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Mps1 defines a proximal blastemal proliferative compartment essential for zebrafish fin regeneration

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SUMMARY

One possible reason why regeneration remains enigmatic is that the dominant organisms used for studying regeneration are not amenable to genetic approaches. We mutagenized zebrafish and screened for temperature-sensitive defects in adult fin regeneration. The *nightcap* mutant showed a defect in fin regeneration that was first apparent at the onset of regenerative outgrowth. Positional cloning revealed that *nightcap* encodes the zebrafish orthologue of *mps1*, a kinase required for the mitotic checkpoint. *mps1* expression was specifically induced in the proximal regeneration blastema, a group of cells that normally proliferate intensely during outgrowth. The *nightcap* mutation caused severe defects in these cells. However, *msxb*-expressing blastemal cells immediately distal to this proliferative region did not induce *mps1* and were retained in mutants. These results indicate that the proximal blastema comprises an essential subpopulation of the fin regenerate defined by the induction and function of Mps1. Furthermore, we show that molecular mechanisms of complex tissue regeneration can now be dissected using zebrafish genetics.

Key words: Zebrafish, Fin, Regeneration, Blastema, Mps1

INTRODUCTION

Human organs are subjected to a variety of insults and injuries, but have a limited ability to heal and regenerate damaged or lost tissue. Natural scientists have actively pursued the problem of regeneration since the 17th century, largely by utilizing lower vertebrate species possessing exceptional regenerative capacities (Dinsmore, 1991). Newts are the primary experimental model used to study vertebrate regeneration, as they can regrow a striking number of adult structures, including limbs, tail, spinal cord, jaws, tongue, lens and optic nerve (Brockes, 1997; Ferretti and Géraudie, 1998). While genetic approaches have been successfully applied to dissect many developmental, physiological and behavioral processes, and could conceivably be applied to reveal factors required for regeneration, newts and other highly regenerative amphibians are not suitable for this analysis. This is because of their long generation times, enormous genomes, and the difficulty of maintaining large numbers of animals.

In contrast, because of their amenability to genetic manipulation, zebrafish have proved to be a valuable laboratory model for understanding many aspects of vertebrate embryogenesis. Small- and large-scale mutagenesis screens have yielded hundreds of interesting mutants, from which dozens of genes essential for ontogeny have been identified (see Driever et al., 1996; Gaiano et al., 1996; Haffter et al., 1996; Zhang et al., 1998). Somewhat overlooked is the fact that zebrafish can regenerate an impressive number of structures as adults, such as spinal cord, optic nerve, scales, and each of five types of fins (Johnson and Weston, 1995; Bernhardt et al., 1996; Becker et al., 1997).

For several reasons, the fin is an excellent model organ for studying regeneration. First, fins have a simple architecture, consisting of several segmented, bony fin rays composed of concave, facing hemirays that surround connective tissue, nerves and blood vessels (Ferretti and Géraudie, 1998). Second, surgery is nearly effortless, so that hundreds of amputations per hour may be performed. Third, regeneration is rapid and reliable, with most structures replaced within 1-2 weeks. Finally, zebrafish unable to regenerate fins survive normally despite their wounds, allowing the recovery of mutant founders from genetic screens (Johnson and Weston, 1995).

Fin regeneration can be broken down into four stages (Poss et al., 2000a). First, epidermal cells migrate to cover the wound and form a multilayered cap. Second, mesenchymal tissue down to two segments beneath the new epidermis disorganizes, or dedifferentiates, and mesenchymal cells migrate distally toward the amputation plane (Poleo et al., 2001). Third, these cells proliferate and accumulate to form the regeneration blastema, a tissue mass from which the new fin structures are

ultimately derived. Regeneration is completed by a phase of outgrowth, composed of exquisitely integrated proliferation, patterning, and differentiation events. Recent work has demonstrated that the distal regenerate is divided into three compartments during regenerative outgrowth (Nechiporuk and Keating, 2002). Distal blastemal cells are essentially nonproliferative and express *msxb*, a transcriptional repressor gene that is induced during blastema formation and might function to activate cellular dedifferentiation in mesenchyme underlying the wound epidermis (Akimenko et al., 1995; Poss et al., 2000a; Odelberg et al., 2000). More proximal blastemal cells are highly proliferative, and show high bromodeoxyuridine (BrdU) incorporation and mitotic indices with a very brief G_2 cell cycle phase. Proximal to this region is a patterning zone, where proliferation is less intense and bone-depositing scleroblasts align. We have postulated that the distal blastema might function to specify the direction of regenerative outgrowth, while the proximal blastema drives regeneneration (Nechiporuk and Keating, 2002). However, these regeneration zones have not been functionally dissected, as no agents or mutations that affect a specific region of the blastema have been discovered.

To find genes that mediate fin regeneration we treated zebrafish with *N*-ethyl-*N*-nitrosourea (ENU) and screened mutagenized families, as adults, for individuals unable to regenerate amputated caudal fins. We identified the *nightcap* (*ncp*) mutant, a strain that fails during regenerative outgrowth owing to severe blastemal proliferative defects. We then used positional cloning to demonstrate that this regenerative block is caused by a temperature-sensitive mutation in the zebrafish orthologue of *mps1*, a cell cycle regulator that is specifically upregulated in the proximal fin blastema. Our findings indicate that proximal blastemal cells are required to support proliferation through *mps1* expression and function, and that compartmentalization of the blastema is crucial for regeneration. Thus, through a molecular genetic approach, we propose a molecular and cellular model for blastemal function during regeneration.

MATERIALS AND METHODS

Mutagenesis and screen for fin regeneration mutants

Males of the C32 inbred zebrafish strain were mutagenized using published protocols and mated to females of the SJD inbred strain (Mullins et al., 1994). Eggs were squeezed from F_1 females (heterozygous for mutated genomes) and subjected to early pressure parthenogenesis (Streisinger et al., 1981). The resulting families were raised at 24-25°C until 2-3 months of age. At this point, one-half of the caudal fin was amputated from each fish, and families were shifted to a recirculating system heated at 33-34°C, for 1 week. After this period, fish were analyzed individually for regeneration defects, using a dissecting microscope. One of the two *ncp* founders was initially outcrossed to a C32 female; the *ncp* mutation was then outcrossed twice to the WIK zebrafish strain and maintained through *ncp* crosses, *ncp*/+ crosses, or *ncp* × *ncp*/+ crosses.

Immunohistochemistry

Hematoxylin staining and whole-mount in situ hybridization were performed as described previously (Poss et al., 2000a). To generate digoxigenin-labeled probes for this study, we used a full-length 3.2 kb *mps1* cDNA (EST fi61a12) and a full-length 1.2 kb *msxb* probe (Akimenko et al., 1995). Immunohistochemistry on sectioned tissue was performed as described previously (Poss et al., 2000b), using the monoclonal antibody Zns-5 (Johnson and Weston, 1995). For simultaneous detection of *mps1* mRNA and PCNA, a monoclonal anti-PCNA antibody (Oncogene, 1:100 dilution) was added following in situ hybridization during fin incubation with anti-digoxigenin antibody coupled to alkaline phosphatase. Fins were then washed for at least 2 hours in multiple changes of phosphate-buffered saline (PBS)-0.1% Tween 20 (PBT), followed by a final wash in PBT with 2 mg/ml bovine serum albumin (PBTs). Fins were treated with anti-mouse secondary antibodies coupled to Alexa-488 (Molecular Probes) in PBTs overnight at 4°C. On the following day, fins were washed in multiple changes of PBT for at least 2 hours and processed for an alkaline phosphatase reaction using the NBT/BCIP substrate. Following the detection reaction, fins were rinsed several times with PBT and processed for cryosectioning as described previously (Poss et al., 2000a). Frozen blocks were sectioned at 14 μm, mounted using Vectashield with DAPI (Vector), and digital images were captured using an Axiocam CCD camera equipped with Axiovision software (Zeiss).

For BrdU incorporation experiments, animals were injected intraperitoneally with a 2.5 mg/ml solution of BrdU. To detect BrdU and H3P by whole-mount immunostaining, fin regenerates were incubated in Carnoy's fixative (60% ethanol, 30% chloroform, and 10% acetic acid) overnight at 4°C and stained as described (Newmark and Sanchez Alvarado, 2000). Fins were washed twice in methanol and rehydrated through a methanol/PBS+0.3% Triton X-100 (PBTx) series. They were then washed twice in 2 N HCl in PBTx and incubated in 2 N HCl in PBTx for 30 minutes, followed by two rinses in PBTx and blocking for 4 hours in (PBTx + 0.25% BSA). Then, fins were incubated with anti-BrdU monoclonal antibodies (Chemicon International Inc., 1:100) and rabbit polyclonal anti-H3P antibody (Upstate Biotechnology, 1:200) overnight at 4°C. The next day, fins were washed in several changes of PBTx (last wash PBTx + 0.25% BSA) and incubated overnight at 4°C in Alexa 594-coupled goat anti-mouse antibodies and Alexa 488-coupled goat anti-rabbit antibodies (Molecular Probes), both diluted 1:200 in PBTx. Whole-mount regenerates were washed several times in PBS and examined by laser confocal microscopy (410 LSM, Zeiss).

The number of mitoses per regenerating ray was counted using three-dimensional projections of confocal images through the entire depth of the fin (100 μm). Epidermal cells developed a nonspecific cytoplasmic fluorescence after H3P staining and secondary antibody detection. This fluoresence was observed at the distal epidermal edges of the regenerate in sections of both wild-type and *mps1* fins (see Fig. 6C). Therefore, it was relatively easy to limit scans to mesenchymal tissue by setting the confocal depth range to just within the highly stained epidermis. In wild-type regenerates during outgrowth, this mesenchymal region was 45-55 μm. Longitudinal sections and work from a previous study confirmed that mesenchymal mitoses far outnumber epidermal mitoses in the distal regenerate (see Nechiporuk and Keating, 2002).

Genetic mapping and positional cloning

We assigned the *ncp* gene to LG16 by centromere-linkage analysis, using CA-repeat markers (Shimoda et al., 1999) to genotype 51 progeny generated by early pressure of *ncp*/+ oocytes (Johnson et al., 1996). CA-repeat markers and ESTs (Hukriede et al., 2001) previously localized to the vicinity of the *ncp* were then utilized to finely map the *ncp* mutation in *ncp* × *ncp*/+ crosses. All progeny were raised to 2-3 months at 25°C, before phenotyping for regeneration defects at 33°C. Genotyping information from both *ncp* and *ncp*/+ progeny were used in mapping experiments.

Zebrafish YAC clone pools (Research Genetics) were screened by PCR for fc38g10 (Clark et al., 2001) and z6506 sequences (Zhong et al., 1998). DNA from YAC43G3 was subcloned into the SuperCos1 cosmid vector (Stratagene) according to manufacturer's protocol. Cosmid clones were screened with labeled zebrafish genomic DNA to select those inserts containing repetitive zebrafish DNA. A cosmid

contig was assembled using recombination data generated from use of cosmid end sequences. After obtaining a critical region of 80 kb, cosmid inserts were labeled and hybridized to cDNA library filters generated from 28-hour embryos or adult fin regenerates. *mps1* and *bckdhb* cDNA clones were obtained through hybridization and, after observing synteny with human 6p14, from commercial sources (Incyte). To find the *ncp* mutation, we amplified 400 bp *mps1* fragments from wild-type and *ncp* mutant cDNAs, and sequenced these using a 3100 Genetic Analyzer (Applied Biosystems).

Mitotic checkpoint analysis

24-hour postfertilization embryos were dechorionated, disaggregated with a kontes pestle in 250 μl trypsin solution, and triturated for 10 minutes. The trypsin was inactivated with 250 μl of 20% FBS/DMEM, and the suspension passed through 100 μm mesh and then 40 μm mesh. Cells were centrifuged for 10 minutes, and the pellet was resuspended in 250 μl PBS/embryo. 10 μl/ml of Triton X-100 was added to the suspension, followed by 20 μl/ml of 0.1 mg/ml DAPI. Cells were kept on ice until flow cytometic analysis. Each sample comprised cells from 1-4 embryos; 10 wild-type samples and 12 *ncp* samples were examined.

RESULTS

Identification of the *ncp* regeneration mutant

To discover zebrafish defective in fin regeneration, we treated male zebrafish with ENU and generated mutagenized families by parthenogenesis, as shown in Fig. 1A. We raised these families to adulthood at 25°C, amputated caudal fins, and shifted them to 33°C for 7 days before assessing regeneration. We looked for conditional, temperature-sensitive effects on regeneration, as we predicted that many genes involved in regeneration also function during embryogenesis. An adult fin regeneration screen designed to detect only strong alleles would fail to recover mutations in these genes. Importantly, wild-type fin regeneration is completed without error at 33°C; in fact, regeneration proceeds nearly twice as quickly at 33°C as at 25°C (Johnson and Weston, 1995).

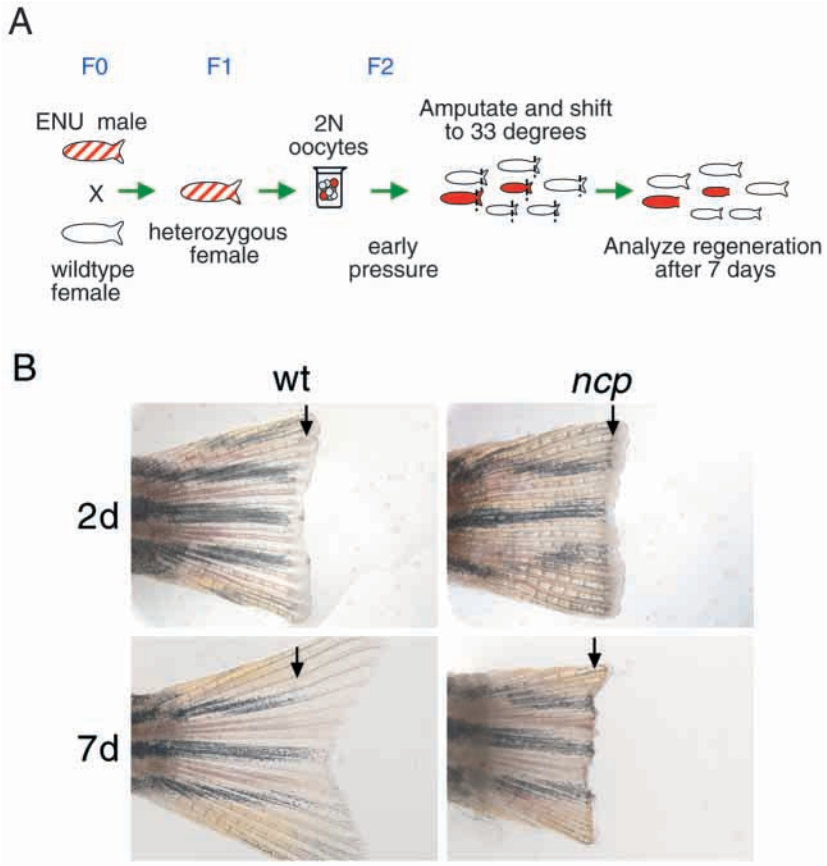

Fig. l. Genetic screen for regeneration and identification of the *ncp* mutant. (A) Illustration depicting mutagenesis and screen for temperature-sensitive fin regeneration mutants. (B) Whole-mount wild-type and *ncp* caudal fin regenerates at 2 and 7 days postamputation. The *ncp* mutant showed a clear defect in fin regeneration by 7 days postamputation. Arrows demarcate amputation plane in each photo. Original magnification is 15×.

The *ncp* mutation was inherited in a recessive manner and identified in a family from which two of seven members displayed regenerative blocks. *ncp* fin regenerates appeared grossly normal through 2 days postamputation at the restrictive temperature of 33°C, but typically had stalled or regressed back to the amputation plane without forming new bone by 7 days (Fig. 1B). Incubation of *ncp* adults at 33°C for long periods (over 3 months) had little or no effect on survival, suggesting that the *ncp* mutation does not hinder general cell survival or adult physiology. All *ncp* mutants regenerated normally at 25°C. Thus, *ncp* is a temperature-sensitive mutation that disrupts fin regeneration.

To test if the *ncp* gene is required for embryonic development, we raised embryos from heterozygous crosses at 33°C. In our studies, wild-type zebrafish embryos raised in a 33°C incubator following fertilization showed considerably reduced viability compared to those raised at 25-28°C. Although 50% of wild-type or heterozygous zebrafish reached swimming stage normally, no homozygous *ncp* mutants attained this stage (data not shown). Defects displayed by *ncp* mutants appeared nonspecific and grossly indistinguishable from those in inviable wild-type or heterozygous siblings, such as cardiac edema, hooked tail and failure to form a swim bladder. *ncp* animals raised at 25°C also appeared to have compromised viability, as only half of the expected numbers of *ncp* from parental backcrosses were recovered during adulthood. This suggested that the *ncp* mutation was hypomorphic at 25°C, but not strong enough to affect regeneration at that temperature. The only consistent observation in *ncp* animals raised at 25°C was that smaller adult fish from parental backcrosses tended to be *ncp* homozygotes. These observations support our initial assumption that genes required for fin regeneration are also required for embryonic or larval development (see also, Johnson and Weston, 1995).

ncp regenerates display proximal blastemal defects at the onset of regenerative outgrowth

To determine the cellular mechanism of the *ncp* regenerative failure, we examined the histology of *ncp* regenerates through different stages of regeneration at 33°C. We found that the

early stages of wound healing and blastema formation appeared normal (Fig. 2A). However, by 2 days postamputation, *ncp* regenerates demonstrated obvious histological abnormalities, particularly in mesenchymal cells distal to the amputation plane. To refine our analysis of the *ncp* defect, we analyzed regenerates for expression of *msxb*. We also examined expression of Zns-5, which recognizes an unknown antigen on scleroblasts, or bone-depositing cells, and thus can be used to visualize patterning events such as scleroblast alignment (Johnson and Weston, 1995). At 1 day postamputation, *msxb* expression was normal in *ncp* regenerates (Fig. 2A). Interestingly, *msxb* expression was maintained in *ncp* regenerates at 2-3 days postamputation, often in an expanded domain, indicating that the mutation spares the distal blastema during outgrowth. The larger *msxb*-expressing domain might be due to (1) failure of blastemal cells to properly condense into the distal blastema, (2) epidermal pinching and separation of the distal blastema, or (3) compensatory expansion of the distal blastema as a result of deficiencies in other portions of the regenerate. Furthermore, we found that scleroblasts were present and correctly patterned in the most proximal regenerate, suggesting that the *ncp* mutation did not directly affect patterning or differentiation functions (Fig. 2B). These data indicate that *ncp* affects a specific subpopulation of cells in the proximal blastema at the onset of regenerative outgrowth.

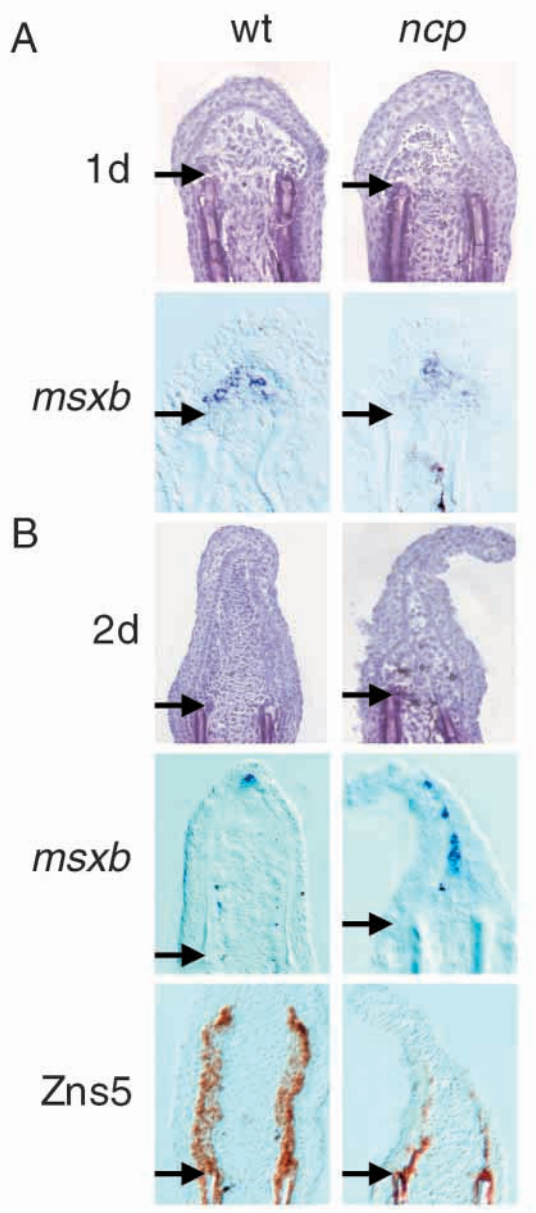

Fig. 2. *ncp* fin regenerates display defects in proximal blastemal cells during regenerative outgrowth. (A) Longitudinal sections of 1-day postamputation fin regenerates during blastema formation, with the distal, regenerating end shown at the top. At this stage, *ncp* regenerates display a typical blastema with normal *msxb* expression (violet stain). (B) Sections of regenerates at the onset of outgrowth. (Top) Hematoxylin stains of *ncp* regenerates indicate a mesenchymal compartment with a reduced number of blastemal cells. (Middle) *msxb* expression is maintained and even expanded in *ncp* regenerates despite blastemal reduction. (Bottom) The antibody Zns-5 detects scleroblasts, or bone-depositing cells (brown stain), which align bilaterally in the patterning zone by 2 days postamputation and begin to deposit mineral. Note that the *ncp* regeneration defect was first apparent at the onset of regenerative outgrowth and had little or no effect on the establishment of the distal blastema or patterning zone. Arrows indicate point of amputation. Original magnification is 250×.

A mutation in the zebrafish orthologue of *mps1* is fully linked to *ncp*

To define the *ncp* gene, we raised 1,751 progeny from *ncp* × *ncp*/+ mapping crosses to adulthood at 25°C, scored for regenerative defects at 33°C, and genotyped these animals using CA-repeat and SSCP markers. We found two closely linked markers that flanked a 0.7 centiMorgan (cM) region containing *ncp* on linkage group 16, and utilized these markers to retrieve a 950 kb yeast artificial chromosome (YAC) clone that spanned the critical region. After making a cosmid library from this YAC clone, we generated additional SSCP markers from cosmid end sequences. We then used these new markers to refine the genetic map to 0.11 cM. These new flanking markers were contained within two cosmids, representing approximately 80 kb (Fig. 3A).

To identify the *ncp* gene, we screened a zebrafish cDNA library with radiolabeled cosmid inserts. We identified cDNA clones representing two genes, the zebrafish orthologues of *bckdhb* (corresponding to zebrafish ESTs fb34c01 and fb54e12) and *mps1* (corresponding to zebrafish ESTs fi32g09, fi61a12, and fl31h05). No other genes were identified using this technique. We then searched the human genome database to identify the human syntenic region. Human *mps1* (known as TTK) and *BCKDHB* are located on chromosome 6p14, approximately 50 kb apart. From a search of GenBank no human mRNAs were located between *TTK* and *BCKDHB*. These experiments indicate that the *ncp* mutation is located in either the *bckdhb* or *mps1* genes.

BCKDHB encodes a subunit of a metabolic enzyme mutated in human maple syrup urine disease (Indo et al., 1987). To test the candidacy of *bckdhb* for the *ncp* mutation, we sequenced the entire *bckdhb* coding sequence from *ncp* mutants. No nucleotide differences were detected from wild-type AB strain sequences (data not shown). These data suggest that *bckdhb* is not the *ncp* gene.

Mps1 is an intracellular kinase important for cell proliferation. cDNA sequence analysis revealed that zebrafish

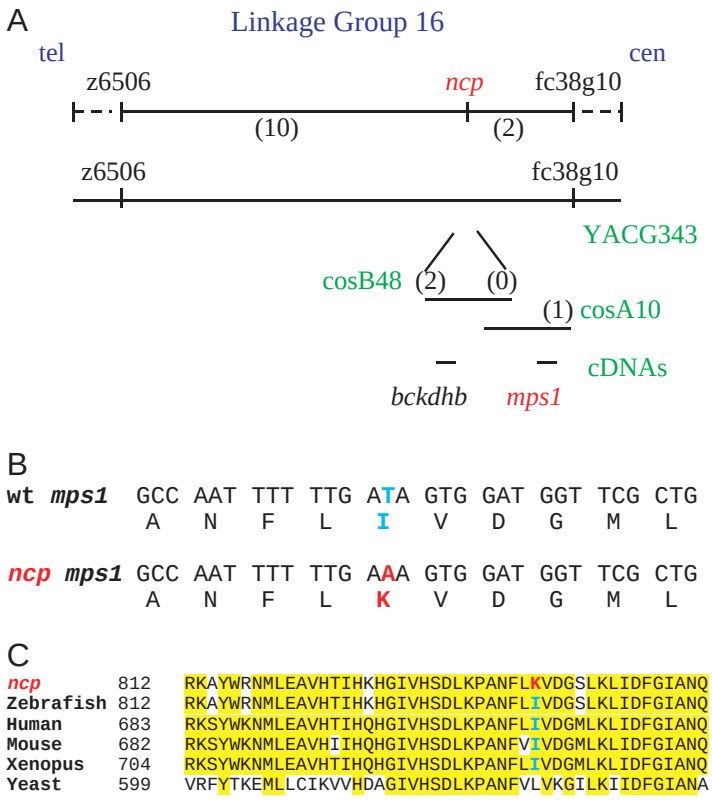

Fig. 3. Linkage of a kinase mutation in *mps1* to *ncp*. (A) Genetic and physical map of *ncp* region indicating genomic DNA, YAC clone, cosmids, and cDNAs. Numbers in parentheses represent recombination events from 1,751 meioses in regions between *ncp* and linked genetic markers. (B,C) Mutational analysis of *mps1*. Sequencing of cDNAs from *ncp* and several wild-type strains revealed a unique thymidine to adenosine mutation that altered isoleucine-843 to lysine (red). A portion of the highly conserved carboxyl terminal kinase domain of Mps1 containing the I843K mutation is shown. This isoleucine is highly conserved among vertebrates. Thus, a mutation in *mps1* is associated with the *ncp* regeneration defect.

mps1 encodes a protein of 983 amino acids. Zebrafish Mps1 is 34% identical at the amino acid level with murine Mps1 (Mpeg1), and 39% identical with *X. laevis* Mps1. The carboxyl terminus encoding the kinase domain (326 amino acids) is 60% and 71% identical, respectively (GenBank accession number AF488735). To test the candidacy of *mps1*, we sequenced cDNAs from wild-type and *ncp* fish. We found one nucleotide difference from wild-type AB strain *mps1* cDNA sequence, a thymidine to adenosine (T to A) transversion in the *mps1* gene of *ncp* fish that converts isoleucine 843 to lysine (Fig. 3B). Isoleucine 843 is identical in zebrafish, amphibians and mammals, and resides within kinase subdomain VII. The comparable amino acid in budding and fission yeasts is leucine, a conservative amino acid substitution (Fig. 3C). The sequence of the corresponding region in the C32 inbred strain was identical to that of the AB-derived cDNA. Since *ncp* was isolated in the C32 inbred background, this indicates that the T to A transversion was caused by the ENU mutagenesis that produced the *ncp* mutation. Furthermore, the point mutation was unique to the *ncp* mutant strain among all additional genetic backgrounds examined, including Ekkwill, WIK and SJD, indicating that the I843K mutation is not a variant or polymorphism among laboratory stocks of zebrafish. These data indicate that *mps1* is the *ncp* gene.

ncp embryonic cells display reduced mitotic checkpoint activity

Mps1 is important for the mitotic checkpoint and centrosome duplication (Abrieu et al., 2001; Fisk and Winey, 2001). *mps1* was initially isolated as a budding yeast mutant that disrupts spindle pole body duplication, leading to its name (*mono*polar spindle) (Winey et al., 1991). Subsequent experiments in yeast revealed a second, distinct role in the mitotic, or spindle, checkpoint (Weiss and Winey, 1996). The mitotic checkpoint is a sensing mechanism that monitors interactions between kinetochores and microtubules and prevents sister chromatid segregation until all chromosomes are properly aligned. In fact, six temperature-sensitive alleles of *mps1* that disrupted mitotic checkpoint activity in *S. cerevisae* changed residues of the kinase domain (Schutz and Winey, 1998). The role of Mps1 in mitotic checkpoint signaling has been confirmed in *S. pombe*, *X. laevis* cell extracts, and human cells in vitro (He et al., 1998; Abrieu et al., 2001; Fisk and Winey, 2001; Stucke et al., 2002). Interestingly, Mps1 was required only for mitotic checkpoint activity and not centrosome duplication in human cells in vitro and *S. pombe* (He et al., 1998; Stucke et al., 2002).

To determine if Mps1 function was affected by the I843K mutation, we examined mitotic checkpoint signaling in *ncp* embryonic cells. We treated 24-hour postfertilization wild-type and *ncp* embryos (raised at 25°C) for 4 hours at 33°C with the microtubule-disrupting agent, nocodazole. Nocodazole destroys mitotic spindles and activates the mitotic checkpoint. Cycling cells with normal mitotic checkpoint activity respond to nocodazole treatment by arresting in mitotic metaphase. In our experiments, wild-type embryonic cells clearly arrested in mitosis (4N nuclear content) in the absence of mitotic spindles. While most cycling *ncp* cells also arrested in mitosis during this period, a subpopulation of cells continued to increase DNA content in the presence of nocodazole, suggesting that these cells entered new cell cycles despite gross spindle defects (Fig. 4). These data indicate that the I843K mutation is associated with abnormal mitotic checkpoint activity in *ncp* cells. Together with genetic data, these functional data indicate that *mps1* is the *ncp* gene. We will refer to *ncp* animals hereafter as *mps1* mutants.

mps1 expression is induced in proximal blastemal cells

To define the timing and pattern of *mps1* expression during fin regeneration, and to determine whether expression is consistent with *mps1* mutant pathology, we performed northern analysis and in situ hybridization experiments. We found that zebrafish *mps1* RNA was undetectable or present at very low levels in all adult somatic tissues examined, including unamputated caudal fins. In contrast, northern analysis of 1, 2, and 4 days postamputation fin regenerates indicated induction of *mps1* (Fig. 5A). In situ hybridization experiments demonstrated *mps1* expression beginning at 18-24 hours postamputation (at 33°C) in the newly formed blastema (Fig. 5B). At this point,

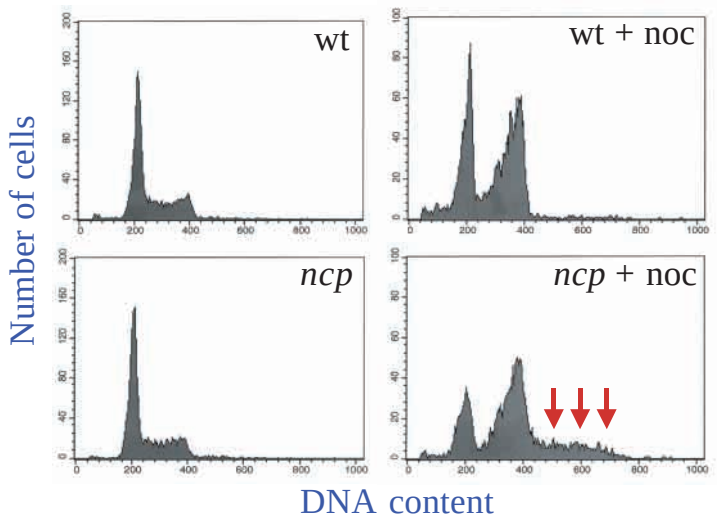

Fig. 4. Reduced mitotic checkpoint activity in *ncp* cells. Histograms from flow cytometric analysis of DAPI-stained cells from 24-hour postfertilization wild-type and *ncp* embryos. (Left) FACs profile of cells collected from embryos (raised at 25°C) after a 4-hour incubation at 33°C. The majority of cells in wild-type and *ncp* samples had a 2N nuclear content. (Right) FACs profile of cells collected from embryos treated with 1 μg/ml nocodazole during the 4-hour 33°C incubation. A large 4N peak, representing cells that have arrested in mitosis, appeared in both wild-type and *ncp* histograms. However, note the substantial >4N subpopulation in the *ncp* histogram (arrows). Such cells presumably reflect those that failed to arrest in mitosis and continued to synthesize DNA despite the absence of mitotic spindles. High DNA content (>2% of cells with DNA content over 4N) was not observed in cell suspensions from nocodazole-treated wild-type embryos (*n*=10), but was seen in 10 of 12 suspensions from treated *ncp* embryos. Thus, mitotic checkpoint activity is reduced in *ncp* embryonic cells, indicating that the I843K mutation disrupts Mps1 function in zebrafish.

mps1 was coexpressed with both *msxb* and proliferating cell nuclear antigen (PCNA), a marker for actively cycling cells (Takasaki et al., 1981) (Fig. 5C). However, during regenerative outgrowth, *mps1* RNA was limited to PCNA-positive proximal blastemal cells, and was not detectable in the PCNA-negative, *msxb*-positive distal blastema or in the patterning zone (Fig. 5C). These data indicate that *mps1* is specifically expressed in a subpopulation of cells in the proximal blastema during regenerative outgrowth, findings that are entirely consistent with *mps1* mutant pathology.

Mps1 is required for blastemal proliferation during outgrowth

To define the mechanism of the *mps1* regenerative defect, we examined cell cycle entry by BrdU incorporation. We also assayed mitosis by the presence of phosphorylated histone-3 (H3P) (Hendzel et al., 1997). *mps1* fin regenerates showed normal BrdU incorporation and H3P staining at 1 day postamputation (data not shown). However, at the onset of regenerative outgrowth (2 days

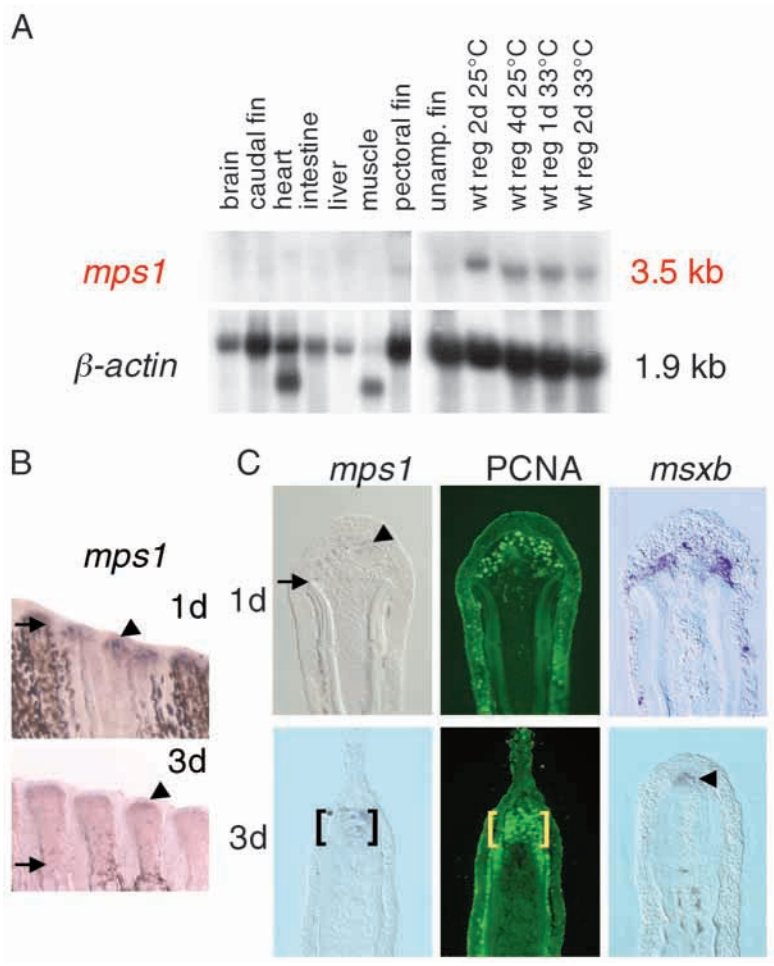

Fig. 5. *mps1* is induced in the proximal blastemal proliferative zone during regenerative outgrowth. (A) Northern analysis of *mps1* expression using several adult tissues as well as regenerating caudal fin tissue. Blots were also probed for *β-actin* expression as a control to indicate the amounts of RNA loaded. (B) Whole-mount in situ hybridization of *mps1* in wild-type 1-day and 3-day postamputation fin regenerates (violet stain indicated by arrowhead). *mps1* RNA levels were increased in the newly-formed blastema at 1 day postamputation (top) and these levels were maintained in the blastema during regenerative outgrowth (bottom). Whole-mount *mps1* signals appeared stronger than section *mps1* signals in 1-day regenerates, an observation that is common at that timepoint for other genes. This likely represents somewhat weak but widespread signals in individual blastemal cells that appear stronger when visualized en masse. (C) (Left and center) Longitudinal sections of wild-type 1- and 3-day fin regenerates co-stained for *mps1* RNA and PCNA protein (green). *mps1* was upregulated in the most highly proliferative cells during outgrowth (brackets), but was absent from the distal blastema. (Right) *msxb* RNA localization (violet, arrowhead at 3 days) in the newly formed blastema at 1 day and the distal blastema at 3 days postamputation. Thus, in the new blastema, *mps1* colocalizes with PCNA and *msxb*. However, *mps1* is specifically induced in the proximal blastema during outgrowth. The morphological difference between 3-day regenerates shown in Fig. 5C represents variation commonly seen in fin regenerates during outgrowth. Original magnifications: 50× in B and 110× in C.

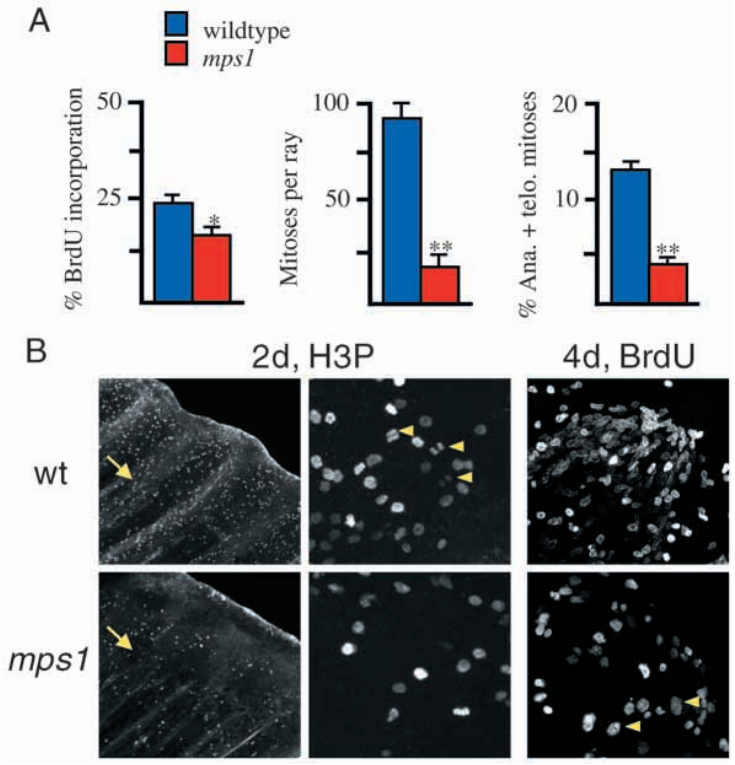

Fig. 6. The *mps1* regeneration defect is caused by severe blastemal proliferative abnormalities. (A) Indices of proliferation at 2 days postamputation. (Left) BrdU incorporation data were obtained from counting 500-3,000 mesenchymal nuclei from 6-10 sections of each of five whole-mount immunostained regenerates. (Middle) A total of 14 regenerating rays from six wild-type fish and 21 rays from eight *mps1* animals were used to count H3P-positive nuclei. (Right) A total of 1,355 H3P-positive nuclei from eight wild-type regenerates and 704 H3P-positive nuclei from 10 *mps1* regenerates were scored for mitotic phases at 500× magnification. Results are shown as mean ± s.e.m. (*t*-test; **P*<0.05, ***P*<<0.001). (B) (Left) Confocal projections of 2-day postamputation fin regenerates stained with anti-H3P to indicate mesenchymal mitoses. The bright points are individual mitotic nuclei, severely reduced in *mps1* regenerates. Both fins show non-specific epidermal fluorescence at the distal edge (see Materials and Methods). (Middle) High magnification confocal images of H3P-positive mesenchymal nuclei. An *mps1* fin ray with an unusually high number of mitoses is shown. Arrowheads point to late phase mitoses, deficient in *mps1* fin regenerates. (Right) Projections of single 4-day postamputation fin rays from animals that have incorporated BrdU for the final 5 hours of regeneration. Note the reduced incorporation, and unusually large nuclei in cycling *mps1* cells that are suggestive of aneuploidy (arrowheads in right image). Original magnification is 150× (left panels) and 945× (middle and right panels).

postamputation), *mps1* blastemal tissue displayed a slight (30%) reduction in mesenchymal BrdU incorporation (Fig. 6A). The decrease in mesenchymal mitoses was more severe, as *mps1* regenerates had approximately one-fifth of the number of mitoses as wild-type at 2 days postamputation (Fig. 6A,B). Microscopic examination of H3P-positive nuclei indicated an unusually low percentage of *mps1* mesenchymal cells progressing into later mitotic phases, suggesting an additional defect in transition to anaphase (Fig. 6A,B). Finally, by 4 days postamputation, at which point gross differences between wild-type and *mps1* outgrowth were apparent, we observed unusually large mesenchymal nuclei in *mps1* regenerates (Fig. 6B). Many of these nuclei continued to incorporate BrdU. Increased nuclear size suggested aneuploidy, and the fact that these enlarged nuclei continued to synthesize DNA indicated a defect in cell cycle regulation. We saw normal TUNEL-staining in *ncp* fin regenerates at 3 days and 7 days postamputation, indicating that programmed cell death did not play a major role in the regenerative block (data not shown).

As Mps1 is required for mitotic checkpoint signaling, and mitotic checkpoint deficiencies have been linked to similar proliferative defects in both invertebrate and vertebrate embryos (Kitagawa and Rose, 1999; Dobles et al., 2000; Kalitsis et al., 2000), the *mps1* proliferative failures likely reflected a reduction in checkpoint activity. Consistent with this notion and the fact that *mps1* embryonic cells showed checkpoint abnormalities, regenerating fin cells from *mps1* animals also had a diminished response when challenged at 33°C to arrest in nocodazole (as assayed by increases in H3P-positive cells; data not shown). Thus, the *mps1* regenerative defect is caused by failed proliferation in proximal blastemal cells during regenerative outgrowth, indicating that *mps1*-expressing proximal blastemal cells have a critical role in regeneration.

DISCUSSION

We initiated a genetic approach to regeneration with a mutagenesis screen for temperature-sensitive fin regeneration mutants in zebrafish. We identified the *ncp* strain, which displayed a deficiency in proximally located blastemal cells at the onset of regenerative outgrowth. The *ncp* defect was associated with a temperature-sensitive mutation in *mps1*, a critical regulator of eukaryotic cell cycle progression. Evidence implicating *mps1* as the *ncp* gene include: (1) localization of the *ncp* mutation to an 80 kb segment that contained only *mps1* and *bckdhb*; (2) no mutations were identified in *bckdhb*; (3) identification of a missense mutation in a conserved amino acid of the Mps1 kinase domain; (4) comparative DNA sequence data indicating that the I843K mutation in *mps1* is not a polymorphism; (5) functional data indicating that *ncp* cells have abnormal mitotic checkpoint activity, consistent with abnormal Mps1 function; (6) expression studies showing that *mps1* is expressed at the time and location one would expect given the *ncp* regenerative phenotype; (7) a *mps1* kinase mutation is consistent with the defects in proliferation observed in *ncp* fin regenerates. Our findings indicate that the proximal blastema is an essential proliferative zone that is defined by the induction and function of Mps1.

A model for blastemal function during fin regeneration

Together with our previous findings (Poss et al., 2000a; Nechiporuk and Keating, 2002) as well as those of others (Laforest et al., 1998; Poleo et al., 2001), a molecular and cellular model of zebrafish fin regeneration is beginning to emerge (Fig. 7). After injury, the first obvious step is formation of the wound epidermis, a non-proliferative event that does not require Mps1. Next, mesenchymal tissue beneath the epidermis

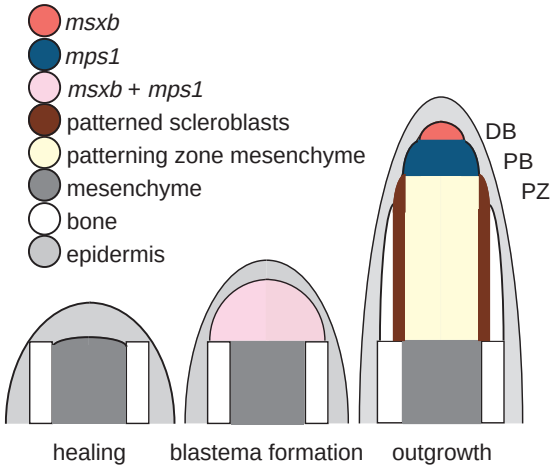

Fig. 7. Cellular and molecular model for fin regeneration. During outgrowth, the distal blastema (DB) is defined by *msxb* (orange), the proximal blastema (PB) by *mps1* (blue), and the patterning zone (PZ) by newly patterned scleroblasts (brown) and differentiating mesenchyme (yellow).

disorganizes; this event may involve cellular dedifferentiation. The first signs of proliferation are apparent at 12-18 hours postamputation, as mesenchymal cells orient longitudinally, begin to migrate distally toward the wound epidermis, and form a rudimentary blastema. Blastema formation involves induction of *msxb* and *msxc* genes, as well as *mps1*, and depends on intact fibroblast growth factor signaling. At 48-72 hours postamputation, the blastema matures to form the distal and proximal blastemal compartments. The *msxb*-positive, distal blastema is approximately 5 cell diameters and is non-proliferative. This region does not induce *mps1* expression and does not require Mps1 function. We believe that the distal blastema may provide a source of undifferentiated cells for proliferation and differentiation. The *msxb*-negative proximal blastema extends 10-20 cell diameters and is highly proliferative. Here, cells are cycling, with a rapid median G_2 cell cycle phase of approximately 60 minutes. *mps1* is induced and required to establish or maintain intense proliferation in this region, ostensibly the engine of regenerative outgrowth. The patterning zone comprises the remaining portion of mesenchymal tissue, and does not express *msxb* or *mps1*. Proliferation is less intense, Mps1 function is not required, and patterning and differentiation events predominate. These three compartments continue to function until the regenerative process is completed.

Our findings indicate that the distribution of blastemal function into distinct domains is essential for regeneration. We suspect that extracellular signaling molecules released from the wound epidermis establish and maintain these domains during outgrowth. Signaling by Fgfs appears to contribute to distal blastemal identity, as pharmacological inhibition of Fgf receptors diminished established blastemal *msxb* expression (Poss et al., 2000a). In addition, recent studies have suggested that Sonic hedgehog might pattern proximal blastemal cells (Laforest et al., 1998; Nechiporuk and Keating, 2002; Quint et al., 2002). It is likely that these molecules or others such as Wnt factors (Poss et al., 2000b) participate in maintaining the proliferative properties of the proximal blastema. The *mps1* strain might be useful for evaluating these candidate signals. For instance, the augmented *msxb*-expressing region in the *mps1* mutant regenerate might represent an expansion of the distal blastema to compensate for compromised proximal function. Potentially, the expression patterns of epidermal signals that maintain this *msxb* expression are also expanded.

A conditional mutation in *mps1*

We predict that genetic dissection of regeneration requires the production of conditional mutations that allow determination of in vivo gene function beyond that gene's earliest requirement. In zebrafish, conditional mutations can be identified by screening for temperature-sensitive phenotypes; thus, experimental regulation of gene function is easily accomplished by changing water temperature. Recently, temperature-sensitive mutations have been identified in developmentally important genes such as *bmp7* and *kit* (Dick et al., 2000; Rawls and Johnson, 2001). One would also predict that the availability of conditional mutations is essential for detailed study of Mps1 function in vertebrates, as Mps1 is required for normal cell division in yeast, and zebrafish *mps1* mutants raised at 33°C fail to complete embryogenesis. This prediction likely applies to all mitotic checkpoint signaling members; for instance, mice containing null mutations in the checkpoint genes *Mad2* or *Bub3* fail to survive past early embryogenesis (Dobles et al., 2000; Kalitsis et al., 2000). Accordingly, we expect that the *mps1* zebrafish strain, containing a temperature-sensitive allele of *mps1*, will be a useful reagent for studying the mitotic checkpoint in vertebrate processes in addition to regeneration, such as gametogenesis, organogenesis, and tumorigenesis.

Regeneration genetics

The study of regeneration holds great promise for the emerging field of regenerative medicine, but to realize this promise, regenerative phenomena must be understood in molecular terms. Genetic analysis of regeneration in zebrafish provides a unique instrument for achieving this goal. Zebrafish are the only genetic model system that reliably regenerates complex tissue. Accumulating technological advances in zebrafish genetics, including the genome sequencing initiative, stand to substantially advance discoveries through regeneration genetics. From our genetic screen for fin regeneration mutants, we expected to find disruptions in wound healing, tissue dedifferentiation, blastema formation and proliferation, and organ patterning. While the *mps1* mutant represents a defect in blastemal proliferation during outgrowth, mutants in other stages of regeneration have also been found and should shed light on these events (Johnson and Weston, 1995) (A. N., K. D. P., S. L. J. and M. T. K., unpublished observations). In future experiments, we will focus on the early events that initiate fin regeneration, and also extend our studies to additional organ systems that have not yet been examined for regenerative potential, such as the heart.

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