)

MOs were obtained by Gene Tools, LLC, OR. The pescadillo MO is a 23-mer MO with the sequence 5′-TTC TCC AGA CCA CCC ATC TCC GC-3′. Wnt-4, Fz3 and p53 MOs were used as recently published (Cordenonsi et al., 2003; Deardorff et al., 2001; Saulnier et al., 2002). Morpholino oligos were resuspended in sterile water and injected unilaterally into a single dorsal-animal blastomere at 8-cell stage at doses of 2–10 ng (pescadillo MO as indicated), 10 ng (Fz3), or 10–20 ng (p53 MO) or 15 ng (Wnt-4 MO) per embryo. As a control MO the standard control MO of Gene Tools was used with the sequence: 5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′. In some experiments, RNA coding for GFP was coinjected as indicated to ensure proper injection. The functionality and binding specificity of pescadillo MO were tested using the *in vitro* transcription and translation assay TNT-Kit (Promega). To further test for the specificity of the pescadillo MO the MO binding site as well as the mutated binding site of Pescadillo_Mut was cloned in front of and in frame with GFP in pCS2+. 1 ng of corresponding RNA was coinjected with 10 ng pescadillo MO and GFP expression was monitored at stage 25.

Whole-mount in situ hybridization

Expression of marker genes were analyzed by whole-mount in situ hybridization using standard procedures (Hemmati-Brivanlou et al., 1990). Embryos were injected unilaterally into one dorsal-animal blastomere at 8-cell stage and cultured until the stages indicated. Embryos were fixed overnight at

4 °C in MEMFA (0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, and 4% formaldehyde) and dehydrated in 100% MeOH. For histology, embryos were postfixed in MEMFA for 1–2 h and embedded in gelatine/BSA. Vibratome sections were cut at 15–20 m and coverglass mounted with glycerol.

RT-PCR

Total RNAs were isolated from *X. laevis* embryos using the Gentra Purescript™ RNA isolation kit following the manufacturer's protocol. Reverse transcription was performed with Superscript II RNase H⁻ reverse transcriptase (Invitrogen) utilizing random primers. Primer sequences for pescadillo were: Pes_RT_L: 5′-CTC TCG TTT CCC TGC AAC TC-3′ and Pes_RT_R: 5′-CAT GCC AAG CAG TCA CTT GT-3′ with an annealing temperature of 55 °C resulting in a product size of 199 bp. Primers for the histone H4-positive control were: H4_l: 5′-CGG GAT AAC ATT CAG GGT ATC ACT-3′, H4_r: 5′-ATC CAT GGC GGT AAC TGT CTT CTT-3′, resulting in an amplification product of 175 bp. Annealing was carried out at 55 °C.

BrdU (5-bromo-2′deoxy-uridin) staining

Cell proliferation was detected with 5-bromo-2′deoxy-uridin (Hardcastle and Papalopulu, 2000). 10 nl BrdU (Roche) was injected bilaterally into the anterior neural tube at the region of the eye at stage 23. Embryos were fixed 2 h later in MEMFA. Incorporation of BrdU into newly synthesized DNA was detected with the BrdU Labeling and Detection Kit II (Roche) according to manufacturers' instruction. For better visualization, embryos were bleached in 30% H_2O_2. Due to the strong proliferation in the eye region (see Fig. 6B), embryos were judged externally for reduced staining as it was done in case of the whole-mount in situ hybridization procedure.

TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling) staining

Whole-mount TUNEL staining of *Xenopus* embryos was done as described (Hensey and Gautier, 1999) by using BM Purple as chromogenic substrate. This technique detects apoptotic cells by using the enzyme terminal deoxynucleotidyl transferase (TdT, Invitrogen) to directly label ends of broken DNA strands with digoxigenin–dUTP (Roche). Embryos were fixed in MEMFA (0.1M MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, and 4% formaldehyde), overnight at +4 °C, dehydrated in 100% methanol, and stored at −20 °C until further processing. After rehydration in PBST (130 mM NaCl, 7 mM Na_2HO_4, 3 mM NaH_2PO_4, 0.1% Tween, pH 7), the embryos were treated with 10 mg/ml Proteinase K (Roche) in PBST for 30 s at room temperature. Proteinase K reaction was stopped 2 times for 5 min by 0,1 M triethanolamin (750 µl 98% triethanolamin, 100 µl 32% HCl, add to 50 ml H_2O) at room temperature. After this, the embryos were rinsed in PBST and postfixed in MEMFA for 1 h at room temperature. The embryos were incubated in TdT-buffer (Invitrogen) for 1 h followed by the overnight TdT-reaction at room temperature (0.5 µM digoxigenin–dUTP+150 U/ml TdT). Next day, the TdT-reaction was stopped by treatment with PBS/1 mM EDTA twice at 65 °C for 1 h. Subsequently, the embryos were washed with PBST and blocked in blocking solution (10% BMB/5× MAB, 0.1% Tween, 0.03% Horse serum) for 1 h. They were incubated in blocking solution with anti-digoxigenin-AP-Fab fragments (Roche, 1:10,000) at room temperature for 2–3 h, rinsed in PBST and were stored at +4 °C overnight. Next day, the chromogenic reaction was performed with BM Purple. To better visualize the TUNEL staining, embryos were bleached in 30% H_2O_2. For statistical evaluations, unilaterally injected embryos were considered to have a phenotype when a comparison between both sides revealed at least a 3-fold difference in TUNEL-positive cells (see Fig. 3A for an example).

Cartilage staining by Alcian blue staining

Embryos were fixed in MEMFA at stage 48 for 2 h at room temperature. Thereafter, they were rinsed in PBS and stained with 1% Alcian blue in 0.5% acetic acid (diluted in H_2O) by incubation for 95 min at room temperature. Overnight, they were stored at room temperature in 80% ethanol/20% acetic acid. The following day, embryos were bleached for 3 h in 30% H_2O_2 and incubated for 2 h in 0.05% Trypsin diluted in saturated sodium tetraborate solution. For better visualization, embryos were rinsed in PBST for several days at +4 °C. For photography, the skin of the embryos was removed and the cartilage expelled.

Results

Pescadillo is enriched in anterior neural structures of Xenopus laevis

We have recently identified the *X. laevis* homolog of pescadillo as a potential Wnt-4 target gene in neuralized animal cap tissue (Maurus et al., 2005). A full-length *X. laevis* pescadillo sequence was deposited before in public databases by others [Acc. No.: BC043950] and the clone was available through the RZPD (Deutsches Resourcenzentrum für Genomforschung, Berlin). Using this clone, we first determined the temporal and spatial expression pattern of *X. laevis* pescadillo by semi-quantitative RT-PCR and whole-mount in situ hybridization. RT-PCR demonstrated that during *Xenopus* embryogenesis, pescadillo is already expressed maternally (data not shown). The first tissue-specific expression of pescadillo could be visualized at stage 18. Strong expression of pescadillo was detected in the anterior neural plate (Fig. 1A). At stage 23, most prominent staining was visible in the migrating cranial neural crest cells as well as in the developing eye (see Fig. 1B). Similar staining was observed throughout later stages 26–31 (Figs. 1C, E, and F). A staining in the pronephros region can also be detected (Fig. 1E). A dorsal view of stage 31 embryos also reveals that pescadillo can be detected in the isthmus, the midbrain–hindbrain boundary (Fig. 1D). Sections of stage 26 and 31 embryos indicate staining in the migrating neural crest cells and the periocular mesenchyme (Figs. 1G–I).

As pescadillo is a potential Wnt-4 downstream factor and as both, Wnt-4 and its putative receptor Frizzled-3 (Fz-3) have been shown to be required for eye development (Maurus et al., 2005; Rasmussen et al., 2001) and axonal guidance of commissural axons (Lyuksyutova et al., 2003), we tested whether the expression of pescadillo depends on either Wnt-4 or Fz-3 using well-characterized antisense morpholino oligonucleotides (MO) against Wnt-4 (Saulnier et al., 2002) or Fz-3 (Deardorff et al., 2001). Indeed, injection of Wnt-4 MO (64%, $N=47$) but not a control MO (17%, $N=54$) resulted in a reduction of pescadillo expression in migrating neural crest cells and the developing eye (Fig. 2A). Similarly, pescadillo expression was impaired after injection of Fz-3 MO (40%, $N=73$) but not a control MO (9%, $N=51$) (Fig. 2B). These data from both knock-down approaches are in agreement with the identification of pescadillo as a potential Wnt-4 downstream target in a neural context and implicate a function of pescadillo during eye and neural crest development, two processes that have been linked to Wnt signaling before (Cavodeassi et al., 2005; De Calisto et al., 2005; Deardorff et al., 2001; Garriock and Krieg, 2007; Lee et al., 2006; Maurus et al., 2005; Rasmussen et al., 2001).

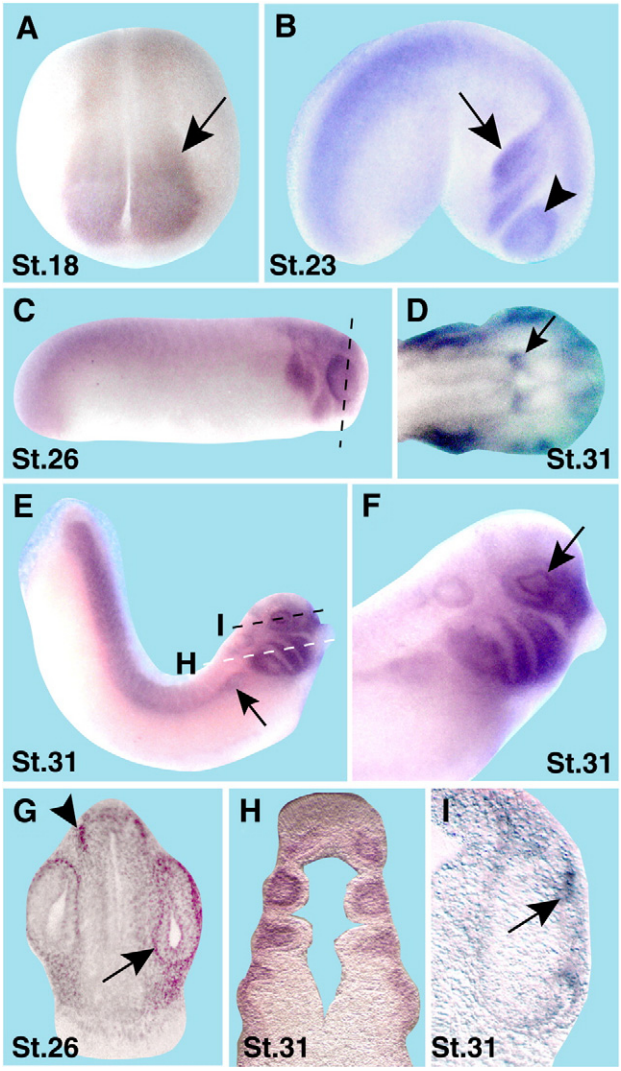


Fig. 1. Spatial expression of *Xenopus laevis* pescadillo. Spatial expression of pescadillo was analyzed by whole-mount in situ hybridization. (A) Specific expression could first be detected at stage 18 in neural tissue with strongest expression in the anterior neural plate (arrow). (B) At stage 23, pescadillo can be detected in migrating cranial neural crest cells (arrow) and the developing eye (arrow head). (C) Similarly, expression of pescadillo at stage 26 can be detected in the neural crest cells and the eye. Dashed line indicates level of cross-section shown in panel G. (D) Dorsal view of an embryo at stage 31. Specific expression can be found at the isthmus, the midbrain–hindbrain boundary (arrow). (E) Expression at stage 31 persists in derivatives of cranial neural crest cells, the eye and the pronephros (arrow). White dashed line indicates level of section as indicated in panel H, whereas the black dashed line indicates level of section as shown in panel I. (F) Magnification of an embryo at stage 31. Pescadillo expression is enriched in the region of the ciliary marginal zone of the eye (arrow). (G) Transverse section of an embryo at stage 26. Expression of pescadillo can be found in the isthmus (arrow head) and outer layer of the eye, the periocular mesenchyme (arrow). (H) Horizontal section at stage 31 indicates expression of pescadillo in the branchial arches. (I) Horizontal section at stage 31 indicates expression to be likely within the periocular mesenchyme (arrow).

Knock-down of pescadillo results in eye and cranial cartilage defects

We next examined the morphological consequences of overexpressing or depleting pescadillo protein. Overexpression of pescadillo RNA in early embryos by mean of RNA injections did not reveal any obvious phenotype. For pescadillo loss of function analyses we designed an antisense morpholino oligonucleotide (MO) against the translation start site of pescadillo (Fig. 3A). Coupled transcription and translation assays (TNT) revealed that the pescadillo MO efficiently blocks translation of pescadillo RNA, whereas a control MO did not (Fig. 3B). For control experiments we introduced silent mutations into the MO binding region of the pescadillo DNA clone (Fig. 3A) which results in the pescadillo_Mut construct. The pescadillo MO was unable to block translation of the corresponding pescadillo_Mut RNA (Fig. 3B). As a second, independent assay to test the specificity of the pescadillo MO, we cloned the wildtype and the mutated MO binding sites in front to and in frame with GFP resulting in pescadillo-GFP and

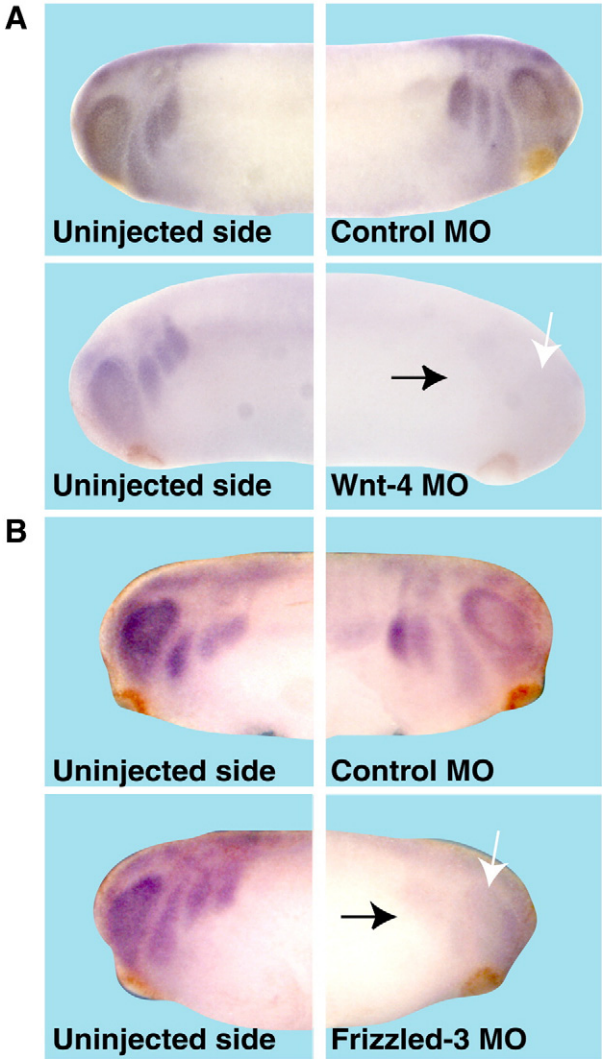


Fig. 2. Expression of pescadillo depends on Wnt-4 and Fz-3. *Xenopus laevis* embryos were unilaterally injected into one dorsal-animal blastomere at 8-cell stage with either antisense morpholino oligonucleotides (MO) against Wnt-4 (A), Frizzled-3 (B) or a control MO (A, B). Expression of pescadillo was visualized by whole-mount in situ hybridization. Expression of pescadillo in the eye (white arrows) and the cranial neural crest cells (black arrows) depends on Wnt-4 as well as Frizzled-3 function.

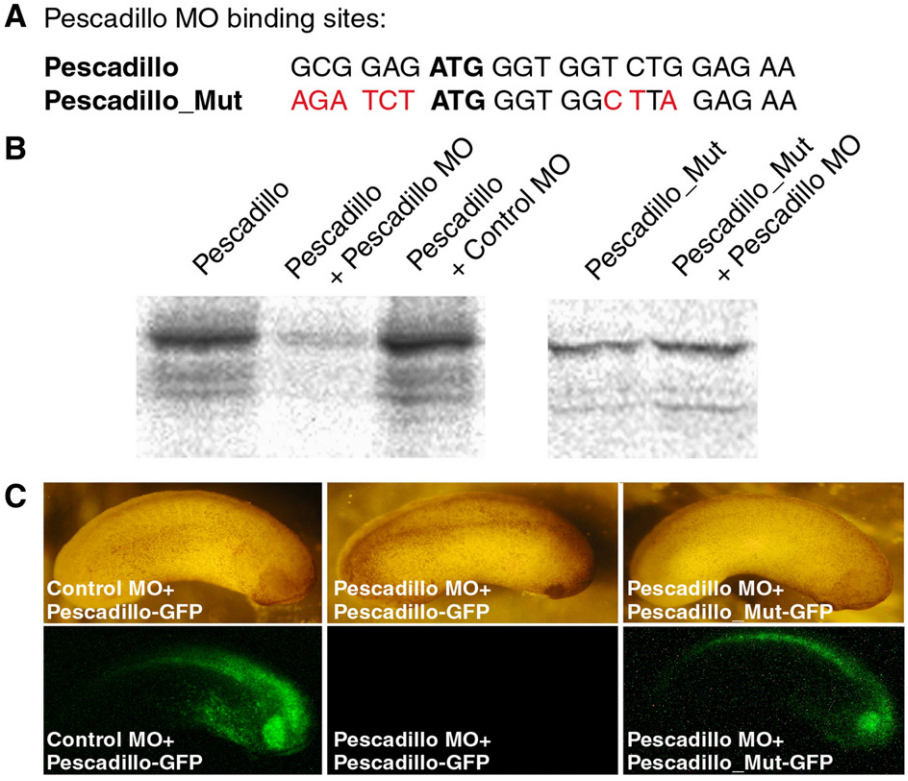
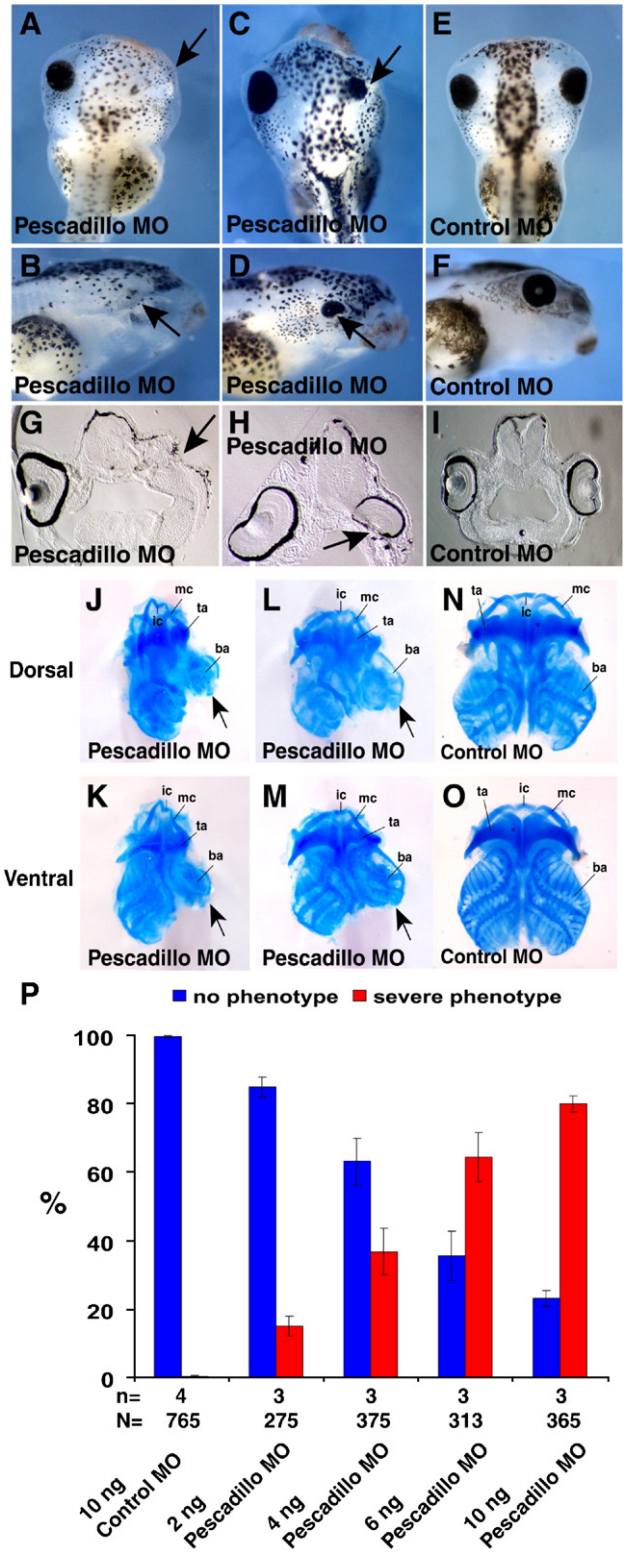


Fig. 3. Characterization of an antisense morpholino oligonucleotide directed against *Xenopus laevis* pescadillo. (A) Binding site of the pescadillo MO within the pescadillo mRNA. The ATG start codon is highlighted. Sequence of the pescadillo_Mut RNA in which 9 nucleotides have been changed as indicated in red. (B) Coupled transcription and translation using radioactively labeled methionine. Plasmids encoding pescadillo or pescadillo_Mut were used as indicated. Morpholino oligonucleotides were added as indicated. Pescadillo MO but not a control MO interferes with translation of the pescadillo RNA. Pescadillo MO does not block translation of Pescadillo_Mut. (C) Efficiency of pescadillo MO was also tested in whole embryos. Oligonucleotides representing the sequences as given in panel A were cloned in frame to and in front of GFP. RNA coding for the corresponding constructs (pescadillo-GFP or pescadillo_Mut-GFP) was injected together with pescadillo MO or control MO as indicated. GFP expression was monitored at stage 25. Pescadillo MO but not control MO interfered with translation of Pescadillo-GFP. Pescadillo_Mut-GFP was not targeted by pescadillo MO.

pescadillo_Mut-GFP. RNA of these constructs was injected together with pescadillo MO or control MO into animal-dorsal blastomeres of 8-cell stage. GFP expression in *Xenopus* embryos was monitored at stage 26. Pescadillo MO but not control MO blocked GFP expression if coinjected with pescadillo-GFP. Pescadillo MO, however, was unable to block GFP expression in presence of the mutated MO binding site in pescadillo_Mut-GFP (Fig. 3C). Thus, both assays used indicate that pescadillo MO interferes with translation of pescadillo RNA but not with pescadillo_Mut RNA, which therefore was considered to be suitable for rescue experiments.

As pescadillo is expressed in anterior neural tissue, we next examined the consequences of depleting endogenous pescadillo protein by the characterized pescadillo MO and unilaterally injected the pescadillo MO into dorsal-animal blastomeres of 8-cell stage *X. laevis* embryos. First we examined pescadillo function on eye development at stage 42. At this stage the retinal pigmented epithelium (RPE) is clearly visible and cross-sections through embryos indicate the lens and the multilayered structure of the retina. After injection of pescadillo MO up to 80% of embryos (for 10 ng of pescadillo MO, number of experiments $n=4$, number of embryos examined $N=365$) displayed severe defects in eye development encompassing a malformed and strongly reduced RPE (Figs. 4A–D, P) in a dose-dependent manner. For these initial experiments GFP RNA (0.5 ng) was coinjected as a lineage tracer. Some embryos also completely lacked eye structures (Figs. 4A, B). Embryos with very modest reduction in RPE were not considered as a phenotype. Injection of a control MO did not reveal these effects on eye development (Figs. 4E, F, P). Histological analysis confirmed these effects of pescadillo but not control MO on eye development (Figs. 4G–I). For further rescue experimentation within this paper we used a dose of 6 ng pescadillo MO as we realized that coinjection of suitable RNA concentrations together with 10 ng of pescadillo MO often resulted in a high death rate of injected embryos (data not shown). Coinjection of the pescadillo_mut RNA (1 ng) together with the pescadillo MO (6 ng) significantly rescued the observed phenotype (Figs. 5B, C) indicating the specificity of the observed effect ($n=3$, $N=282$), whereas the same amount of GFP RNA (1 ng) did not rescue the observed phenotype ($n=6$, $N=470$). We further observed that pescadillo MO (68%, $n=4$, $N=62$) but not control MO (4%, $n=4$, $N=54$) injected embryos display defects of cranial cartilage development which was visualized by Alcian blue staining (Figs. 4J–O). The severity of the cartilage defects in all cases reflected the severity of the observed eye phenotype (see in particular Figs. 4J, K). These

data indicate a conserved function of pescadillo during development as zebrafish mutant for pescadillo also displays small eyes and cartilage defects (Allende et al., 1996). Interestingly, external inspection of the embryos did not indicate a reduced number of melanocytes suggesting a cell-type-specific effect within the neural crest derivatives.



In a next step we asked, which domains of the pescadillo protein were required for its effect on eye development. We generated several deletion constructs missing the BRCT, SUMO or NLS domains, respectively (Fig. 5A). All of these constructs harbor the silent mutations in the ATG region to prevent pescadillo MO binding and therefore are suitable for rescue experiments. After coinjection with the pescadillo MO, the mutants lacking the BRCT and SUMO domains, pescadilloΔBRCT and pescadilloΔSUMO, were able to rescue the pescadillo MO phenotype using the eye as a read out. The mutant lacking the nuclear localization signal, pescadilloΔNLS, in contrast did not rescue the pescadillo MO phenotype (Figs. 5B, C). Taken together, these data indicate that pescadillo is required for proper eye and cranial cartilage development in *X. laevis* and that the nuclear localization of pescadillo is required for this function whereas the BRCT and the potential sumoylation domain are dispensable for this function.

Pescadillo MO affects p53-mediated apoptosis and proliferation

We next examined the molecular basis of the observed phenotype. As a misregulation of ribosomal assembly leads to an activation of p53 (Dai et al., 2004; Pestov et al., 2001; Rubbi and Milner, 2003) we analyzed apoptosis and proliferation in the pescadillo MO injected embryos. TUNEL staining of treated embryos revealed that injection of pescadillo MO results in an increase of apoptosis on the injected side of the embryo (Fig. 6A). If this effect is due to an activation of p53 then coinjection of p53 MO should result in a rescue of the observed phenotype. To check this hypothesis we injected a recently published and characterized p53 MO (Cordenonsi et al., 2003) together with the pescadillo MO. This indeed resulted in a reversal of the apoptosis phenotype of pescadillo MO injected embryos (Fig. 6A). As an increase in apoptosis might be accompanied by a decrease in proliferation, we next analyzed whether the proliferation rate is altered in pescadillo MO injected embryos. Labeling of dividing cells by incorporation of BrdU indicated that proliferation is reduced in pescadillo injected embryos and that this effect could be reverted by coinjection of p53 MO (Fig. 6B). These data strongly suggest that a loss of pescadillo results in an activation of p53 to affect apoptosis and proliferation.

However, when analyzing the overall appearance of the eye phenotype in p53 rescued embryos, we could not detect a

Fig. 4. Loss of pescadillo function results in severe eye and craniofacial cartilage defects. (A–F) Unilateral injection of pescadillo MO but not control MO into one dorsal-animal blastomere of 8-cell stage embryos results in severe eye phenotypes. (G–I) Histological analysis of pescadillo MO injected embryos indicates strong reduction or severe morphological eye defects in comparison to control MO injected embryo. (J–O) Embryos were unilaterally injected into one dorsal-animal blastomere of 8-cell stage embryos and cultured until stage 48. Cartilage development was visualized using an Alcian blue staining. Loss of pescadillo results in cartilage defects of diverse severity that correlates with the eye phenotype. ba: Branchial arches, ta: tectum anterius, mc: Meckel's cartilage, lc: infrarostral cartilage. (P) The effect of pescadillo MO onto eye development is dose dependent. *n*: Number of independent experiments, *N*: number of embryos scored in total. Error bars indicate standard error of the mean.

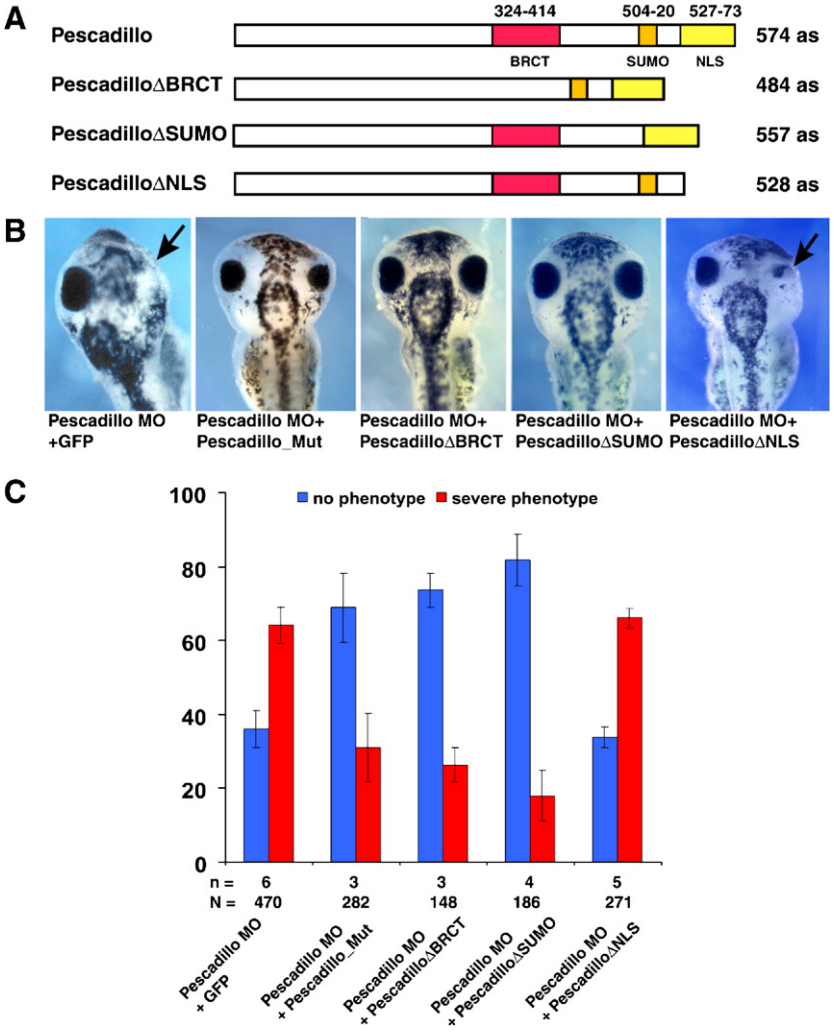


Fig. 5. Analysis of domain requirements for pescadillo function. (A) Schematic drawing of pescadillo deletion mutants. (B) RNAs coding for pescadillo_Mut, pescadilloΔBRCT, or pescadilloΔSUMO are able to revert the pescadillo MO phenotype. PescadilloΔNLS is unable to revert the pescadillo MO phenotype. The eye was used as a read out. Representative embryos are shown. (C) Statistical evaluation of described experiments. Embryos were scored for strong reduction and morphological eye defects as shown in Fig. 4 panels A–D. Mild effects on eye size were not considered to be a phenotype. *n*: Number of independent experiments, *N*: number of embryos scored in total. Error bars indicate standard error of the mean.

statistically significant decrease in the occurrence of this phenotype (Fig. 6C) suggesting that interfering with proliferation and apoptosis is not the major mechanism which is responsible for the observed eye phenotype. To further follow up this possibility, we investigated which of the pescadillo mutants described before might be able to revert this pescadillo/p53-mediated proliferation phenotype. Whereas pescadillo_Mut or pescadilloΔSUMO were able to rescue the proliferation phenotype, peccadilloΔBRCT or pescadilloΔNLS were not (Fig. 6D). It needs to be noted here, that the pescadilloΔBRCT mutant is unable to rescue the effect of pescadillo MO on proliferation (Fig. 6D) although it is able to rescue the overall pescadillo MO phenotype in the eye (Figs. 5B, C). As the BRCT domain of pescadillo has recently been shown to be involved in regulating peccadillo's function in proliferation (Lerch-Gaggl et al., 2002; Prisco et al., 2004), this again indicates that the effect of pescadillo MO on proliferation is not the major cause of the observed phenotype. These results furthermore implicate additional, previously unidentified functions of pescadillo during embryonic development.

Pescadillo MO affects cellular differentiation in the eye field

In a next step, we therefore performed whole-mount in situ hybridization analysis using different eye specific marker genes including *Pax-6*, *Rx*, and *Otx-2*. Unilateral injection of pescadillo MO into animal-dorsal blastomeres was accompanied by a strong reduction in eye marker gene expression (Fig. 7A) on the injected side at stage 23. For these experiments GFP was coinjected with the pescadillo MO and embryos were selected for successful injection during earlier stages. This reduction was not observed after injection of the control MO. Expression of MyoD, a skeletal muscle marker, as well as Emx1, a forebrain marker, was also not affected and thus served as negative control (Fig. 7A, Fig. 8B). We finally tested whether eye-specific marker gene expression could be restored when pescadillo function was

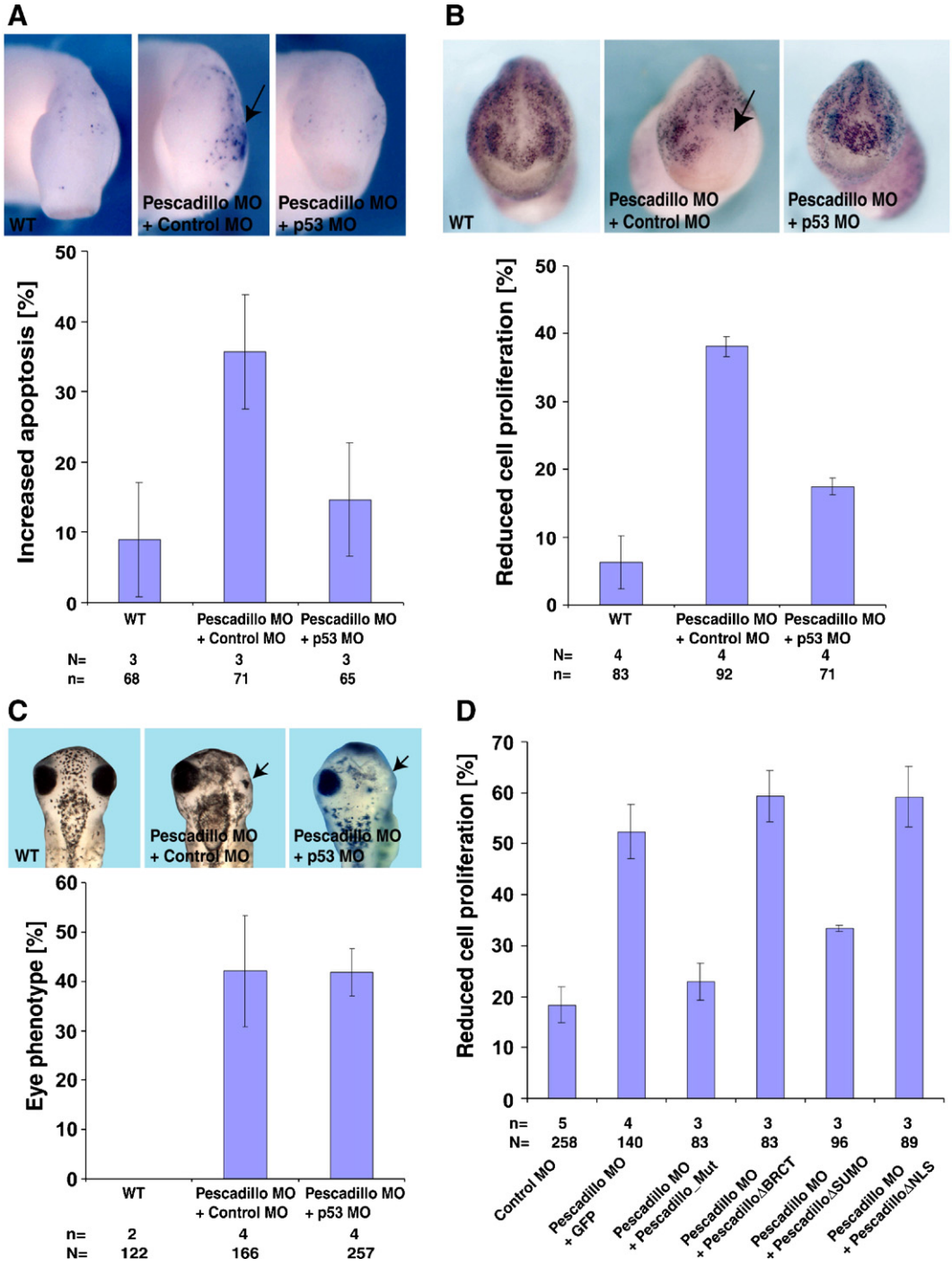
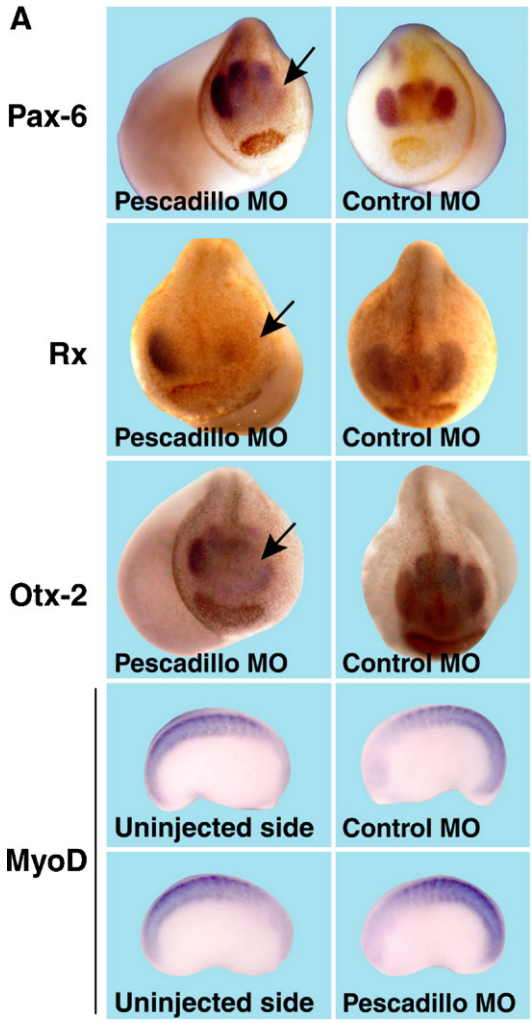


Fig. 6. Pescadillo regulates apoptosis and proliferation through p53. Embryos were injected at 8-cell stage into one dorsal-animal blastomere with pescadillo MO (6 ng), control MO or p53MO (10 ng) as indicated. (A) Embryos were analyzed for apoptosis using TUNEL staining at stage 26. A statistical evaluation of embryos with an increased apoptosis is given. Coinjection of p53 MO together with pescadillo MO can reduce the number of embryos with an increased apoptosis. (B) Embryos were analyzed for changes in proliferation by BrdU assays at stage 25. A statistical evaluation of embryos with decrease of proliferation is given. Coinjection of p53 MO together with pescadillo MO can reduce the number of embryos with decreased proliferation. (C) Coinjection of pescadillo MO (6 ng) and p53MO (10–20 ng) does not rescue the pescadillo loss of function eye phenotype. Both doses tested (10 ng, $n=2$; 20 ng, $n=2$) had no rescuing activity. (D) Pescadillo MO was injected unilaterally into one dorsal-animal blastomere of 8-cell stage embryos and proliferation was monitored using BrdU assay at stage 25. Embryos were scored for unilateral reduction in BrdU incorporation as shown in Fig. 5B. Coinjection of RNAs coding for pescadillo_Mut or pescadilloΔSUMO but not GFP, pescadilloΔBRCT or pescadilloΔNLS were able to rescue the pescadillo MO phenotype on proliferation. n=Number of independent experiments. N=number of embryos examined in total. Error bars indicate standard error of the mean.

reactivated. We therefore monitored Rx expression after injecting pescadillo MO together with RNA coding for the different pescadillo constructs. Injection of pescadillo MO resulted in 54% of treated embryos in a strong reduction of Rx expression ($n=3$, $N=60$). Rescue experiments indicate, that pescadilloΔNLS is unable to restore Rx expression after loss of

pescadillo function (53% of Rx phenotype, $n=3$, $N=68$). Pescadillo_Mut RNA reverted the observed reduction in Rx expression to 28% ($n=3$, $N=81$) and, similarly, the pescadilloΔBRCT and pescadilloΔSUMO constructs reduced the number of embryos with a reduced Rx staining to a similar extent (Fig. 7B). These molecular data are in agreement with the analysis of morphological phenotypes described above and further support the idea that a nuclear localization of pescadillo is important for its function during eye development.



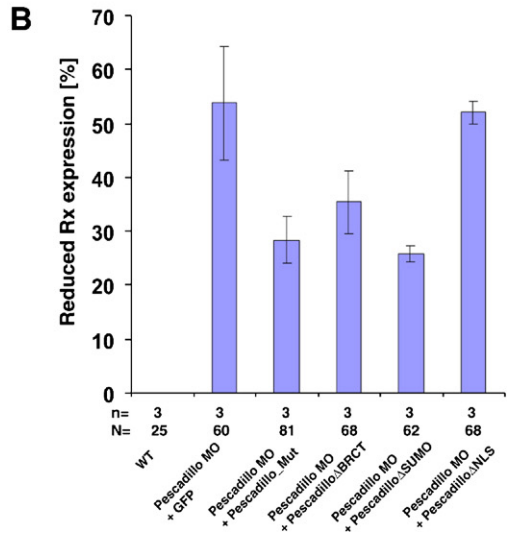


Fig. 7. Pescadillo affects differentiation within the early eye field. Pescadillo MO or control MO was injected unilaterally into one dorsal-animal blastomere of 8-cell stage embryos. (A) Expression of marker genes *Pax-6*, *Rx*, *Otx-2*, or *MyoD* was analyzed by whole-mount in situ hybridization at stage 23, as indicated. Expression of Pax-6, Rx, or Otx-2 in the eye field was reduced whereas expression of MyoD was not affected. (B) Statistical evaluation of reduced Rx expression in pescadillo MO injected embryos. Coinjection of pescadillo_Mut, pescadilloΔBRCT, or pescadilloΔSUMO but not pescadilloΔNLS was able to reduce the number or embryos with an unilateral reduction in Rx expression. n=Number of independent experiments. N=number of embryos examined in total. Error bars indicate standard error of the mean.

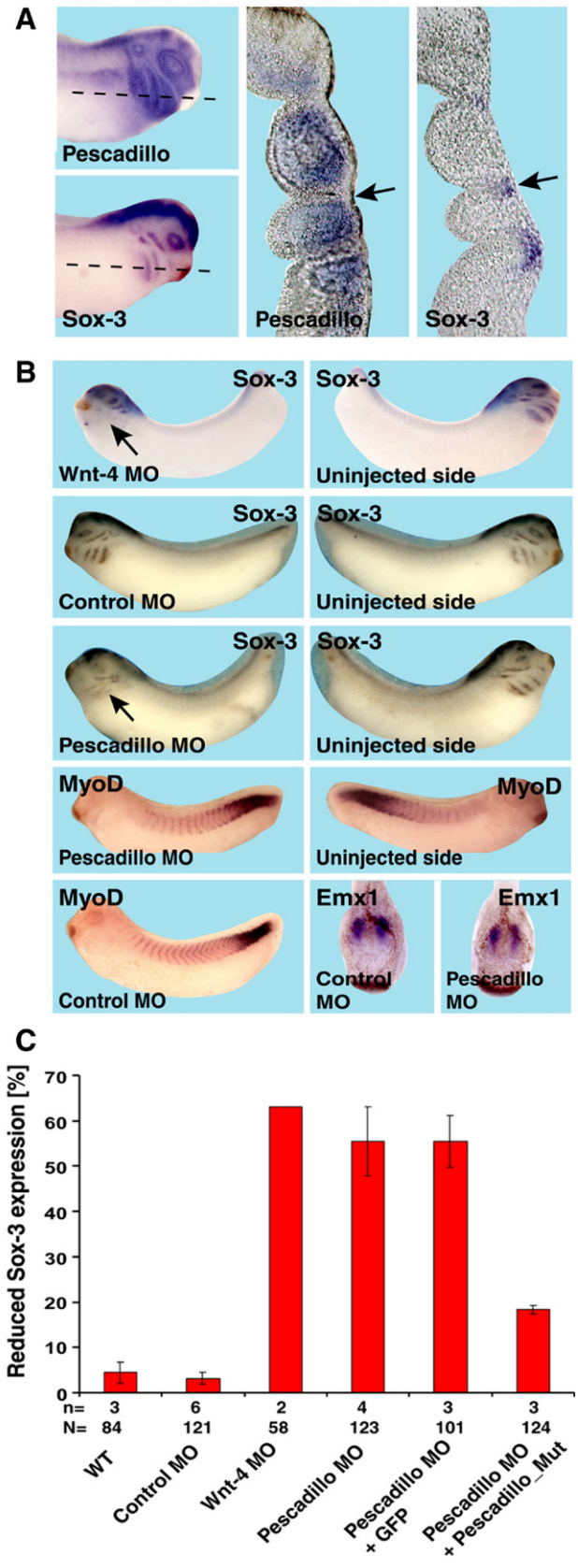
Downregulation of pescadillo interferes with neural crest cell development

In a last set of experiments we focused on the observed cranial cartilage phenotype. Cranial cartilage is derived from neural crest cells that migrate through the branchial arches (Baltzinger et al., 2005; Sadaghiani and Thiebaud, 1987). Furthermore, expression of pescadillo in these cells depends on Wnt-4 (Fig. 2A) and Frizzled-3 (Fig. 2B) and Wnt signaling through different intracellular signaling pathways has been implicated in neural crest induction (Deardorff et al., 2001) as well as neural crest migration (De Calisto et al., 2005; Garriock and Krieg, 2007). As shown before (Fig. 1H), in the *X. laevis* embryo pescadillo is expressed in the migrating cranial neural crest cells of the branchial arches. *Sox-3* (Penzel et al., 1997) is a marker gene that labels the pharyngeal pouches of the branchial arches at stage 32 (Fig. 8A). We hypothesized that interfering with branchial arch development should also interfere with Sox-3 expression. Interestingly, we observed a downregulation of Sox-3 on the injected site after pescadillo MO or Wnt-4 MO injections (Fig. 8B). Pescadillo MO injections, however, did not interfere with expression of the muscle specific marker gene *MyoD* or the forebrain marker Emx-1 at stage 32 indicating the specificity of the observed effect. This effect of pescadillo MO could be reverted by coinjection of pescadillo_Mut but not GFP RNA (Fig. 8C). These data further support the hypothesis that pescadillo is required for proper development of cranial neural crest cells and are clearly in agreement with the before mentioned cartilage phenotype (Figs. 4J–M).

In the following experiments we therefore focused on earlier aspects of neural crest development. At stage 19 when neural crest cells are induced, Wnt-4 is expressed in close vicinity to cranial neural crest cells as indicated by double whole-mount in situ hybridization studies using digoxigenin/fluorescein labeled antisense riboprobes against Wnt-4 and the neural crest marker slug (Fig. 9A). In addition, Frizzled-3 is expressed throughout the whole anterior neural tissue with different expression levels.

In a first set of experiments we analyzed expression of two neural crest marker genes, *slug* and *FoxD3*, at stage 17 when neural crest cells are specified (Mayor et al., 1995; Pohl and Knochel, 2001; Sasai et al., 2001). Frizzled-3 has been shown to be required for neural crest induction through involvement in a

canonical Wnt pathway (Deardorff et al., 2001). In agreement with these earlier findings, unilateral depletion of Frizzled-3 resulted in a loss of slug-positive neural crest cells at stage 17. In contrast, neither pescadillo MO nor Wnt-4 MO injections resulted in a downregulation of slug. This result was in addition confirmed by the finding that pescadillo MO injection did also not result in a loss of FoxD3 expression (Fig. 9B). In a following set of experiments we focused on marker gene expression during later stages (stage 20) when cranial neural crest cells are migrating (Fig. 9C), a process that has been shown to be regulated through non-canonical Wnt pathways (De Calisto et al., 2005; Garriock and Krieg, 2007). At this stage, downregulation of pescadillo or Wnt-4 MO results in defects in neural crest cell migration as indicated by slug-positive cells that do not leave the dorsal neural tube. In case of pescadillo MO, we confirmed this observation by staining for FoxD3. At stage 25, both pescadillo MO and Wnt-4 MO inhibit migration of Krox-20 (Bradley et al., 1993)-positive neural crest cells (Fig. 9C). These data are clearly supporting the observation that pescadillo is required for proper neural crest migration and cartilage development in *Xenopus*.



In summary, we here have provided data that the Wnt-4 downstream target pescadillo is required for proper eye development and neural crest cell migration in *X. laevis*, two aspects that have been linked to Wnt signaling before (Abu-Elmagd et al., 2006; Cavodeassi et al., 2005; De Calisto et al., 2005; Deardorff et al., 2001; Lee et al., 2006; Maurus et al., 2005; Rasmussen et al., 2001). Pescadillo might affect as different aspects as apoptosis/proliferation, differentiation and cell migration in these settings. Further analysis will indicate how these different processes may be connected through a common multifunctional protein, pescadillo.

Discussion

Interfering with pescadillo function during early embryonic development in *X. laevis* results in small to absent as well as deformed eye structures and defects in cranial neural crest cell derived structures. This phenotype is evolutionary conserved as zebrafish mutant for pescadillo shows similar defects (Allende et al., 1996). This raises the question what might be the molecular mechanisms underlying these phenotypes.

Pescadillo as a regulator of apoptosis and proliferation

Pescadillo has recently been described as a regulator of proliferation and apoptosis (Du and Stillman, 2002; Grimm et al., 2006; Haque et al., 2000; Killian et al., 2004; Kinoshita et al.,

Fig. 8. Pescadillo affects Sox-3 expression in branchial arches. (A) Pescadillo is expressed in the neural crest cell derived part of the branchial arches, whereas Sox-3 is expressed in the pharyngeal pouches within the endodermal layer that touches the outer ectodermal layer. More dorsally, Sox-3 is expressed in the lateral line placode and the otic placode (Schlosser and Ahrens, 2004). (B) Unilateral injection of Wnt-4 MO or pescadillo MO but not control MO results in a reduction of Sox-3 expression in the branchial arches. Neither pescadillo MO nor control MO affects expression of MyoD or Emx1 at stage 32. (C) Statistical evaluation of embryos with reduced Sox-3 expression at stage 32 after unilateral injection of pescadillo or Wnt-4 MO. Pescadillo _Mut RNA can revert the pescadillo MO effect on Sox-3 expression. *n*=Number of independent experiments. *N*=number of embryos examined in total. Error bars indicate standard error of the mean.

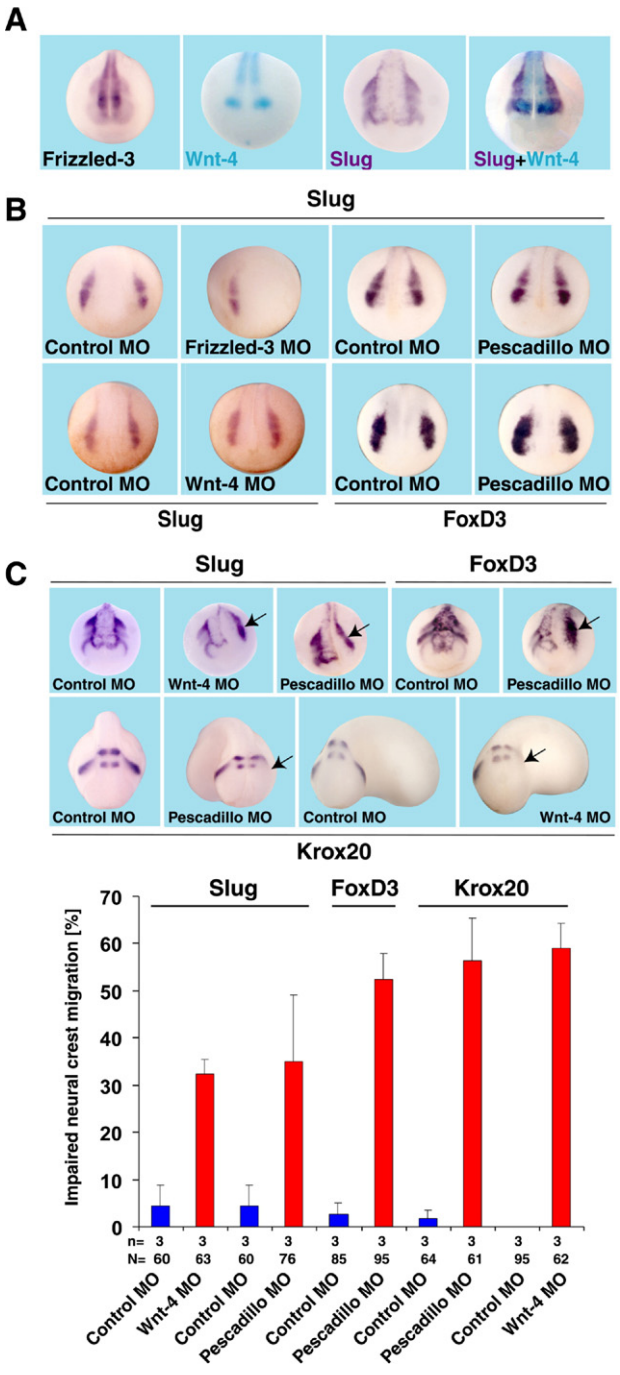


Fig. 9. Pescadillo interferes with cranial neural crest migration but not specification. (A) Whole-mount in situ hybridization indicates a partial coexpression of Slug and Wnt-4 at stage 19. Frizzled-3 is expressed throughout the whole anterior neural plate with different intensities. (B) Frizzled-3 MO but not Wnt-4 MO, pescadillo MO or control MO interferes with slug expression at stage 17. Pescadillo MO does not interfere with FoxD3 expression at stage 17. (C) Unilateral injection of Wnt-4 MO and pescadillo MO results in a neural crest cell migration defect as indicated by staining for slug (stage 20) and Krox20 (stage 23/24). In case of pescadillo MO, FoxD3 was used as an additional marker (stage 20). Staining for FoxD3 indicates that neural crest cells stay in the dorsal part of the embryo and do not migrate towards the ventral side. A statistical analysis of these experiments is given at the bottom of the panel C. *n*=Number of independent experiments. *N*=number of embryos examined in total. Error bars indicate standard error of the mean.

2001; Lerch-Gaggl et al., 2002; Maiorana et al., 2004; Oeffinger et al., 2002; Prisco et al., 2004). Our here described experiments indicate that loss of pescadillo function in *Xenopus* indeed results in a decrease in proliferation as measured by BrdU incorporation assays. In parallel we were able to detect an increase in apoptosis by TUNEL staining. This effect seems to be stronger in the eye region than in the neural crest region. It needs to be noted though that this issue has not been analyzed in detail here and would require additional experimentation in the future. The observed ability of a p53 MO to rescue the effect of a pescadillo MO on proliferation and apoptosis is in agreement with previously published data that pescadillo is involved in pre-rRNA processing, as a failure of this process results in defects in 60S ribosomal subunit assembly, accumulation of free ribosomal proteins, inhibition of the E3 ubiquitin ligase Mdm2 and finally accumulation and activation of p53. This effect of pescadillo in *X. laevis* depends on the presence of the BRCT domain as described previously in other cell systems (Haque et al., 2000). Similar to the observed effect of pescadillo, it has recently been shown that the perturbation of rRNA synthesis in bap28-deficient zebrafish embryos results in p53-mediated apoptosis in the central nervous system during early development (Azuma et al., 2006). Taken together, our data and those published by Azuma et al. (2006) indicate that proper pre-rRNA synthesis is required to ensure correct proliferation and apoptosis during early development and that pescadillo and bap28 are involved in this process. The failure of the p53 MO to rescue the pescadillo MO induced morphological phenotype, however, suggest that the major reason for the observed eye phenotype is not the effect of pescadillo on proliferation and apoptosis. This does not, however, exclude the possibility that a change in proliferation/apoptosis contributes to the observed eye phenotype or that so far unobserved effects of pescadillo in other tissues might be primarily due to this mechanism.

Why do *X. laevis* embryos survive a loss of pescadillo function whereas pescadillo-deficient mice die during preimplantation stages of development? An important reason might be the different principles underlying early development of *Xenopus* and mice. In *Xenopus* but not mice, huge maternal stores of RNAs, proteins, lipids but also ribosomes are established that are used up by the early embryo (Brown and Dawid, 1968; Dawid and Sargent, 1988). Therefore, the effect of a loss of pescadillo in early *X. laevis* embryos (likely due to deficits in ribosomal assembly) seems to be milder than in the mouse embryo that cannot rely on these storages. A deficiency in ribosomal assembly during mouse embryogenesis therefore results in severe defects, a block of proliferation in preimplantation stages. Similar to *X. laevis*, zebrafish embryos also have maternal storages for early development and a defect in ribosomal assembly is not necessarily lethal (Allende et al., 1996; Azuma et al., 2006).

Pescadillo as a regulator of differentiation

Pescadillo has recently been shown to be able to act as a transcription factor due to direct binding to DNA (Sikorski et al.,

2006) raising the possibility that pescadillo might also affect gene expression during early embryogenesis. A binding motif for pescadillo has been defined which has also been functionally tested in reporter gene assays (Sikorski et al., 2006). Using whole-mount in situ hybridization we were able to show that a loss of pescadillo function indeed leads to a downregulation of marker gene expression in the early eye field. Interestingly, this could be rescued by introducing non-targetable pescadillo RNA that lacks the BRCT domain which is required for the effect of pescadillo on proliferation/apoptosis. This finding underscores the hypothesis that pescadillo directly affects gene expression in the eye field. It remains unclear, however, whether the regulation of the marker genes analyzed (*Pax-6*, *Rx*, *Otx-2*) occurs in a direct manner by pescadillo. In a first inspection we were not able to detect a pescadillo response element within the −3 kb promotor regions of those genes. In the future, more detailed analyses will be required to clarify the detailed molecular mechanism how pescadillo regulates eye specific marker genes.

Pescadillo as a regulator of cell migration

As an additional phenotype after loss of pescadillo function we observed a defect in cranial neural crest cell migration which results in cartilage defects. Marker gene analysis revealed that the initial formation of neural crest cells as indicated by the expression of the marker genes *slug* or *FoxD3* is not disturbed after pescadillo MO injection. The location of slug and FoxD3-positive cells within the early embryo of stage 20 rather suggests that cranial neural crest cells are unable to migrate after loss of pescadillo. In addition we observed a loss of Krox20-positive migratory neural crest cells. Furthermore, Sox-3 expression in the pharyngeal pouches of the branchial arches was reduced after pescadillo MO injection. In line with these observations is the observed cartilage defect which was obtained frequently in later stages. What might be the molecular mechanism underlying this phenotype? Several explanations come into mind. First, based on the findings concerning marker gene expression in the eye one might speculate that pescadillo could be involved in regulating yet unidentified genes required for neural crest cell migration. Considering this hypothesis one would rely on a single uniform molecular mechanism for the different phenotypes observed. A systematic screen for gene expression in neural crest cells will indicate whether this hypothesis holds true and corresponding experiments are under way. Second, pescadillo might interfere with signal transduction pathways involved in regulating neural crest cell migration. Just recently, pescadillo has been shown to interact with the insulin-receptor substrate 1 (IRS1) which is involved in insulin and insulin-like growth factor signaling (Maiorana et al., 2004). This indicates that pescadillo might be able to directly interfere with signal transduction pathways. Third, one cannot exclude an unexpected function of pescadillo in regulating cell migration. Just recently, an elegant study of Nguyen et al. (2006) showed that the cell cycle regulator protein p27kip1 not only regulates cell proliferation but also affects differentiation and cell migration in the cerebral cortex indicating an unexpected behaviour and additional functions of a well-characterized protein. Clearly, more detailed work will be necessary to fully understand the function of pescadillo in this context.

Pescadillo is a Wnt-4/Frizzled-3 downstream factor

Pescadillo has recently been identified as a potential downstream target of Wnt-4 (Maurus et al., 2005). Our herein described experiments further support this observation. Expression of pescadillo in the eye as well as in migrating neural crest cells depends on Wnt-4 as well as Frizzled-3. These data are in full agreement with previous observations that non-canonical Wnt signaling is required for proper eye development (Cavodeassi et al., 2005; Lee et al., 2006; Maurus et al., 2005; Rasmussen et al., 2001) as well as neural crest migration (De Calisto et al., 2005; Garriock and Krieg, 2007). In an initial set of experiments, however, we failed to show that pescadillo might be a direct target of non-canonical Wnt signaling. For these experiments we made use of previously established cell lines that were stably transfected with inducible Frizzled receptors that couple to either canonical or non-canonical Wnt signaling (Maurus et al., 2005). These cells were stimulated to activate Wnt signaling pathways and expression of pescadillo was monitored through RT-PCR experiments without any positive results (data not shown). These data suggest that pescadillo lays rather downstream in a chain of different events triggered by non-canonical Wnt signaling.

In summary, our data provide the first characterization of pescadillo function during early embryogenesis on a molecular level. We here provide evidence that pescadillo serves different functions during early *X. laevis* embryogenesis: (1) Pescadillo is involved in regulating cell proliferation and apoptosis, most likely due to its role in ribosomal assembly, (2) it regulates gene expression in the early embryo, and (3) it regulates neural crest cell migration. This study will foster additional, more detailed analysis of pescadillo function during embryogenesis in the future. Due to the unique features of *X. laevis*, this model organism will be a suitable tool to further characterize the function of pescadillo. As pescadillo is also involved in tumor formation (Maiorana et al., 2004), these future experiments will clearly shed some light on these novel functions of pescadillo also during tumor formation.

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