Int. J. Dev. Biol. 53: 525-533 (2009)
doi: 10.1387/ijdb.082720yy

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

The *mob as tumor suppressor (mats1)* gene is required for growth control in developing zebrafish embryos

YUAN YUAN[1], SHUO LIN[1,2], ZUOYAN ZHU[1], WENXIA ZHANG*[,1] and ZHI-CHUN LAI*[,1,3,4]

[1]*Center of Developmental Biology and Genetics, School of Life Sciences, Peking University, Beijing, China,* [2]*Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles, CA USA,* [3]*Department of Biology and* [4]*Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA USA*

ABSTRACT The *mob as tumor suppressor (mats)* family genes are highly conserved in evolution. The *Drosophila mats* gene functions in the Hippo signaling pathway to control tissue growth by regulating cell proliferation and apoptosis. However, nothing is known about whether *mats* family genes are required for the normal development of vertebrates. Here we report that zebrafish has three *mats* family genes. Expression of *mats1* is maternally activated and continues during embryogenesis. Through a morpholino-based knockdown approach, we found that *mats1* is required for normal embryonic development. Reduction of *mats1* function caused developmental delay, a phenotype similar to that of *Drosophila mats* homozygous mutants. Both cell proliferation and apoptosis were defective in *mats1* morphant embryos. Moreover, *mats1* morphant cells exhibited a growth advantage in chimeric embryos, similar to *mats* mutant cells in mosaic tissues in *Drosophila*. Therefore *mats1* plays a critical role in regulating cell proliferation and apoptosis during early development in zebrafish, and the role of *mats* family genes in growth regulation is conserved in both invertebrates and vertebrates. This work shows that zebrafish can be a good model organism for further analysis of Hippo signaling pathway.

KEY WORDS: *zebrafish, growth control, mob as tumor suppressor, hippo signaling*

Introduction

Hippo (Hpo) signaling plays a crucial role in controlling cell proliferation and apoptosis, and disruption of this growth regulatory mechanism causes tissue overgrowth in *Drosophila* (reviewed in Hariharan and Bilder, 2006; Harvey and Tapon, 2007; Pan, 2007; Saucedo and Edgar, 2007). While Hpo signaling is mediated through several tumor suppressor proteins such as Hippo (Hpo) protein kinase to activate Warts (Wts)/Large tumor suppressor (Lats) protein kinase, a Mob family protein Mats (Mob as tumor suppressor) is critical for activating the catalytic activity of Wts kinase (Lai *et al*., 2005; Wei *et al.,* 2007). Consequently, a growth-promoting transcription coactivator Yorkie (Yki) and the *Drosophila* ortholog of mammalian Yes-associated protein (YAP) are inhibited by Wts/Lats protein kinases via phosphorylation and cytoplasmic retention (Huang *et al.,* 2005; Dong *et al.,* 2007; Wei *et al*., 2007; Zhao *et al*., 2007; Hao *et al*., 2008). When Yki is present in the nucleus, the TEAD family transcription factor Scalloped (Sd) is turned on to promote tissue growth by forming a complex with Yki to directly activate transcription of target genes such as the *Drosophila inhibitor of apoptosis* (*diap1)* gene (Wu *et al*., 2008; Zhang *et al*., 2008). Although the Hpo signaling pathway has been extensively studied in *Drosophila*, much less is known about its components and physiological function in vertebrates.

The first Mob family protein was discovered in yeast as "Mps one binder protein" and shown to be a binding partner as well as a coactivator of protein kinases of the Ndr (nuclear Dbf2-related) family in regulating mitotic exit and cytokinesis (reviewed in Hergovich *et al*., 2006b). Mob proteins also have been studied in fly and mammalian cells in recent years. In *Drosophila*, Mats (also called dMob1) was discovered in 2005 as a coactivator of an Ndr family serine/threonine protein kinase Wts to control cell proliferation and apoptosis (Justice *et al*., 1995; Xu *et al*., 1995; Lai *et al*.,

Abbreviations used in this paper: Mats, mob as tumor suppressor.

***Address correspondence to:** Wenxia Zhang. College of Life Sciences, Peking University, Beijing, 100871, China. Fax: +86-10-6275-6185. e-mail: wxzhang@pku.edu.cn or Zhi-Chun Lai. Department of Biology, The Pennsylvania State University, 201 LSB, University Park, PA 16802, USA. Fax: +1-814-863-1357. e-mail: zcl1@psu.edu

Supplementary Material for this paper is available at: http://dx.doi.org/10.1387/ijdb.082723yy

Accepted: 15 July 2008. *Published online:* 20 January 2009. *Edited by:* Chrisopher Wylie.

ISSN: Online 1696-3547, Print 0214-6282

2005). Recently, Mats has been shown to be phosphorylated and activated by Hpo/Mst protein kinases in both fly and human cells (Wei *et al.*, 2007; Praskova *et al.*, 2008). Interestingly, while loss of *mats* function causes tissue overgrowth in mosaic flies (Lai *et al.*, 2005), mutants homozygous for *mats* are developmentally delayed and die at an early larval stage (He *et al.*, 2005; Shimizu *et al.*, 2008). *Drosophila* Mob family proteins also genetically interact with *tricornered* (*trc*), which is another Ndr family protein kinase in *Drosophila* and is required for the normal morphogenesis of a variety of polarized outgrowths (He *et al.*, 2005). In human, LATS1 interacts with MATS/MOBKL1, and hLATS1 activation may be mediated through rapid recruitment to the plasma membrane by hMATS (Hergovich *et al.*, 2005; Hergovich *et al.*, 2006a). Functionally, hLATS1/hMATS complex appears to be required for cytokinesis and mitotic exit (Yang *et al.*, 2004; Bothos *et al.*, 2005). Although a human *mats* ortholog *hMATS1* can rescue the lethality and tumor phenotypes of *Drosophila mats* mutants (Lai *et al.*, 2005), nothing is known about the physiological function of *mats* family genes during vertebrates development.

We chose zebrafish to investigate the role of *mats* in vertebrate development, since zebrafish provides a genetic model system to study early development and cancer-related genes (Amaruda *et al.*, 2002; Stern *et al.*, 2003; Berghmans *et al.*, 2005; Shepard *et al.*, 2005). Two *mats* orthologs have been identified in zebrafish (Lai *et al.*, 2005). Here, we report that the zebrafish genome has one more *mats* ortholog. We show that zebrafish *mats1* is maternally expressed and is also expressed throughout embryogenesis. Using a morpholino-based gene knockdown approach, we found that *mats1* is required for normal embryonic development, and is involved in regulating both cell proliferation and apoptosis. Similar to what was observed in *Drosophila*, *mats1* morphant cells seem to have a growth advantage over wild-type cells in chimeric zebrafish embryos. Our results suggest that growth regulatory properties of *mats* are conserved in vertebrates.

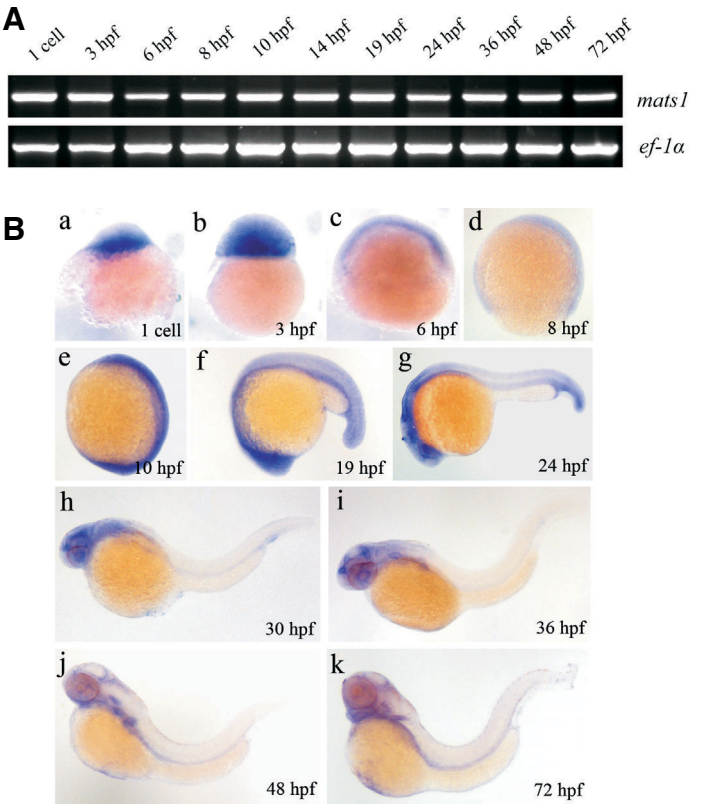

Fig. 1. *mats1* is expressed during zebrafish early development. (A) *Temporal expression of* mats1 *detected by RT-PCR.* ef-1a *is shown on the bottom panel as an internal control.* **(B)** *Expression of* mats1 *during early development detected by* in situ *hybridization. The animal pole is towards the top in (a-d). Anterior is towards the top and dorsal is towards right in (e). Anterior is towards left and dorsal towards top in (f-k). They are all showed by lateral view. Before 24 hpf, expression of* mats1 *is ubiquitous. After 24hpf,* mats1 *expression was observed in the head region of the body.*

Results

Three mats *orthologs exist in zebrafish*

Through a phylogenetic analysis, two orthologs of the *Drosophila mats* gene have been identified in vertebrates (Lai *et al.*, 2005). In zebrafish, *mats1* (also named *mobkl1b* for *Mps One Binder kinase activator-like 1b*) and *mats2* (also named *mobkl1a* for *Mps One Binder kinase activator-like 1a*) genes encode protein products that share 85 and 88% identity with the *Drosophila* Mats protein, respectively (Lai *et al.*, 2005; Supplementary Fig. S1A). Through synteny analysis, the arrangement of genes in the flanking regions of *mats1* and *mats2* was found to be highly conserved in zebrafish, mouse and human (Supplementary Fig. S1B and S1C). These results confirmed the orthologous relationships of *mats1* and *mats2* genes in these vertebrates. By searching the updated zebrafish genome database, we found that zebrafish has an additional *mats* ortholog, *mats3* (also named *mob4b*), whose intron-exon structure is identical to other vertebrate *mats* genes while other *mob* family genes have distinct intron-exon structures (X. Ye and Z.-C. Lai, unpublished results). Similar to Mats1 and Mats2, the zebrafish Mats3 protein is 88% identical to *Drosophila* Mats. As *mats3* is not found in other vertebrates, it is likely a product of gene duplications occurred after divergence of fish from other vertebrates. For clarity, the terms *mats1, mats2* and *mats3* refer to the above genes are used throughout this paper. In this study we have focused on *mats1* to investigate its developmental role in zebrafish embryos.

mats1 *mRNA is maternally stored and expressed during early embryonic development*

To facilitate functional analysis of *mats* genes, expression of *mats1* during early development was examined through RT-PCR and *in situ* hybridization. RT-PCR results showed that *mats1* mRNA was detected at the one-cell stage of embryonic development (Figure 1A). Thus, *mats1* mRNA is maternally provided. Moreover, *mats1* was continuously expressed throughout the first three days after fertilization, with some reduction at 6 hours post fertilization (hpf) (Figure 1A). RNA whole-mount *in situ* hybridization confirmed this result, and provided information about the spatial distribution of *mats1* mRNA (Figure 1B). Before 24 hpf, *mats1* was broadly expressed in the embryo (Figure 1B, a-g). After 24 hpf, expression of *mats1* was stronger in the head than in the trunk (Figure 1B, h-k). This expression

analysis suggests that *mats1* plays a role during embryonic development.

mats1 *is required for normal embryonic development*

Morpholino-based antisense oligonucleotides provide an efficient and specific means to block protein translation in zebrafish embryos (Nasevicius and Ekker, 2000; Draper *et al*., 2001). To investigate the function of *mats1* during embryogenesis, a translation-blocking morpholino (MO1) and a splice-blocking morpholino (MO2) were designed to knock down *mats1* expression (Figure 2A). Interestingly, both MO1 and MO2 caused a phenotype with developmental delay. MO1 was less effective since only 20-30% of morphant embryos exhibited the delay phenotype. In contrast, MO2 was much more effective; over 70% of the morphant embryos showed the delay phenotype when injected with 8.5 ng of *mats1* MO2. Among these abnormal *mats1* morphants, over 50% of them showed 16.5 hpf morphology, 20-30% with morphology between 16 and 18 hpf stages, and 10-20% with morphology between 18 and 20 hpf stages. This effect was concentration-dependent (data not shown). Consequently, *mats1* MO2 was used throughout this study.

To determine the efficiency and specificity of *mats1* morpholino treatment, RT-PCR was done at both 10 hpf and 24 hpf with primers corresponding to exons flanking the MO2 target site (Figure 2A). We found that MO2 treatment caused 70-80% reduction of–*mats1* expression in embryos showing severe developmental delay (Figure 2A, lanes 4 and 8), whereas normal-looking morphant embryos had less reduction of *mats1* expression (40-50% of the wild-type level) (Figure 2A, lanes 3 and 7). While MO2 binding appears to block correct splicing of *mats1* transcript, no aberrant splicing was observed. It is possible that the splice-modified *mats1* mRNA cannot be exported and consequently degraded in the nucleus. As internal controls, expression of the two other *mats* orthologs *mats2* and *mats3* was not affected

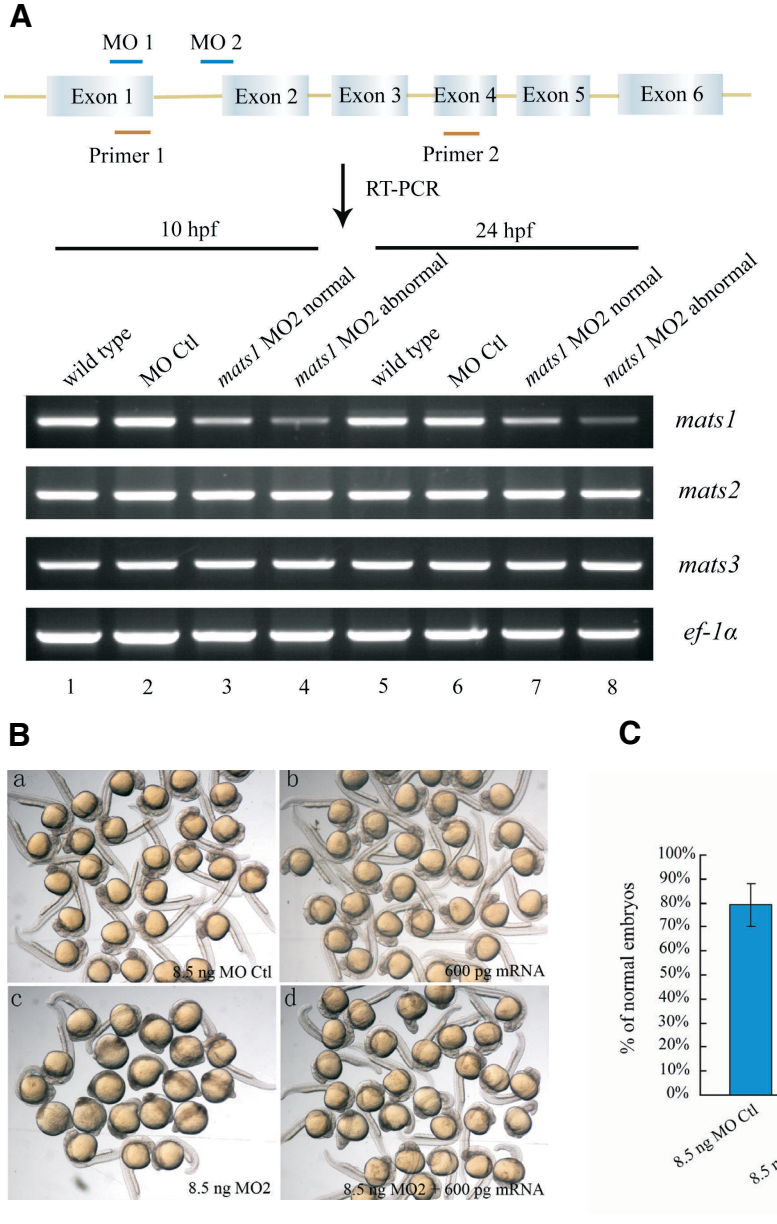

Fig. 2. Expression of *mats1* is reduced by morpholino treatment. (A) *Location of* mats1 *MOs and effect of* mats1 *MO2 on* mats1 *mRNA levels. The schematic structure of* mats1 *gene is shown, and the size is not in scale.* mats1 *MO1 binds to ATG site and* mats1 *MO2 binds to the intron1-exon2 boundary. RT-PCR was done with primers 1 and 2 to detect* mats1 *mRNA levels at 10 hpf and 24 hpf.* mats1 *MO2 morphants showing abnormal phenotype and normal phenotype were grouped separately. Expression of* mats1 *was reduced in* mats1 *MO2 morphants (lane 3-4 and lane 7-8) compared to wild-type (lane 1 and 5) and MO Ctl morphant (lane 2 and 6) embryos. Degree of the reduction was positively associated with severity of abnormal phenotype. As controls, mRNA levels of* mats2 *and* mats3 *were not affected.* ef-1α *was used as an internal control.* **(B)** *Rescue of* mats1 *MO2-induced developmental delay phenotype by* mats1 *mRNA. (a) Embryos injected with 8.5 ng MO Ctl as a control. (b) Embryos injected with 600 pg* mats1 *mRNA exhibited normal phenotype. (c) Embryos injected with 8.5 ng* mats1 *MO2 showed severe developmental delay. (d) Most embryos co-injected with 600 pg* mats1 *mRNA and 8.5 ng* mats1 *MO2 showed normal or less severe abnormal phenotype.* **(C)** *Rescue of* mats1 *morphants by* mats1 *mRNA is dosage-dependent. At 24 hpf, only 19% (n=181) of the* mats1 *MO2 morphant embryos were normal. However, co-injection of 300 pg* mats1 *mRNA with* mats1 *MO2 made 37% (n=142) of the embryos to become normal. When 600 pg* mats1 *mRNA was co-injected, 55% (n=196) of the embryos showed a normal phenotype. Although many remaining embryos still exhibited a developmental delay phenotype, the severity was decreased. Embryos injected with 8.5 ng MO Ctl were used as a control (n=134). All the statistical data included dead embryos. All the living embryos injected with 8.5 ng MO Ctl were normal. Standard errors were shown by the error bars.*

by *mats1* MO2 (Figure 2A). These results indicated that *mats1* mRNA levels can be effectively and specifically reduced by morpholino treatment.

Moreover, *mats1* mRNA was co-injected with *mats1* MO2 to test whether *mats1* mRNA is able to rescue the abnormal phenotypes induced by *mats1* MO2. While injection of *mats1* mRNA alone did not cause any abnormal phenotype (Figure 2B-b), co-injection of *mats1* mRNA with MO2 effectively rescued MO2 morphant embryos (Figure 2B, compare image (d) with (c)). With 300 or 600 pg *mats1* mRNA co-injection, normal-looking embryos increased from 19% to 36% and 55%, respectively (Figure 2C). Since 11 nucleotides which can be recognized by *mats1* MO2 still remained in the *in vitro* transcript *mats1* mRNA, titration of *mats1* MO2 by *mats1* mRNA might exist. To further confirmed that the developmental delay phenotype was specifically caused by *mats1* knock down, a putative *mats1* MO2-binding defective (MO2-bd in short) mRNA in which 5 nucleotides in the *mats1* MO2 binding region were mutated based on degeneracy of codons was also synthesized to do rescue experiment. With 20 pg MO2-bd mRNA co-injection, proportion of normal-looking embryos increased from 19 to 65% (Supplementary Figure S2 compare A with C). Consistently, while injected with 20 pg MO2-bd mRNA alone, no abnormal phenotype was observed (Supplementary Figure S2B). It seemed that the putative MO2-bd mRNA can rescue the MO2 morphant embryos much more effectively than wild type *mats1* mRNA. One explanation is that wild type *mats1* but not the mutant *mats1* mRNA can be targeted by MO2. These results support the idea that MO2-induced abnormalities were due to the reduction of *mats1* function.

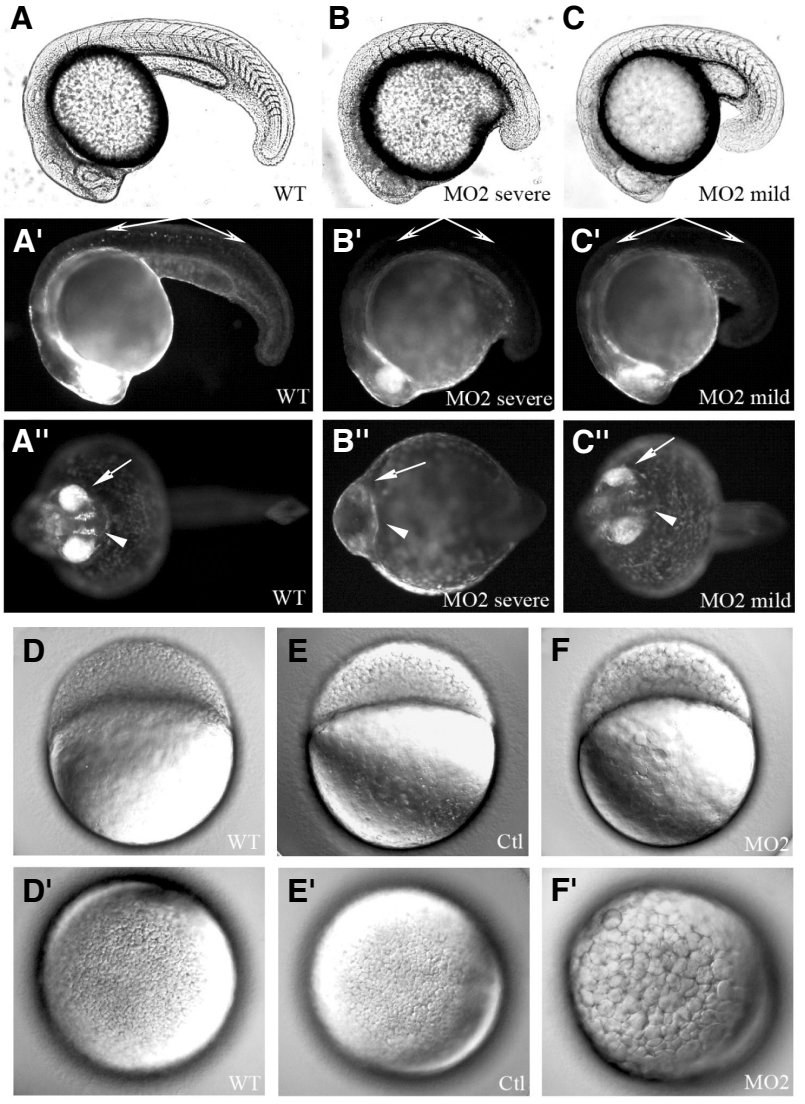

Fig. 3. The *mats1* gene is required for normal development of zebrafish embryos. (A-C") *24 hpf embryos of a transgenic line 1040 whose CNS is marked by GFP. Bright-field images in* **(A-C)**. *Fluorescent images of the same embryos shown in* **(A'-C")**. *Lateral view in (A-C'). Ventral view in (A"-C"). "Severe" represents* mats1 *MO2 morphants which showed severe developmental delay, while "mild" represents those showed a weaker developmental delay phenotype. At 24 hpf, some neurons in trunk (indicate by long arrow) had already emerged in wild-type embryos, but they were not observed in* mats1 *MO2 morphants. Eyes (indicated by short arrow) were either not visible* **(B")** *or less developed* **(C")** *in* mats1 *MO2 morphants. Similarly, the brain (indicate by arrow head) was less developed in* mats1 *MO2 morphants* **(B",C")**. **(D-F')** *Embryos at 3.5 hpf.* mats1 *MO2 morphants show developmental delay at very early stage. Lateral view with anterior towards left in* **(D-F)**. *Top view to see animal pole in* **(D'-F')**. mats1 *MO2 morphant embryos had fewer but bigger cells* **(F,F')** *compared to wild-type embryos* **(D,D')** *and embryos injected with MO Ctl* **(E,E')**, *suggesting that* mats1 *MO2 morphant cells divided less than control cells during the same period of time.*

Some morpholinos are known to activate the p53 pathway by an off-target effect (Robu *et al.*, 2007). As *mats1* morphant embryos exhibited elevated expression of *Δ113p53* (a truncated version of *p53*) and *p21* (a direct target of p53), we tested whether activation of p53 pathway contributes to the developmental defects of *mats1* morphants. To do this, we co-injected *p53* MO with *mats1* MO2 and confirmed that *p53* MO can effectively reduce *p21 and Δ113p53* (*p53* MO binds to *p53* start codon, so it can block *p53* translation without affecting RNA expression of *p53*) expression. However, *mats1*/*p53* morphant embryos still exhibited the developmental delay phenotype (Supplementary Figure S3A). These results further support that reduction of *mats1* function disrupted normal embryogenesis.

Reduction of *mats1* expression and function causes developmental delay. At 24 hpf, delayed *mats1* morphants only had 14-22 somites just like 16-20 hpf wild-type embryos, whereas wild-type siblings had 26 somites. More than 50% of *mats1* morphant embryos that showed severe developmental delay phenotype only had less than 16 somites. The trunk of *mats1* morphants was shorter and more curved (Figure 3, A-C). Given more time, *mats1* morphants developed more somites although their overall morphology was still abnormal. Development of the central nervous system was also delayed (Figure 3, A'-C' and A"-C"). Consistently, the head and eyes of *mats1* morphants usually were smaller. Assessment of marker genes like *no tail*, *goosecoid*, *frb35*, *pax2*, *myoD* showed that expression of these genes was delayed without changing their expression patterns (data not shown). The delayed phenotype was also found in *mats1* morphant embryos

at earlier stages. At 10 hpf, epiboly of siblings injected with MO Ctl was already complete but *mats1* morphants showed only 50-90% epiboly (Figure 4K). At about 3 hpf, when wild-type siblings reached the 1000 cell stage, the cleaving morphant embryos had fewer but larger cells, demonstrating that they divided less often than their wild-type siblings (Figure 3, compare F-F' with D-D' and E-E'). Thus, reduction of *mats1* function impedes embryonic development.

Knockdown of *mats1* function also reduced viability of the morphants. About 50-80% *mats1* morphants showing abnormalities survived five days post fertilization (dpf), while others died along the way. When wild-type embryos normally hatched from 48 hpf to 72 hpf, the *mats1* morphants did not hatch from the chorion, and consequently, only survived up to 5 dpf before using up the yolk. Those that successfully hatched were unable to escape and swim away when touched. Instead, they could only circle at the same location, likely due to defects in their neural and muscular systems. Moreover, some *mats1* morphants also exhibited defects such as pericardial expansion, reduced number of otoliths, and decreased density of blood cells. Thus, *mats1* function appears to be required throughout development in many tissues.

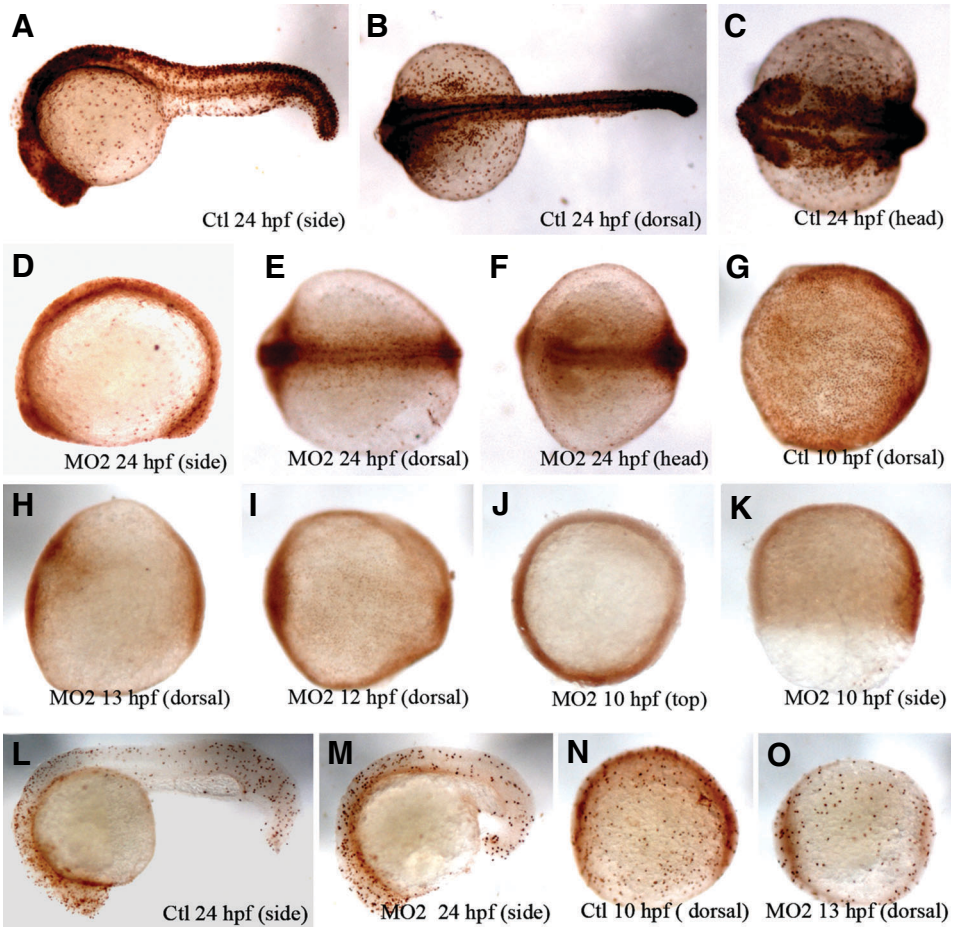

Fig. 4. Cell proliferation was decreased in *mats1* morphant embryos. (A-K) *BrdU staining results at 24 hpf and tail bud stage. At the 24 hpf stage, BrdU levels were reduced in* mats1 *morphant embryos* **(D-F)** *compared to control wild-type embryos* **(A-C)**. *While epiboly in wild-type embryos is finished at 10 hpf* **(G)**, *this process is not completed in* mats1 *MO2 morphant embryos with the same age* **(J, K)**. *Two to three more hours were needed for* mats1 *MO2 morphant embryos to reach tail bud stage (equivalent to 10 hpf of wild-type embryos at 28.5 °C) (H, I).* **(L-O)** *PH3 antibody staining results. PH3 antibody staining results at tail bud stage and 24 hpf were consistent with BrdU staining, although the decreased degree of marked cell is not as distinctive as BrdU staining results. Anterior is towards left in all panels except for* **(J,K)**. *Top view to see animal pole in (J), animal pole is towards top in (K).*

Defective cell proliferation in *mats1* morphant embryos

Our analysis has focused on growth defects of *mats1* morphant embryos. Because *mats1* morphants had fewer cells than wild-type siblings at the same age, cell proliferation and/or apoptosis were likely aberrant due to the reduction of *mats1* function. To test this idea, we first determined whether cell proliferation in *mats1* morphant embryos was defective. For this purpose, we used BrdU staining to mark S-phase cells and phosphohistone H3 (PH3) antibody staining to mark M-phase cells. At 24 hpf, BrdU incorporation decreased in *mats1* morphant embryos (36/41) compared to wild-type siblings and siblings injected with MO Ctl (Figure 4, A-F). This reduction of S-phase cells was more evident in *mats1* morphant embryos showing severely delayed phenotype than normal-looking morphant embryos. The same experiment was repeated with 10 hpf embryos, and the results were consistent with those of 24 hpf embryos (Figure 4, G-K). Since *mats1* morphant embryos were developmentally delayed, the decrease of S-phase cell number could be attributed to age differences between *mats1* morphant and control embryos. To test this, *mats1* morphant embryos were cultured for a few more hours until they reached the tail bud stage (equivalent to10 hpf in wild-type embryos at 28.5°C). Embryos that showed severe developmental delay needed 13 h to reach this stage, while those that showed a mild phenotype needed 11-12 h. Interestingly, these embryos still did not have the same number of S-phase cells as control embryos at 10 hpf (Figure 4, compare H-I with G).

PH3 antibody staining was done to identify mitotic cells in embryos. At an early stage (10-13 hpf), the number of mitotic cells in severely delayed *mats1* morphant embryos was decreased compared with uninjected siblings and embryos injected with MO control (about 50% of control embryos) (Figure 4, N-O). But at 24 hpf, the PH3 staining results didn't show marked difference when comparing *mats1* morphants with control embryos. The difference between BrdU and PH3 staining suggests that *mats1* may be involved in cell cycle control. To further test this idea, fluorescence-activated cell sorting (FACS) analysis was done with *mats1* morphant and control embryos at 24 hpf to see whether the ratios of cells at different phases of the cell cycle changed. We

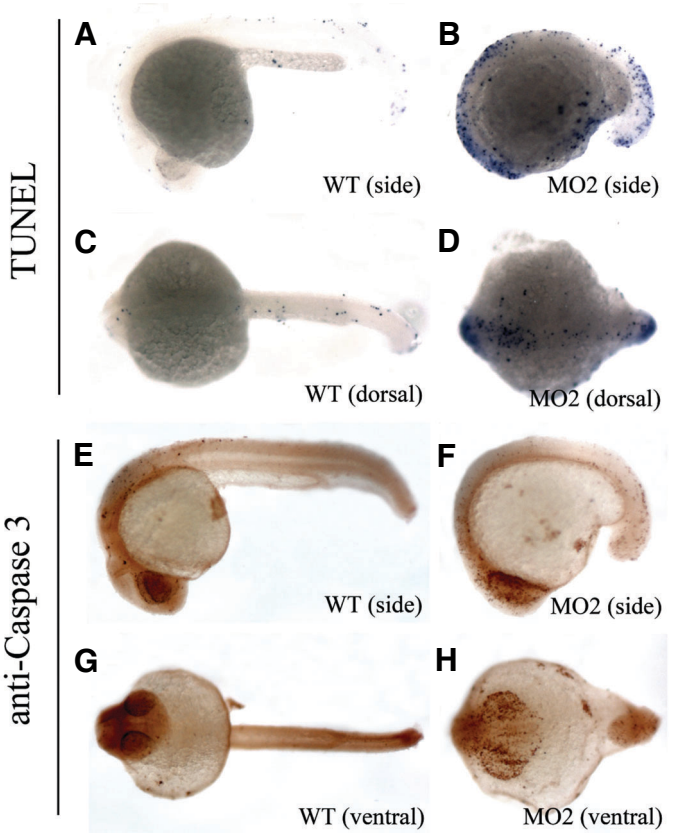

Fig. 5. Apoptosis was increased in *mats1* morphant embryos. (A-D) *TUNEL staining results at 24 hpf derived from wild type embryos* **(A,C)** *and MO2-treated embryos* **(B,D)**. **(E-H)** *Cleaved Caspase 3 antibody staining results at the same developmental stage with wild type embryos* **(E,G)** *and MO2 treated embryos* **(F,H)**. *Anterior is towards left in all panels. Both of TUNEL and cleaved Caspase 3 antibody-staining results showed that apoptosis increased in* mats1 *morphant embryos.*

found that the ratio of cells at the M-phase in *mats1* morphant embryos was more than that of the control embryos (13.33±0.57* vs. 9.60±0.20*, "*" represents standard error, t-test, p<0.005), while the ratio at S-phase didn't change much in *mats1* mrophant embryos compared to control embryos (29.68±1.00* vs. 30.82 ± 0.74*, "*" represents standard error). These results indicate that loss of *mats1* function may have mitotic defects that cause the accumulation of mitotic cells.

Apoptosis increased in mats1 *morphant embryos*

To test whether *mats1* is involved in regulating cell death, the TUNEL assay was done at 24 hpf, when apoptosis normally occurs in developing embryos. Compared to control embryos (Figure 5, A and C), *mats1* morphant embryos showed an increase in TUNEL-positive cells (29/38) (Figure 5, B and D). Thus, more cells died in *mats1* morphant embryos. The results are similar when coinjected with *p53* MO. Thirty-nine out of 46 *mats1*/*p53* morphant embryos still exhibited excessive apoptosis with decreased signal in head region (Figure S3B), which are consistent with a former report (Robu *et al.*, 2007).

To determine whether cell death in *mats1* morphant embryos is mediated through apoptosis, cleaved Caspase 3 antibody staining was done to specifically label apoptotic cells. The results of cleaved Caspase3 staining were consistent with the TUNEL results (Figure 5, E-H). Compared to control embryos (Figure 5, E and G), *mats1* morphant embryos clearly exhibited increased apoptosis at 24 hpf (50/50), mainly in the head and caudal parts (Figure 5, F and H). Thus, knockdown of *mats1* leads to increased cell death, and this occurs mainly through apoptosis.

mats1 *morphant cells have a growth advantage in chimeric zebrafish embryos*

Loss of *mats* function causes mutant cells to overproliferate in mosaic fruit flies (Lai *et al.*, 2005). To determine how *mats1*-deficient cells might behave when surrounded by normal cells in zebrafish, we carried out cell transplantation experiments to generate *mats1* chimeric embryos (Supplementary Figure S4). From three independent experiments, four hundred thirty one *mats1* morphant cells and 522 cells from embryos injected with MO Ctl were transplanted into more than 130 embryos at the 3-4 hpf stage. By 10 hpf, they had proliferated to generate 3,010 and 2,840 cells, respectively. Therefore, their respective proliferation index (PI) was 6.98 and 5.44 (t-test, p <0.0001; Table 1). From two other control experiments, the proliferation index of cells from embryos injected with FITC or Rhodamine were 5.84 and 5.59 respectively, similar to cells from embryos injected with MO Ctl (Table 1). Similar to control normal cells, *mats1* morphant cells distributed throughout the embryo. These results suggested that *mats1* morphant cells in chimeric embryos had a growth advantage and proliferated more than normal cells. Thus, the growth inhibitory activity of *mats* family genes appears to be conserved in zebrafish.

Discussion

All vertebrate Mats proteins share extremely high levels of sequence identity with *Drosophila* Mats. Yet functional significance of *mats* genes in vertebrate development has not been investigated. On the basis of functional analysis of *mats* in *Drosophila* (Lai *et al.*, 2005; Wei *et al.*, 2007; Shimizu *et al.*, 2008), we hypothesized that zebrafish *mats1* plays a critical role during embryogenesis. Supporting this idea, *mats1* is expressed in developing embryos. Using a morpholino-based loss-of-function analysis, we found that *mats1* plays a critical role in regulating cell proliferation and apoptosis in early embryos. Similar to *Drosophila* homozygous *mats* mutants (He *et al.*, 2005; Shimizu *et al.*, 2008), reduction of *mats1* expression caused severe develop-

TABLE 1

***MATS1* MORPHANT CELLS PROLIFERATE FASTER THAN WILD-TYPE CELLS IN CHIMERIC EMBRYOS**

	N1 (total)	N2 (total)	PI on average
mats1 MO2	431	3010	6.98 ± 0.37*
MO Ctl	522	2840	5.44 ± 0.27*
FITC Ctl	69	395	5.72 ± 0.08*
Rhodamine Ctl	50	282	5.64 ± 0.13*

Proliferation index (PI, defined by N2/N1) of four types of cells. N1 refers to the cell number at 3-4 hpf, and N2 refers to the cell number at 10 hpf. *mats1* MO2:*mats1* MO2 morphant cells. MO Ctl: cells from embryos injected with control MO. FITC Ctl: cells from embryos which were only injected with FITC. Rhodamine Ctl: cells from embryos which were only injected with Rhodamine. "*" represents standard error. It is calculated with average proliferation index derived from three independent transplantation experiments, and represents the deviation of PI on average.

mental delay of the zebrafish morphant embryos, which exhibited reduced cell proliferation and increased apoptosis. This is the first time that a *mob* family gene is shown to be required for normal embryogenesis in vertebrates. Further studies shall reveal how Hippo signaling might function to control early development in vertebrate animals.

Growth inhibitory role of mats *appears to be conserved in zebrafish*

mats was first discovered in *Drosophila* as a tumor suppressor because loss of *mats* function caused tissue overgrowth in mosaic flies (Lai *et al*., 2005). In this study we have tested whether the growth inhibitory activity of *mats* family gene is conserved in vertebrates by generating chimeric zebrafish embryos through cell transplantation. Interestingly, chimeric analysis showed that *mats1* morphant cells proliferated faster than control normal cells, just like *mats* mutant cells in mosaic fly tissues. It is less likely that the increase of *mats1* morphant cell number was caused by inhibition of cell death, since cell death barely occurs before 10 hpf. Thus, this growth-inhibitory role of *mats* appears to be evolutionarily conserved between invertebrates and vertebrates. *mats1* morphant cells in chimeric embryos had a growth advantage likely through competition with neighboring wild-type cells. To acquire such a growth advantage, *mats1* morphant cells may need to be stimulated by surrounding wild-type cells, or capable of inhibiting growth of neighboring wild-type tissues. Although the mechanism by which *mats1* morphant cells acquire growth advantage in the context of chimeric embryos is presently unknown, cell-cell interaction should play a critical role in this process. A future challenge is to reveal the molecular basis of this intercellular interaction critical for tissue growth control during animal development.

Materials and Methods

Animals

The wild-type Tübingen strain or Tübingen/AB stain zebrafish and a transgenic line 1040 whose central nervous system is marked by GFP were used in this study. Zebrafish embryos were obtained by natural spawning, raised at 28.5 °C (±0.5 °C) in Holtfreter's solution and the developmental stage determined as described by KIMMEL *et al.* (1995).

Whole-mount in situ *hybridization*

Digoxigenin-labeled antisense RNA probes were generated *in vitro* by using a zebrafish *mats1* full-length cDNA as template with T7 or SP6 RNA polymerase (Promega, Madison, WI). Whole-mount RNA *in situ* hybridizations were performed essentially as described in The Zebrafish Book (Westerfield *et al*., 1995) on embryos at the following developmental stages: 1 cell, 3 hours after fertilization (hpf), 6 hpf, 8 hpf, 10 hpf, 19 hpf, 24 hpf, 30 hpf, 36 hpf, 48 hpf and 72 hpf.

In vitro *mRNA synthesis*

Capped mRNAs were transcribed from linearized DNA using T7, T3 and SP6 RNA polymerase *in vitro* transcription kits according to the manufacturer's instruction (mMESSAGE, mMACHINE; Ambion). The pCMV-SPORT6 and pXT7 vectors were used. Moreover, site-directed mutagenesis through fusion PCR was done to make five silent nucleotide replacements within the MO2-binding site of *mats1* to convert 5'-CCATTTGATTTCAGC GGA AAC CGT T-3' to 5'- CCATTTGATTTCAGt GGg AAt aGg T -3'. This mutant *mats1* still encodes a wild-type Mats protein product and its mRNA was synthesized *in vitro* as described above for the rescue experiment.

Microinjection

Capped mRNAs and morpholinos were dissolved and diluted in nuclease-free water to an appropriate concentration. They were injected into 1- to 2-cell-stage embryos in yolk. The amount of injection was determined by measuring the volume of liquid injected into a 1-μl capillary glass (34 mm long) using a ruler, and volume per microinjection was thereby calculated.

Morpholino design and phenotypic analysis

One translation-blocking morpholino (MO1) and one splice-blocking morpholino (MO2) of *mats1* were synthesized by Open Biosystems, Inc. (Huntsville, AL). The sequence of MO1 is:
5'-TTCCGAATAAGAAACTCATCTCCGC-3', which corresponds to the start codon region, and the sequence of MO2 is:
5'-AACGGTTTCCGCTGAAATCAAATGG-3', which corresponds to the putative intron1-exon2 boundary of *mats1*.

A translation blocking morpholino targeting *p53* from Gene Tools (Philomathe, OR) was used to eliminate *p53* dependent off-target effect, its sequence is 5'-GCGCCATTGCTTTGCAAGAATTG-3'. A standard control morpholino oligo (MO Ctl) from Gene Tools (Philomathe, OR) was used as control. Its sequence is 5'-CCTCTTACCTCAGTTACAATTTATA-3'.

Injected embryos were cultured in Holtfreter's solution at 28.5 °C (±0.5 °C). An embryo was considered abnormal if it showed less than 90% epiboly when its siblings injected with the same mount of MO Ctl exhibited complete epiboly at the 10 hpf stage, or if it appeared younger than 20 hpf (defined by its overall look and somite number) compared to its siblings injected with the same mount of MO Ctl at the 24 hpf stage.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and quantification

For detection of expression pattern of *mats1* during early development, total RNA was extracted from wild-type embryos at various stages with Trizol reagent (Invitrogen, Carlsbad, CA). To detect splice-blocking variants of *mats1* mRNA with RT-PCR, total RNA was extracted from the morphant and control embryos (injected with MO Ctl at the same quantity and uninjected wild-type siblings) at 10 hpf and 24 hpf. Reverse transcription was carried out with random nonamer by using M-MLV reverse transcriptase (Promega, Madison, WI). Two rounds of PCR were performed. Primers used for detection of zebrafish *mats1* mRNA [GenBank: BC045979] were *mats1* forward (base pairs 273-297): 5'-GAAGAAGAAGGACAAGCGGAGATG -3', and *mats1* reverse (base pairs 758-734): 5'-CAGACGCTTCAGGATCGTTTTAGC-3'. The product size of *mats1* RT-PCR is 485 bp. Zebrafish *mats2* [GenBank: BC045952] and *mats3* [GenBank: NM_214783] mRNA levels were determined with following primers: *mats2* forward (base pairs 1-20): 5'-AGAAGTTTTCCACGGGCAGG-3', and *mats2* reverse (base pairs 393-374): 5'-GCAGCTTTCCTCAGTGCAGA-3', the product size of *mats2* is 393 bp; *mats3* forward (base pairs 105-126): 5'-AAGCCGAAGAAGAATA TTCCTG-3', *mats3* reverse (base pairs 612-591): 5'-AAGAGGTGTTGAGGTGAGCTTC -3', and the product size of *mats3* is 508 bp. Zebrafish *elongation factor-1α* (*ef-1α*) [GenBank NM_131263] was used as an internal control. The *ef-1α* primers were *ef-1α* forward (base pairs 496-516): 5'-TCACCCTGGGAGTGAAACAGC-3', and *ef-1α* reverse (base pairs 1188-1168): 5'-ACTTGCAGGCGATGTGAGCAG-3'. The product size of *ef-1α* is 692 bp.

The quantification of RT-PCR bands was done with BandScan 5.0.

BrdU staining

BrdU (5-bromo-2-deoxyuridine) (Roche Diagnostics, Mannheim, Germany) staining was performed to label S-phase cells. It was done essentially according to the protocol in Methods in Cell Biology (Detrich

et al., 2004). Embryos were dechorionated and chilled 15 minutes on ice in Holtfreter's solution, then placed in cold 10 mM BrdU/15% Dimethylsulfoxide (DMSO) in Holtfreter's solution and chilled on ice for 20 minutes to allow uptake of BrdU. Then embryos were changed into warm Holtfreter's solution and incubated exactly 5 minutes at 28.5 °C. They were fixed 2 hours at room temperature in PFA (4% paraformaldehyde buffered with 1 x PBS), dehydrated in graded methanol:PBS series (1:3,1:1, 3:1) and preserved in methanol at -20 °C at least overnight. Rehydrated in graded methanol:PBST [1xPBS with 0.1% (v/v) Tween-20] series (3:1,1:1,1:3) for 5 minutes each, followed by in PBST twice, 5 minutes each. Embryos were digested in 10 μg/ml proteinase K for 10 minutes, washed in PBST for 5 minutes, and refixed in PFA for no more than 20 minutes, followed by 3 times quick washes in H_2O with 0.1% (v/v) Tween-20 and 2 times in 2N HCl. Embryos were incubated 1 hour in 2N HCl to denature the labeled DNA to expose the BrdU epitope, rinsed several times in PBST to bring the pH back up to approximately 7 before adding blocking solution, and then blocked for at least 30 minutes in blocking solution (1% DMSO + 0.1% Tween-20 + 1% BSA + 2% serum in PBS). After that, embryos were incubated in monoclonal anti-BrdU antibody (Zhong Shan Jin Qiao) at a dilution of 1:100 in blocking solution overnight at 4 °C. Subsequently, embryos were washed in PBST five times, 10 minutes each, and incubated in horseradish peroxidase-conjugated anti-mouse secondary antibody about 2 hours at room temperature. Finally, embryos were washed in PBST five times, 10 minutes each. Color reaction was developed with diaminobenzidine.

PH3 and cleaved Caspase 3 antibody staining

Mitotic cells were stained with a rat polyclonal anti phosphorylated histone H3 antibody from Upstate Biotechnology (Charlottesville, VA). It was performed essentially as described in The Zebrafish Book (Westerfield *et al*., 1995), with a dilution of 1:1000, and the horseradish peroxidase-coupled secondary antibody was diluted 1:250. Cleaved caspase3 antibody staining was performed to detect apoptotic cells. The method is basically the same with PH3 staining, and the cleaved Caspase-3 (Asp175) antibody from Cell Signaling Technology, Inc. (Beverly, MA) was diluted 1:100 and the horseradish peroxidase-coupled secondary antibody was diluted 1:250.

TUNEL staining

For detection of cell death, terminal desoxynucleotidyl transferase mediated biotinylated UTP nick end labeling (TUNEL) was performed, using the *In situ* Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). Embryos were dechorionated and fixed overnight at 4 °C in PFA, dehydrated in a graded ethanol:PBS series (1:3,1:1,3:1), then preserved in ethanol at -20 °C for at least 30 minutes. Embryos were rehydrated in a graded ethanol:PBST series (3:1,1:1,1:3) for 5 minutes each, washed in PBST for 5 minutes, then digested in proteinase K (10 μg/ml) at room temperature for 10 minutes. After being washed twice in PBST, embryos were postfixed in PFA for 20 minutes at room temperature. With 5 times washes in PBST, 5 minutes each, embryos were postfixed for 10 minutes at -20 °C with pre chilled ethanol:acetic acid (2:1). After being washed in PBST three times, 5 minutes each at room temperature, embryos were used for TUNEL assay according to manufacture's instruction.

Cell transplantation

Eggs were collected and raised in Holtfreter's solution with 50 units penicillin and 50 μg streptomycin per ml (Gibco). Eggs that were used as donors were microinjected with Fluorescein isothiocyanate-dextran (FTIC) (Sigma Chemical Co., St. Louis, MO) or tetramethylrhodamine-dextran (TMR) (Invitrogen) (5% in nuclease-free water) or one combination of *mats1* MO2 or MO Ctl with one of these two dyes. Cell transplantation began at the 1000-2000 cells stage. Since *mats1* MO2 could cause developmental delay started before the 1000 cell stage, embryos injected with *mats1* MO2 were raised at 28.5 °C (±0.5 °C) while the sibling embryos injected with MO Ctl and embryos without injection were raised at 25.5 °C (±0.5 °C) to adjust their development stage. The temperature was calculated using the formula described in (Kimmel *et al*., 1995). To maximally eliminate the difference of proliferation ability among cells from different location, all the transplanted cells were fetched in the center of the deep layers of injected embryos. One to five transplanted cells from each donor embryos were loaded by suction, and then were injected among the deep cells at the center of the same wild-type sibling at the same stage, without damaging the yolk cell. The numbers of each marked cells were counted (record as N1) under fluorescent microscope after cell transplantation was done (usually about 1 hour from the first transplant was done) one by one in the order of transplantation, and counted again at about 10 hpf (record as N2). N2 divided with N1 is the index of cell proliferation ability.

Imaging

Images of zebrafish embryos were acquired by using an Imager Z1 or Stemi 2000-C microscope equipped with an AxioCam digital camera and AxioVission software (Zeiss, Oberkochen, Germany), and then edited with PhotoShop CS2 9.0 (Adobe Systems, San Jose, CA).

Acknowledgement

We would like to thank Qichang Fan, Bo Zhang, Chuanmao Zhang and Shiqiang Wang at Peking University, Feng Shao at the National Institute of Biological Science, Beijing, Anming Meng at Tsinghua University for discussion, reagents, zebrafish lines and apparatus support. We thank Xing Ma and Yongfei Yang for participation in part of the project, and Li-Lun Ho for help order morpholinos. We also thank Iain Bruce for revising manuscript. This work was supported by the National Natural Science Foundation of China (Grant No. 30270186 and No. 30510403188) and by a grant to Z.-C.L. from Pennsylvania Formula Tobacco Settlement Funds.

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