Medaka *simplet* (FAM53B) belongs to a family of novel vertebrate genes controlling cell proliferation

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The identification of genes that regulate proliferation is of great importance to developmental biology, regenerative medicine and cancer research. Using an in situ screen on a cortical structure of the medaka fish brain, we identified the *simplet* gene (*smp*), which is homologous to the human FAM53B gene. *smp* was expressed in actively proliferating cells of the CNS throughout embryogenesis. It belongs to a family of vertebrate-specific genes with no characterized biochemical domains. We showed that FAM53B bound 14-3-3 chaperones, as well as SKIIP proteins, adaptor proteins connecting DNA-binding proteins to modulators of transcription. *smp* inactivation with morpholinos led to delayed epiboly and reduced embryonic size. Absence of *Smp* activity did not induce apoptosis, but resulted in a reduced cell proliferation rate and enlarged blastomeres. Moreover, *smp* was shown to control the expression of the pluripotency-associated *oct4/pou5f1* gene. We propose that *smp* is a novel vertebrate-specific gene needed for cell proliferation and that it is probably associated with the maintenance of a pluripotent state.

KEY WORDS: Fish, Zebrafish, *Oct4*, *Pou2*, *Ol-pou5f1*, Epiboly, Pluripotency

INTRODUCTION

Although much progress has been accomplished in recent years towards understanding cell proliferation at the genetic level, many key genes involved in this process still remain to be discovered. A combinatorial network of cell cycle regulators, signaling and metabolic pathways, and also, probably, of uncharacterized genes, underlies the biogenesis of proliferating neural progenitors (Karsten et al., 2003). Novel genes involved in the proliferation machinery will likely be discovered among the 41% of predicted human genes corresponding to proteins with unknown function (Venter et al., 2001).

Our experimental model is the Japanese medaka *Oryzias latipes*, a currently emerging vertebrate model (Wittbrodt et al., 2002) with features similar to those of the zebrafish *Danio rerio* (high fecundity, small size and external development of transparent embryos). The telost optic tectum (ot) is a cortical structure of the dorsal midbrain (Nguyen et al., 1999), which grows by the successive addition of open rings of progenitor cells originating from a population of stem cells located at the periphery of the organ. In this structure, mitotic cells are located at the margin, differentiating cells at the center, and in between lies an ‘arrest zone’, where cell cycle-exit genes are expressed (Nguyen et al., 2001a). We took advantage of this topographically oriented growth mode to undertake a systematic whole-mount in situ hybridization (WMISH) screen on the medaka CNS (Deyts et al., 2005; Nguyen et al., 2001b), aimed at identifying novel molecules linked to cell proliferation in the CNS and, potentially, in other tissues. These studies demonstrated the predictive value of this approach, at least for the genes expressed in the arrest zone; there is a very strong correlation between the expression pattern and gene function (in that case, downregulation of the cell cycle). Among the candidate genes isolated in the course of our screen, a previously uncharacterized gene was called *simplet* (*smp*). Although Smp has no characterized biochemical domains, it binds to the 14-3-3 adaptor proteins. In addition, the human homolog FAM53B binds to the SKIIP protein, known to modulate the activity of transcriptional regulators implicated in cell proliferation. Based on *smp* expression patterns during embryonic development, and on gain- or loss-of-function experiments, we propose that *smp* is involved in the regulation of cell proliferation and, possibly, in the maintenance of pluripotency.

MATERIALS AND METHODS

Fish strain

Medaka embryos of the Carbio strain (kindly provided by Jochen Wittbrodt, EMBL, Heidelberg) were raised at 26°C under a reproduction regime (14 hour light/10 hour dark). Fertilized eggs were collected after spawning (at the onset of the light) and incubated in Yamamoto’s embryo rearing medium (Yamamoto, 1975). In all experiments, embryos were placed at 26°C and staged according to Iwamatsu (Iwamatsu, 1994; Iwamatsu, 2004) and Furutani-Seiki (Furutani-Seiki and Wittbrodt, 2004).

Cloning and sequence analysis

A 1025 bp fragment of *simplet* 3' cDNA end was isolated from a library of medaka anterior brain (35-somite, stage 30-31) in the course of the in situ hybridization screen (Nguyen et al., 2001b). To isolate the 5' cDNA sequence, total RNA was extracted from medaka anterior brain at 18-19 somites (stage 25) and RT-PCR was further performed, following the SMART RACE cDNA Amplification Kit instructions (Clontech, CA, USA). Synthesized cDNA was used as template for PCR amplification of the full-length *simplet* coding region using the following primers: forward, 5'-CATGCCATGGCCCGAT-3' and reverse, 5'-ATAGTTTACG-GCCGCTTTCCATGAGCTCTTCAAGACAT-3'. A fragment (~1.5 kb) was purified with the QIAEX II Gel Extraction Kit (QIAGEN) and subcloned into pCRII-TOPO (Invitrogen). The *simplet* sequence was compared with the GenBank and SwissProt databases using BLAST (National Center for Biotechnology Information, USA). Homologous protein sequences of fugu were downloaded from the JGI website (http://genome.jgi-psf.org/Takra4/Takra4.home.html). Alignments were created using MultAlign (http://prodes.toulouse.inra.fr/multalin/multalin.html) (Corpet, 1988). For phylogenetic analyses, we used the CLUSTAL X Multiple Sequence Alignment Program (Thompson et al., 1997) and MEGA version 2.1 (Kumar et al., 2001). To isolate medaka *Ol-pou5f1*, we blasted the medaka genome

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sequence using the NIG DNA sequencing center website (http://dolphin.lab.nig.ac.jp/medaka) to identify the closest medaka relative to zebrafish FAM53B. PCR primers were: forward, 5′-CTGTTGGGGCAGAAGCTACATTGATG-3′; and reverse, 5′-TGCAGTTGAGGTGGGTCTCAGC-3′. A 162 bp fragment containing the whole ORF was cloned; the medaka protein is 56% identical to the zebrafish protein.

Yeast two-hybrid screen

Yeast two-hybrid screen using the entire ORF of the human smp related gene FAM53B was carried out by Dualsystems Biotech AG (Zurich, Switzerland). For the screen using the mutated FAM53B ORF, the underlined serine of each 14-3-3 binding domain was changed into alanine to generate mutations sites (RSXPS). Library plasmids were isolated from positive clones and assayed in a FAMS3B dependency test with (1) the bait plasmid and (2) a control bait encoding a LexA-lamin fusion using a mating strategy (Kolonin et al., 2000). Bait dependent interactors were further analyzed for homologies with BLASTp.

Plasmids

For immunoprecipitation, the smp cDNA insert was excised from the pCRII-TOPO plasmid and inserted into a pTracer plasmid (Invitrogen). A FLAG epitope was fused to the N-terminal protein extremity by insertion between the KpnI and PpuMI restriction sites of the PCR amplified product obtained with a 5′ primer containing the FLAG sequence, CGGTGACCCGGCCCACCACATGGAATCAGCATGGAGGAGGAGGACGAGACGACAGTCCAGCTTCGCG, using pTracer/Ol-smp as template. To amplify human FLAG/FAM53B, we used a cDNA library from MCF-7 cells. PCR product was obtained with the FLAG-containing 5′ primer GGTACCCTGGAAGATGTTGAGGTGGGTCTCAGC and a 3′ primer, TTGTTGGAGGAAAGGTGCTCCGAG, using pTracer/Ol-smp and EcoRI restriction enzymes and inserted into the pTracer plasmid in the same orientation.

Immunoprecipitation

HCT-116 cells were transfected with empty pTracer, pTracer/FLAG/Ol-smp and pTracer/FLAG/FAM53B with lipofectamine 2000 (Invitrogen). After 72 hours, cells were lysed by RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Deoxycholate, 1% Triton, 0.2% SDS] with a proteasine inhibitor cocktail tablet (Roche), and immunoprecipitated with M2 ELISA/Beads (Sigma). After protein denaturation, immunoprecipitated complexes were separated by 15% SDS-PAGE and transferred onto a nitrocellulose membrane. FLAG/OL-Smp and FLAG/FAM53B proteins were revealed with anti-FLAG M5 antibody (Sigma), and 14-3-3 proteins with polyclonal rabbit antibody (Upstate), followed by HRP-coupled secondary antibodies, before ECL treatment (Amerham). EGF-stimulated A431 cell lysate was used as a positive control for 14-3-3 proteins. Detection of SKIIP proteins was performed using an anti-human SKIIP antibody (a gift of Dr L. Banks) (Prathapan et al., 2002).

Whole-mount in situ hybridization (WMISH)

WMISH were performed with an Intavis automat. Embryos from the one-cell stage to the late gastrula stage (stage 17) were fixed overnight in 4% paraformaldehyde (PFA) and 0.05% glutaraldehyde in 0.12 M phosphate buffer (PBS, pH 7.4); from the late gastrula stage onwards, embryos were fixed overnight in 4% PFA. Sense and antisense digoxigenin-UTP RNA probes were prepared according to Joly et al. (Joly et al., 1997). From the 256/512-cell stage (stage 9), embryos were subjected to Proteinase K treatment (25°C for 35-somite embryos (stage 30). Control sense probe did not lead to mis-paired control morpholinos (smpMOIC and -IIC). To use them as in vivo dyes, 3′-end modifications of morpholinos with carboxyfluorescein or lissamine were performed. Morpholinos were resuspended to 20 mg/ml (about 2 nm) in sterile RNase-free water. Knockdown experiments were performed with 2 to 10 mg/ml of smpMO, and clonal and rescue experiments with 8 mg/ml of smpMO. Embryos injected with RNase-free water or a balanced salt solution gave the same results.

For overexpression, capped mRNAs encoding Smp or EGFP were in vitro synthesized (Ambion mRNA/Message Machine kit). EGF RNA was injected either alone or together with smpRNA. For rescue experiments, five independent experiments were performed with more than 50 embryos in each batch.

TUNEL assay

The development of smpMOI- or smpMOIC-injected embryos (6 mg/ml) was arrested in 4% PFA, 0.05% glutaraldehyde (for 48 hours at 4°C). Fixed-embryos were mechanically dechorionated, permeabilised (0.1% sodium citrate) and labelled for DNA strand breaks using the In Situ Cell Death Detection Kit (TUNEL, Roche). Staining substrate was Fast-Red (Roche).

DAPI staining

Following MO injection into two- or 32-cell-stage embryos (8 mg/ml), development was arrested in 4% PFA, 0.05% glutaraldehyde (2 hours). Embryos were dechorionated and incubated for 30 minutes with DAPI fluorescence (300 nM in PBS/Tween 1%). Blastoderm was detached from the yolk and mounted on PPD-glycerol (1,2-Phenylendiamin, Merck) for microscopy observation. For clonal analyses, nuclear morphology was analyzed under a laser scanning confocal microscope.

Flow cytometry

smpMOI-injected (10 mg/ml) and uninjected embryos were frozen in liquid nitrogen at early gastrula stage (13 hours post fertilization, hpf) and dechorionated in Galbraith buffer (Galbraith et al., 1983). Nuclei were separated from debris by filtration through a 50-μm nylon mesh. Filtrates were treated with RNase A (100 μg/ml) and stained with 300 μl propidium iodide (50 μg/ml). Nuclei (10,000) were analyzed with an EPICS Elite ESP flow cytometer. Nuclei from adult medaka liver cells (G1 arrested) were used for calibration. Histograms were obtained using MultiCycle AV software.

RESULTS AND DISCUSSION

smp belongs to a small family of vertebrate genes

Sequence analysis indicated that simplet contains a putative 407 amino acid ORF. Alignment with databases revealed the existence of several potential homologous genes in fish (zebrafish, fugu, burmese pufferfish) and tetrapods (human, mouse, chicken, frog). It is noteworthy that the smp sequences did not reveal any significant homologous genes in invertebrate genomes, including non-vertebrate chordates. Thus, simplet-related genes may have arisen during vertebrate evolution. In this respect, it has already been documented that some cell cycle regulators are restricted to vertebrate genomes (for a review, see Roberts, 1999), despite the conservation of fundamental cell division processes in eukaryotes. For example, cyclin F (Bai et al., 1994) has a specific role in coordinating crucial cell cycle events in vertebrates (Kong et al., 2000; Tetzlaff et al., 2004).
Two conserved domains [named homology regions I and II (HRI and HRII)] were found in Smp proteins (Fig. 1A). The HRI domain was shown to contain a putative nuclear localization signal (NLS). We used HRI and HRII to construct a phylogenetic tree with the identified set of smp-related genes (Fig. 1B). These genes appeared to be organized into two main groups. A first group (A) contains two human predicted proteins (Hs XP094066 and Hs AA637666) and the previously identified chicken DNTNP [Dorsal Neural Tube Nuclear Protein, Gg AAL76115 (Jun et al., 2002)]. Globally, two genes are found in mammals, whereas a single member was identified in amphibians and chicken, suggesting that an additional duplication event may have occurred in the mammalian lineage. In the other group (B), which contains smp, we identified only one gene in each tetrapod species. The human gene (Hs Q14153) was successively named KIAA0140, by the Kazusa DNA Research Institute, and FAM53B by HUGO Gene Nomenclature Committee after its identification by systematic genome data mining. By contrast, medaka, fugu, and tetraodon have two genes in this group. This probably results from the well-known increase in gene number in fish following a major duplication event that occurred in the teleostean lineage. Zebrafish presents only one gene, either as a result of a later loss of one of the paralogs, or because the sequence of a second gene is not present in the current release of the zebrafish genome.

**smp is expressed predominantly in proliferating cells throughout embryonic development**

**Maternal expression**

The *smp* expression pattern was examined by WMISH. Maternal transcripts were detected at the one-cell stage and were found to be evenly distributed among all undifferentiated blastomeres during the first five cleavages (Fig. 2A,B). In teleost embryos, three individual cell layers are progressively determined before the mid-blastula transition (MBT), which corresponds to the onset of zygotic transcription and which begins around the 1000-cell stage (stage 10) (Aizawa et al., 2003; Iwamatsu, 2004). The central blastomeres, which produce the embryo proper, are not specified [except for the germinal precursors (Yoon et al., 1997)] and proliferate actively until MBT. By contrast, the marginal blastomeres, which constitute the future extra-embryonic tissues [the enveloping layer (EVL) and the yolk syncytial layer (YSL) (Kimmel and Law, 1985)], are specified early, although they are not irreversibly committed to their fate (Ho, 1992; Kane et al., 1992; Kimmel et al., 1990). Strikingly, by the seventh cell cleavage (64/128-cell stage, stage 8), *smp* transcripts were no longer detected in peripheral blastomeres, but only in central ones (Fig. 2C-E). The maternal *smp* expression suggests that it is associated with a cellular undifferentiated state and/or with an active cell proliferation.

To our knowledge, few genes exhibit such a restricted expression to the central blastomeres. One of these is the zebrafish *pou2* gene (Hauptmann and Gerster, 1995; Takeda et al., 1994), which is related to the mammalian transcription factor *Pou5f1* (formally Oct3/4) (Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1989; Scholer et al., 1990). Numerous studies showed that *pou2* is implicated in the maintenance of an active proliferation state and in the maintenance of cell pluripotency (Burgess et al., 2002; Takeda et al., 1994). Moreover, mice *Pou5f1* regulatory regions are activated in blastulae and embryonic stem cells of the medaka (Hong et al., 2004). We cloned a medaka homolog (*Ol-pou5f1*) by searching the genome database and performing RT-PCR (see Materials and methods). WMISH confirmed that *Ol-pou5f1* is expressed maternally in embryo deep cells (Fig. 2F), with an unexpected additional expression in the EVL, not found in zebrafish (Hauptmann and Gerster, 1995; Takeda et al., 1994). With the exception of EVL cells, *Ol-pou5f1* and *smp* seem to be associated with both pluripotent and proliferating cells in the medaka embryo.

**Zygotic expression**

Although WMISH was not sensitive enough to detect *smp* expression after MBT in the thin cells layers of the gastrula, real-time PCR experiments allowed us to clearly detect zygotic *smp* transcripts at MBT and later (data not shown). WMISH on 2-somite (stage 19) and 16-somite (stage 24) embryos revealed that zygotic *smp* transcripts are restricted to the anterior midline of the telenencephalon and to the prospective midbrain-hindbrain boundary (mhb, Fig. 2G,H). In 35-somite embryos (stage 30), transcripts were detected in retinal progenitor cells and other parts of the CNS, particularly in proliferative zones of the forebrain, the optic tectum, the mhb and the hindbrain rhombic lips (Fig. 2I). At these stages, WMISH also revealed an expression in the developing somites, but we focused subsequent analysis on the neural expression. In the CNS, we carefully compared the areas of *smp* expression with proliferative zones, as revealed by WMISH with a PCNA probe (Proliferating Cell Nuclear Antigen, Fig. 3). Transversal sections in the CNS after WMISH at the 34/35-somite stage (stage 29-30) showed that *smp* and PCNA expression domains are similar in the
forebrain (Fig. 3B-E) and in the midbrain (Fig. 3F,G). In the hindbrain, such comparisons were difficult: the smp expression pattern is highly dynamic and differentiation events take place very early. Overall, our results suggest that smp is expressed in CNS mitotic cells.

smp expression in the CNS is reminiscent of that of the chick DNTNP gene, which belongs to the same family. DNTNP has been described as a dorsal CNS-specific gene (Jun et al., 2002). smp expression appears likewise to be predominantly dorsal (Fig. 2), but we feel that this mostly reflects the dorsal position of most CNS proliferative zones (Wullimann and Knipp, 2000). Assuming that the smp mRNA pattern reflects protein distribution, smp expression appears indeed to be correlated with cell proliferation zones rather than with ‘dorsalness’, as shown by its strong expression in the (proliferative) ciliary marginal zone of the retina (a ventrally derived region).

FAM53B interacts with 14-3-3 and SKIIP proteins

To identify molecular partners of Smp proteins, a yeast two-hybrid screen was performed. We used the human smp gene (FAM53B) as bait to screen a human adult brain cDNA library. Among the clones analyzed, the majority (14 out of 17) corresponded to 14-3-3 chaperone proteins, which represent a large and highly conserved family [from yeast to mammals (Fu et al., 2000; Kultz et al., 2001; Wang and Shakes, 1996)] of sequence-specific phosphoserine-binding proteins. These proteins are ubiquitously expressed and are especially abundant in the CNS (Takahashi, 2003). To confirm that both medaka and human Smp interact with 14-3-3 proteins, we performed an immunoprecipitation experiment using FLAG-tagged medaka and human Smp proteins (Fig. 1C). Analysis of the complex clearly showed that 14-3-3 proteins were co-immunoprecipitated only when Ol-Smp or FAM53B are produced in cells. We also observed a degradation profile of medaka Smp, probably indicating a protein instability in human cells. However, the functional property to interact with 14-3-3 was conserved between fish and human. A closer analysis of the Smp sequence identified a conserved consensus 14-3-3 recognition motif (RSXpSXP) (Muslin et al., 1996) in both HRI and HRII conserved domains (Fig. 1A), further strengthening the hypothesis that 14-3-3 interaction is important for Smp function.

This motif has been demonstrated to mediate interactions between 14-3-3 and the yeast Raf-1 kinase (Clark et al., 1997). Recently, in vivo analysis in Drosophila showed that 14-3-3 proteins are involved in the correct timing and progression of normal cell cycle through the regulation of sub-cellular protein localization (Su et al., 2001). In addition, studies in Xenopus showed that 14-3-3 proteins are important in vertebrate embryonic development (Wu and Muslin, 2002). Therefore, Smp represents a new partner of 14-3-3 proteins, which are involved in the control of cell signaling, cell division and apoptosis (Fu et al., 2000; Hermeking, 2003).

To identify other Smp partners, a second yeast two-hybrid screen was performed using the human FAM53B bait with an inactivating mutation in each 14-3-3 binding domain (see Materials and methods). Of the 34 clones showing a specific interaction with the bait, 29 corresponded to known human proteins (see Table S1 in the supplementary material). Among them, 20 still corresponded to the previously characterized 14-3-3 β, ξ and ε interactors. The presence of these prey, despite the fact that their canonical binding domains were mutated, indicated that their interaction with FAM53B is not only mediated by the canonical phosphoserine-containing motif. Recent work has demonstrated that 14-3-3 can indeed bind to other motifs, including unphosphorylated sites (Aiiken, 2002; Fuglsang et al., 2003). Two clones were identified as unknown genes (KIAA hypothetical proteins), while three others presented no significant similarity with sequences in public protein databases. These latter three clones corresponded to human cDNA 3’ regions. We focused our attention on one clone corresponding to the Ski-interacting protein (SKIIP). Co-immunoprecipitation experiments confirmed that the human SKIIP and FAM53B proteins (but not the medaka Smp) interact with each other (Fig. 1C, see also Materials and methods). Interactions between these two families of proteins are potentially important, as SKIIP is involved in signaling pathways controlling cell proliferation along with vitamin D, retinoic acid, oestrogens, glucocorticoids, Notch1-IC and TGFβ (Prathapam et al., 2002). For example, Ski binds to the DNA-binding protein Smad and recruits a repression complex including HDAC. The Ski/SKIIP complex was also shown to overcome pRb-mediated cell cycle arrest in G1 (Prathapam et al., 2002). SKIIP proteins are part of the spliceosome and can have either an activator or a repressor role in transcription, as...
an adaptor that connects DNA-binding proteins to other transcriptional regulators (Prathapam et al., 2002). FAM53B might therefore be a new component in this complex that was undetected in screens for proliferation regulators performed in protostome models because of its vertebrate-specific occurrence. It will be interesting to test whether Smp, and/or the protein encoded by the other medaka ortholog to FAM53B (Olf 11143), interact with medaka Ski/SKIIP. This is necessary to establish whether Smp function can be mediated by an interaction with SKIIP. Because Ski is a muscular proto-oncogene and Smp is expressed in somites, it would be interesting to analyze their functional relationships in mesodermal tissue.

**smp loss-of-function leads to epiboly delays and smaller embryos**

To investigate the role of smp during medaka embryogenesis, we performed loss-of-function experiments by microinjecting antisense MOs to inhibit translation (Nasevicius and Ekker, 2000). After microinjection of smpMOI into one blastomere of two-cell embryos (leading to a ubiquitous MOs distribution within the two blastomeres), dose-dependent developmental defects were observed at different times of development (hpf). smpMOI-injected embryos (smp-morphants) displayed no apparent morphological defects before the onset of epiboly, at the early gastrula stage (stage 13, 13 hpf). However, about one third of injected embryos displayed a phenotype consisting of fewer, but enlarged, nuclei (as shown by DAPI staining) just after MBT in the late blastula (stage 11-12, 9 hpf; Fig. 5A,B), but not before MBT in 512-cell embryos (stage 9, 6 hpf; data not shown). The lack of pre-MBT detectable defects might be due to an absence of Smp protein activity at this stage of development (for example, if Smp belongs to a transcriptional complex but transcription is not yet active), or to a previous accumulation of sufficient amounts of maternal Smp protein, or to the presence of a related protein with redundant functions (the product of the smp paralog being a likely candidate).

Two categories of gastrulation defects were distinguishable at 25 hpf, corresponding to the late gastrula stage (stage 17; Fig. 4A-C and Table 1). The most strongly affected embryos (class I: strong) were characterized by arrested development at the onset of epiboly (Fig. 4C). Mildly affected embryos (class II: weak) developed up to the 50%-70% epiboly stage (Fig. 4B). These embryos, in addition to the epiboly effects, also displayed a dorso-vegetal accumulation of abnormally large cells (Fig. 4B, inset). A third class (class III: wild type) included embryos that completed epiboly normally (Fig. 4A). Additional observations of all of these injected embryos at 44 hpf, corresponding to the 16-somite stage (stage 24), showed important subsequent developmental abnormalities (Fig. 4D-F, Table 1).
Moderate developmental defects ranged from a smaller cerebellum to complete deletion of the ot, mhb and hindbrain regions (Fig. 4E). In extreme cases, severely affected embryos were much smaller than controls and so abnormal that their developmental stage could not be determined (Fig. 4F).

Because gastrulation delays often lead to axial embryonic defects, we checked whether delays of epiboly observed at 25 hpf (in class I or class II embryos) were responsible for late defects at 44 hpf (Fig. 4G). Strongly delayed embryos (class I) failed to gastrulate, did not survive until 44 hpf or gave rise to severely affected neurulae. Class II and class III embryos continued to develop. Among class II embryos (mildly delayed), nearly half displayed moderate midbrain-hindbrain defects (48%) and about half exhibited severely affected neurulae (44%). By contrast, among class III embryos (wild type), half developed normally (48%) and half displayed moderate midbrain-hindbrain defects (52%). Because this late phenotype also appeared in embryos that had successfully gastrulated (class III embryos), we concluded that it is a direct consequence of smpMO-injection, occurring independently of earlier gastrulation defects. Because of the similarity of these phenotypes to that of the zebrafish spieble ohne grenzen mutant (Belting et al., 2001; Burgess et al., 2002), which bears mutations in the oct4/pou2/pou5f1 gene, the name simplet was given, the French name for Dopey, the foolish dwarf in Snow White.

Phenotype specificity was assessed by injecting a second non-overlapping morpholino (smpMOII) and the corresponding control containing base-pair mismatches (smpMOIC and smpMOIC; Table 1). Phenotypes similar to those observed with smpMOI were obtained with smpMOII (with reduced efficiency). No defects were observed following injection of control MOs, indicating a high specificity of both smpMO.

Rescue experiments were performed by co-injecting smpMOI (8 mg/ml) and mRNA (50 and 100 µg/ml) encoding Smp but devoid of the 5’ UTR. The resulting numbers of wild-type embryos at 25 hpf (late gastrulation) were significantly larger (55%) than when injecting MO alone (37%; P<0.003 by Student’s paired t-test). These data indicated a partial rescue of epiboly delay, and confirmed that the defects are a specific consequence of smpMO knockdown. At greater smpRNA concentrations (150 to 500 µg/ml) no rescue was observed. At such concentrations, smpRNA injections led to specific defects, including epiboly delays (see below), which may mask the rescue effect.

**smp does not interfere with embryonic patterning and does not induce apoptosis**

To determine whether smpMO injections produce abnormal patterning events during neurogenesis, we examined the expression patterns of several brain markers (fgf8, wnt1, pax2 and otx2) along the anteroposterior and dorsoventral axis of mhb-affected embryos at the 18-somite stage (stage 25, data not shown). For these markers, no major patterning defects were detected and we thus focused our subsequent analysis on cell proliferation, apoptosis and cell pluripotency.

We first analyzed the effect of smpMO injection on apoptosis, when the smp knockdown effect becomes detectable, e.g. at the late blastula stage (stage 11-12, 9 hpf, post-MBT), when apoptotic pathways become active (Carter and Sible, 2003; Hensey and Gautier, 1997) (Fig. 5). Two-cell-injected embryos were allowed to develop until late blastula stage and were stained with DAPI to detect apoptotic bodies (Huynh and Teel, 2000; Zong et al., 2003).

<table>
<thead>
<tr>
<th>Morpholino</th>
<th>Concentration (mg/ml)</th>
<th>Number of embryos</th>
<th>Wild type (Class III)</th>
<th>Weak (Class II)</th>
<th>Strong (Class I)</th>
<th>Phenotypic response at 25 hpf (%)</th>
<th>Phenotypic response at 44 hpf (%)</th>
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<tr>
<td>smpMOI</td>
<td>8</td>
<td>60 (2)</td>
<td>100.0</td>
<td>0.0</td>
<td>0.0</td>
<td>100.0</td>
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<tr>
<td>smpMOI</td>
<td>2</td>
<td>48 (1)</td>
<td>91.0</td>
<td>8.0</td>
<td>0.0</td>
<td>49.0 45.0 6.0 0.0</td>
<td>10.0 62.0 22.6 5.4</td>
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<tr>
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<td>221 (4)</td>
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<td>45.6</td>
<td>4.7</td>
<td>15.5 41.5 33.0 10.0</td>
<td>100.0 0.0 0.0 0.0</td>
</tr>
<tr>
<td>smpMOII</td>
<td>6-8</td>
<td>485 (15)</td>
<td>31.6</td>
<td>41.2</td>
<td>27.2</td>
<td>57.0 62.0 22.6 5.4</td>
<td>9.0 34.6 37.5 13.2 14.6</td>
</tr>
<tr>
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<td>119 (2)</td>
<td>72.0</td>
<td>17.0</td>
<td>11.0</td>
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<td>52.0</td>
<td>16.0</td>
<td>32.0</td>
<td>100.0 0.0 0.0 0.0 0.0 0.0 0.0 0.0</td>
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</table>

Injected embryos were scored at 25 hpf (late gastrula) and at 44 hpf (16-somite) according to the severity of developmental defects. For each smpMO concentration, the number of independent experiments is indicated in parentheses. Larger amounts of smpMO lead to more severe developmental defects.
Nuclear morphology analysis revealed no sign of apoptosis, not at this stage (Fig. 5A,B) nor during early gastrulation (data not shown). We then analyzed the effect of the smpMO on apoptosis during neurulation (Fig. 5C-G). MO-injected embryos were allowed to develop until late gastrula and 18-somite stage (stage 17 (24 hpf) and stage 25 (49 hpf), respectively), and were analyzed for apoptosis by the TUNEL method. No apoptosis above basal levels was detected either at late gastrula stage (in class II embryos, Fig. 5D) or at 18-somite stage (in mhb-defective embryos), although the level of apoptosis was slightly increased in extra-embryonic tissues (Fig. 5G). A slight increase in apoptotic cell number was observed in a few class II embryos (Fig. 5E). However, this was probably not a direct effect of the smpMO on apoptosis, but rather a secondary phenomenon due to earlier developmental defects initiated during gastrulation.

These results indicate that smpMO-induced defects do not result from patterning modifications or from a massive induction of apoptosis. Therefore, we investigated whether smp phenotypes could be the result of an impairment in cell proliferation, and/or in cell specification.

_smp-morphant cells have a reduced cell proliferation but a normal cell cycle pattern_

DAPI staining of two-cell stage smpMO-injected embryos suggested that smp knockdown does not affect cell division before MBT (Fig. 5A,B; data not shown). To analyze the cycling properties of smp-morphant cells after MBT, smp-morphant and control embryos were subjected to flow cytometry at early gastrula stage (stage 13, Fig. 6).

Whole-embryo DNA content was almost identical in both injected and uninjected embryos, indicating no phase-specific cell block. In addition, no endo-duplication phenomenon was observed (i.e. no cells with a DNA content higher than 4N; data not shown).

To determine whether smp knockdown affects the cell proliferation rate, we performed a clonal analysis: we microinjected a single central blastomere of 32-cell stage embryos with a fluorescent smpMO or its corresponding control. In all cases, fluorescence remained confined to the injected blastomere and to its progeny, thus allowing us to follow proliferation activity in a wild-type context. Progeny of the injected blastomere was followed under a dissecting microscope until the end of gastrulation (Fig. 7). From the time of injection to MBT, both smpMO-injected and control-injected blastomeres would be expected to divide about five times and thus would display about 32 fluorescent descendent cells. A quantitative analysis was performed after MBT, at early gastrula stage (stage 14). Control embryos exhibited from 50 to 150 distinct fluorescent cells, indicating that about two or three additional rounds of division occurred after MBT (Fig. 7A,B,G). Within a single batch of embryos injected with smpMOI or smpMOII, embryos typically displayed a correct timing of epiboly but less...
**smp knockdown leads to enlarged cells and to altered cell motility**

The nuclear morphology after clonal injections was examined by DAPI staining at early gastrula stage (stage 14; Fig. 7LN). Control labelled cells (smpMOC-containing cells) and non-labelled cells both displayed interphasic (Fig. 7I-K, arrow and inset) or mitotic nuclei (Fig. 7I-K, arrowhead). By contrast, smp morphant cells displayed abnormally large blastomeres and large nuclei (Fig. 7L-N, arrow; see also Fig. 4B and Fig. 5B) containing decondensed chromatin (Fig. 7M, inset). It is unlikely that the reduced cell number results from mitosis arrest, as no mitotic nuclei were observed. As flow cytometry revealed no specific modification of the cell cycle pattern, we propose that smp knockdown slows down cell proliferation by increasing the length of the G1, S and G2 (but not M) phases. This is not an unusual situation: other cell cycle regulators that do not control a particular step of the cell cycle have recently been identified in *Drosophila*. For example, *shar-pei* and *hippo* mutant cells both show an even acceleration of cell cycle progression (Kango-Singh et al., 2002; Tapon et al., 2002). Finally, analysis of the integrity of nuclear DNA (Fig. 7J,M), cell death analysis by TUNEL (data not shown) and flow cytometry profiles (Fig. 6) confirmed that smp-morphant cells do not present apoptotic characteristics.

In clonal experiments, we noticed the presence of clusters of cells in smpMO-injected embryos (Fig. 7D,E). Eventually, at the end of gastrulation, their descendants were excluded from the embryonic axis (Fig. 7F), whereas smpMOC cells were found all along the embryonic axis (Fig. 7C). Thus, smpMO-containing cells also exhibit altered cell movements, which might be linked to modified adhesion properties, and/or to the large size of blastomeres. This could be one of the main factors causing delayed epiboly. Indeed, in the course of a Tübingen screen for new zebrafish mutants (Haffter et al., 1996), several mutants identified as affecting the cell cycle also displayed an early epiboly arrest phenotype (Early Arrest Group Class I mutants) (Kane et al., 1996). Furthermore, overexpression of the *XCS1* (*Xenopus* cleavage signal protein) gene in *Xenopus* embryos disturbs mitosis and leads to abnormal gastrulation due to cell enlargement (Nakamura et al., 2000), and the mouse *Rnf2* (*Ring 1b*) (Ring finger protein) null mutant causes cell cycle inhibition together with gastrulation arrest (Vonken et al., 2003). Our results thus provide an additional illustration of the tight link between cell proliferation and the morphogenetic movements of epiboly: in absence of Smp, the cycling activity of cells slows down and cells fail to move normally over the yolk sphere, precluding further morphogenetic movements at gastrulation.
smp regulates expression of Ol-pou5f1, homologous to pou2/oct4

Because early smp expression has features reminiscent of Ol-pou5f1 (Fig. 2E,F), we tested whether smp regulates Ol-pou5f1 expression. We first examined its expression in smpMO-injected embryos. Decreased expression of Ol-pou5f1 was observed after MBT in deep cells of early gastrula stage embryos (stage 13; 10 out of 14 embryos), but not in the enveloping cells (not expressing smp, Fig. 8A,B). Later during gastrulation, no difference could be detected (data not shown). These data suggest that smp might be part of a network triggering the activation of Ol-pou5f1 zygotic expression soon after MBT, whereas, at later stages, the expression of Ol-pou5f1 might be under the control of other factors, including redundant proteins.

Another cue for the link between smp and Ol-pou5f1 was provided by smp gain-of-function experiments. When smpRNA (500 µg/ml) was injected at the two-cell stage, most embryos displayed a perturbed and sometimes delayed gastrulation, as well as abnormal body axes during early somitogenesis; they eventually recovered, but showed midbrain and hindbrain defects (data not shown). When smpRNA (500 µg/ml) was injected at the 32-cell stage, gastrulation movements were not affected (Fig. 8C,D). In injected embryos (54 out of 109), the cell progeny was excluded from the embryo axis at the 2somite stage (Fig. 8E,F), but it revealed no apparent modification of the cell number (Fig. 8C,D).

Ol-pou5f1 expression was further analyzed into smp-overexpressing embryos at the 2somite stage (stage 19) after two-cell stage injections (Fig. 8G-O). In control embryos, Ol-pou5f1 is predominantly expressed in the pluripotent cells of the tailbud and in the telencephalon (Fig. 8G). A massive ectopic expression of Ol-pou5f1 could be observed in the forebrain, in the mhb and in the hindbrain, and for pre-gastrula morphogenesis. Development 129, 905-916.

Development of a novel vertebrate-specific cell proliferation modulator

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Supplementary material
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