

# Bone Growth in Zebrafish Fins Occurs via Multiple Pulses of Cell Proliferation

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Fin length in the zebrafish is achieved by the distal addition of bony segments of the correct length. Genetic and molecular data provided evidence that segment growth uses a single pulse of growth, followed by a period of stasis. Examination of cell proliferation during segment growth was predicted to expose a graphical model consistent with a single burst of cell division (e.g., constant, parabolic, or exponential decay) during the lengthening of the distal-most segment. Cell proliferation was detected either by labeling animals with bromodeoxyuridine (during S-phase) or monitoring histone3-phosphate (mitosis). Results from both methods revealed that the number of proliferating cells fluctuates in apparent pulses as a segment grows (i.e., during the growth phase). Thus, rather than segment size being the result of a single burst of proliferation, it appears that segment growth is the result of several pulses of cell division that occur approximately every 60 microns (average segment length ~ 250 microns). These results indicate that segment lengthening requires multiple pulses of cell proliferation. *Developmental Dynamics* 236: 2668–2674, 2007. © 2007 Wiley-Liss, Inc.

**Key words:** ontogeny; saltations; bone growth; segment length; zebrafish

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## INTRODUCTION

The diversity of life forms is most clearly manifested as differences in shape and structure. Variations in morphology arise as organs and limbs grow to different proportions with respect to each other and to the body. The final form is the result of these different growth rates. Understanding the pattern of growth provides insight into the underlying mechanisms of development. For example, the finding that human bone growth occurs by saltations (i.e., discrete pulses of growth) rather than in a continuous process, suggests the existence of a previously unknown hormonal synchronizing mechanism regulating

growth (Lampl et al., 1992). Such discoveries will lead to the identification of the cellular and molecular mechanisms involved in size regulation, which remain largely unknown.

Short generation time, high fecundity, ease of maintenance, and a predominantly sequenced genome (<http://www.sanger.ac.uk/>) make the zebrafish a popular model for evaluating underlying molecular pathways in vertebrate systems. More particular to the questions of growth or size, the caudal fin is easily measured and amputated for histochemical analyses. Furthermore, as the fin is nonessential for viability (especially in a laboratory setting) and has a comparatively simple structure with

few cell types, it has been the focus of many studies (reviewed in Poss et al., 2003; Akimenko et al., 2003).

The caudal fin is bi-lobed and composed of 16–18 fin rays of varying length. There is a single shortest fin ray in the medial or central part of the fin, and symmetric lobes extending laterally in both dorsal and ventral directions. Each fin ray grows by the distal addition of bony segments (Goss and Stagg, 1957; Haas, 1962) added synchronously in the adult fin (Goldsmith et al., 2003, 2006). Fin rays are composed of two opposed concave hemirays of bone matrix, or lepidotrichia, surrounding mesenchymal cells including undifferentiated fibro-

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blasts, nerves, and blood vessels (Santamaria et al., 1992). Proliferating cells in the distal mesenchymal compartment contribute to outgrowth by condensation along specialized collagen-like fibers called actinotrichia, differentiation as bone-forming cells (osteoblasts), and ultimately deposition of bone matrix by intramembranous ossification (Landis and Geraudie, 1990; Goldsmith et al., 2003).

Recent analyses revealed that ontogenetic fin growth is a saltatory process, and suggest that the bony segment is the unit of growth. Thus, fin length is achieved by discrete pulses of growth (when a segment is produced) separated by phases of rest, or stasis. The occurrence of saltations was revealed by the discovery that an anonymous growth marker *fa93e10* (a gene-based marker shown to correlate with distal mesenchymal cell proliferation) is expressed episodically and with decreasing frequency as animals grow or age (Goldsmith et al., 2003). Indeed, 100% of young fins expressed *fa93e10* (at the growing ends of all fin rays), suggesting either that young fins are always in a state of growth or that rest phases are very short. Older animals, 24 weeks of age, expressed *fa93e10* only 5% of the time (again, across all fin rays). Since fin growth occurs by the synchronous addition of new segments, it has been suggested that one saltation is responsible for the growth of one segment (Iovine and Johnson, 2000; Goldsmith et al., 2003).

Growth in vertebrates is regulated primarily by changes in cell number (Conlon and Raff, 1999), indicating that cell proliferation may be used to gauge growth. Thus, the next level of processes to consider during fin growth is the pattern of cell division that results in the formation of an individual bone segment. This examination was predicted to expose a graphical representation of proliferation consistent with a single burst of cell division (e.g., constant, parabolic, exponential decay) during the lengthening of the distal-most segment. To assess these possibilities, we monitored cell proliferation using markers for recently dividing cells (BrdU) and mitosis (H3P) and followed the growth of the ultimate segment in each the longest and shortest fin rays in the caudal

fin. Interestingly, our data do not support the initial hypothesis that segment growth is the result of a single saltation. Instead, multiple apparent fluctuations of cell proliferation are identified during the growth of an individual segment, revealing multiple bursts of cell division. We find evidence for four pulses in the longest rays (average segment length ~250 microns) and three pulses in the shortest rays (average segment length ~200 microns), suggesting that the number of saltations (but not the amplitude) controls the length of segments. One intriguing possibility for how fin shape is regulated is that a systemic factor coordinates saltations across the fin, but individual fin rays respond to that factor differentially.

## RESULTS AND DISCUSSION

### Cell Proliferation During Segment Growth Is Pulsatile

Recently, Goldsmith et al. (2006) identified a physiological transition from juvenile (i.e., paddle-shaped caudal fin) to adult (i.e., bi-lobed caudal fin) form during ontogenetic fin growth. Segment addition was found to be asynchronous until after the bi-lobed shape is established, when segments are added concurrently across the fin. We chose to monitor cell proliferation after the transition to adult growth, when segment addition is synchronous, and when fins are growing rapidly (~12–14 weeks of age). It was possible to confirm that fin growth had transitioned to adult growth by counting the number of segments from the longest and shortest rays (i.e., at least 12 segments in the longest rays and at least 8 segments in the shortest ray). Furthermore, because segment length tends to be longer in the lobe fin rays than in other regions of the fin (Iovine and Johnson, 2000), we completed our analyses in both the longest (i.e., “lateral”) and shortest (i.e., “medial”) fin rays to provide insight into how differences in segment length across the fin are attained.

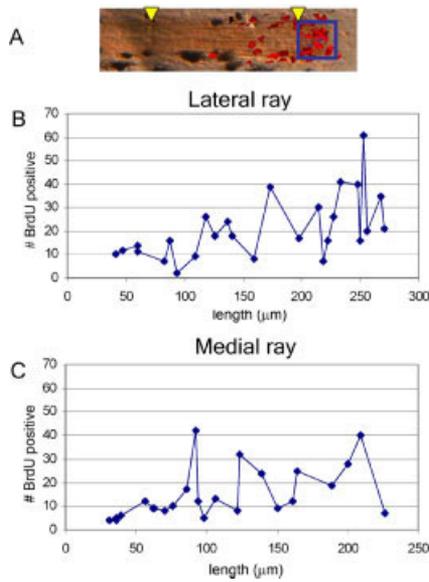
Vital markers for dividing cells have not been developed, so we examined cell proliferation from a population of 40 animals using histochemical techniques, and reasoned that growing segments of varying lengths would

be identified. Cell proliferation during growth of the ultimate fin ray segment was assessed by labeling animals with the thymidine analog bromodeoxyuridine (BrdU), which incorporates into DNA during the S-phase of the cell cycle and is detectable using a monoclonal antibody. Initially, we evaluated the number of mesenchymal BrdU-labeled cells with the length of the ultimate (i.e., incomplete, or growing) segment using Confocal microscopy to identify labeled cells from the entire distal mesenchymal compartment (Fig. 1). Labeled cells were counted at level with or distal to the ultimate joint. Of interest, the pattern of proliferating cells was not consistent with the predicted patterns for a single pulse of dividing cells mentioned above. Rather, we identified multiple apparent pulses during growth in both the longest and shortest fin rays (Fig. 1). Furthermore, the overall slope of the data is positive, suggesting an increase in the number of dividing cells per pulse as the segment grows longer.

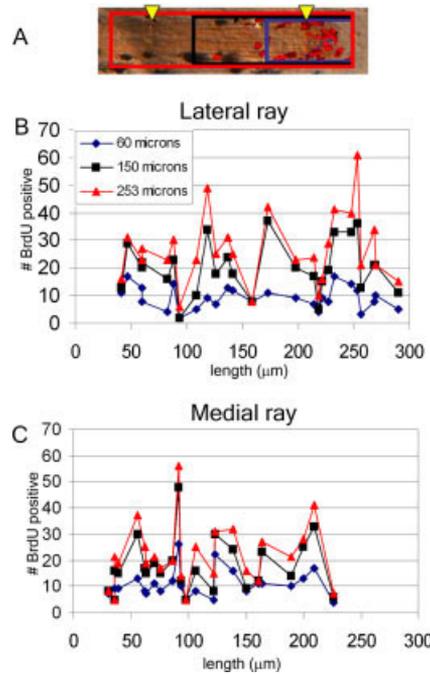
### Cells Proximal to the Growing Segment Contribute to Outgrowth

One explanation for the increasing number of BrdU-labeled cells with increasing segment length is that the newly forming segment recruits an increasing number of dividing cells as it grows. If true, this would suggest that the rate of segment growth increases with increasing length. Although this remains a formal possibility, we do not find a preponderance of fins with short ultimate segments (as would be predicted if the segment grew slowly initially). Alternatively, the positive slope in Figure 1 may simply reflect that, as a segment lengthens, we would naturally identify BrdU-positive cells from a larger area.

One way to correct for the latter possibility is to count BrdU-positive cells over fixed distances (i.e., 60, 150, 250 microns) from the distal end of the fin, even if this means counting cells proximal to the most distal joint. Indeed for any fixed distance, the fluctuations in cell proliferation are still apparent (Fig. 2), but the overall slope of the data no longer appears positive. Of interest, the difference in labeled cells

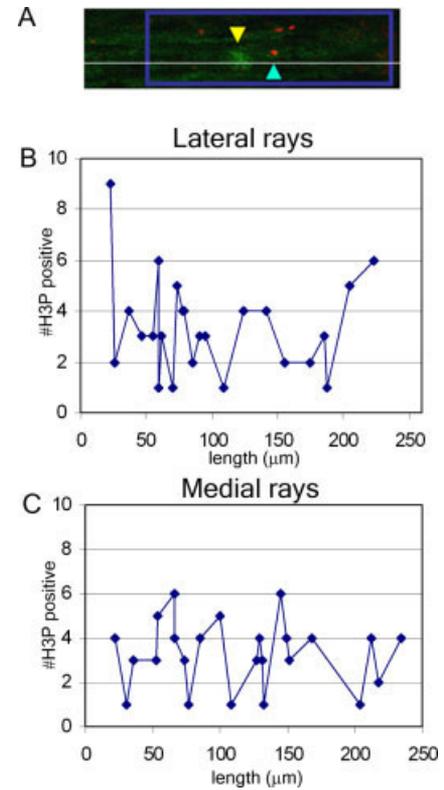


**Fig. 1.** Cell proliferation during segment growth appears pulsatile. **A:** Overlaid images of ZNS5+ cells (osteoblasts) in brown and bromodeoxyuridine-positive (BrdU+) cells in red. Arrowheads indicate joints. BrdU+ cells distal to the most distal joint were counted (boxed region) from individual fin rays and plotted against the length of the ultimate segment from the same fin. **B:** Number of BrdU+ cells in the longest fin ray in the ventral lobe. **C:** Number of BrdU+ cells in the shortest fin ray in the fin. Each data point in B and C represents a single fin ray. N = 28 rays.



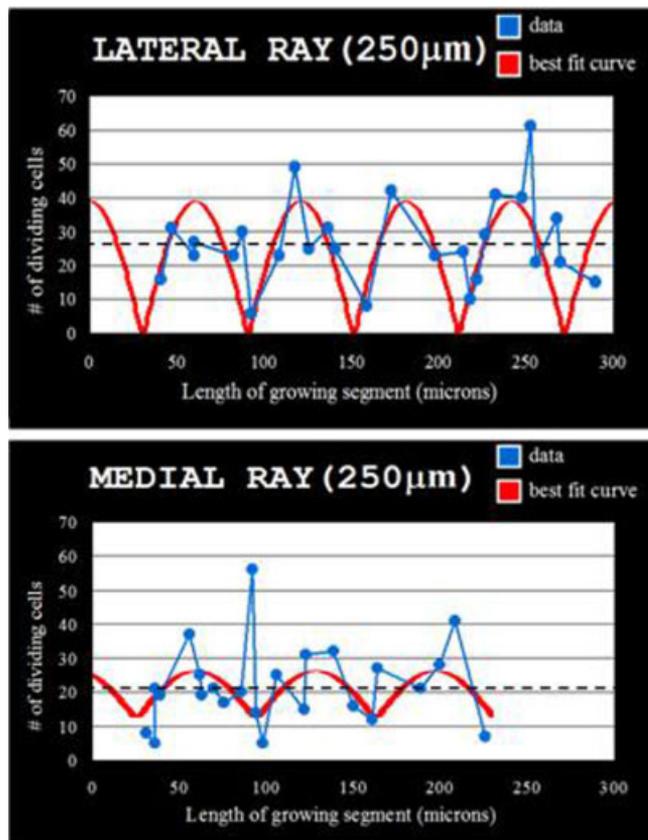
**Fig. 2.**

**Fig. 2.** Cells proximal to the most distal joint appear to contribute to segment growth. **A:** Overlaid images of ZNS5+ cells (osteoblasts) in brown and bromodeoxyuridine-positive (BrdU+) cells in red. Arrowheads indicate joints. BrdU+ cells were counted from three areas: 60 μm from the end of the fin (blue box), 150 μm from the end of the fin (black box), 250 μm from the end of the fin (red box); and plotted against the length of the ultimate segment in B and C. **B:** BrdU+ cells counted in each of the three areas, from the longest fin ray in the ventral lobe. **C:** BrdU+ cells counted in each of the three areas, from the shortest fin ray in the fin. Each data point in B and C represents a single fin ray. N = 28 rays.



**Fig. 3.**

**Fig. 3.** The number of mitotic cells during segment growth appears pulsatile. **A:** Confocal image of a single section with ZNS5+ cells in green and H3P+ cells in red. The yellow arrowhead indicates the joint; the blue arrowhead indicates a single H3P+ cell in this section (and in this fin ray). The boxed region represents an area 250 μm from the end of the fin. **B:** H3P+ cells in the longest fin ray in the ventral lobe. **C:** H3P+ cells from the shortest fin ray in the fin. Each data point in B and C represents a single fin ray. N = 25 rays.



**Fig. 4.** Mathematical representation of cell proliferation during segment growth in the longest (top) and shortest fin rays (bottom). The dotted line represents the mean number of bromodeoxyuridine-positive (BrdU+) cells.

**Fig. 4.**

increases appreciably when measured from 60 microns to 150 microns, indicating that many cells within this area likely contribute to outgrowth. In contrast, there is not a notable difference in the number of labeled cells from 150 microns to 250 microns, suggesting that cells more proximal than 250 microns do not add significantly to the number of proliferating cells. To account for all potential proliferating cells involved with segment growth, we used data from the 250 micron population for further analyses.

The distinction between counting cells over fixed or variable distances may seem arbitrary since pulses of BrdU-positive cells are apparent using either method of counting dividing cells. Therefore, the method for counting proliferating cells does not alter this conclusion. The next question to consider is whether or not it is reasonable to conclude that the labeled cells proximal to the ultimate joint contribute to the outgrowth of the ultimate segment. Fin regeneration studies have demonstrated that proliferating mesenchymal cells one to two segments proximal to growing segments in fact contribute to outgrowth (Poleo et al., 2001; Nechiporuk and Keating, 2002). Our findings are consistent with the recruitment of more proximal cells contributing to the growth of more distal segments, and further suggest that underlying mechanisms of cell proliferation are similar during fin regeneration and ontogeny (Mari-Beffa et al., 1996).

We next used an independent method to detect and follow actively dividing cells during segment growth. Phosphorylation of serine 10 on histone H3 occurs only during mitosis (Hendzel et al., 1997), and the antibody against this phosphorylated residue serves as a specific marker for mitotic cells (H3P, Wei et al., 1999). We followed the number of H3P-positive cells and found results consistent with our analysis of BrdU-labeled cells. Specifically, the number of mitotic cells fluctuates in apparent pulses as the ultimate segment increases in length (Fig. 3). Thus, proliferating cells detected by means of BrdU or H3P reveals pulsatile patterns of cell division during segment growth.

### Mathematical Representation Describing the Pattern of Cell Proliferation

To provide additional support for the periodicity of cell proliferation, we conducted a statistical analysis to test our conclusions. Indeed, the apparent fluctuations in the number of BrdU-positive cells may represent actual fluctuations in cell proliferation, or may simply represent normal variation for a model of constant cell proliferation. Therefore, we tested the fit of our data to a periodic curve (alternate hypothesis) and to a horizontal line (null hypothesis). We began with an equation representing the cosine function, which is a periodic curve fluctuating in both the positive and negative quadrants. To account for both the discontinuous and necessarily positive nature of the data, we used the absolute value cosine curve (i.e., choosing not simply to shift the cosine curve into the positive quadrant).

The equation for the alternate hypothesis is  $y = a|\cos(bx-c)| + d + \epsilon$ ; the equation for the null hypothesis is  $y = k$ . An  $F$ -ratio test statistic of both the alternate hypothesis and the null hypothesis resulted in a  $P$  value of 0.034 for the longest fin rays and  $P = 0.035$  for the shortest fin rays, indicating that the alternate hypothesis (i.e., absolute value cosine curve) is a more valid representation of the data than a horizontal line (Fig. 4). The most likely source of error in our data collection is in distinguishing between newly forming segments and recently completed segments. Therefore, we repeated the test of variance omitting the first interval for the lateral rays. In this case, the  $P$  value for the longest fin rays was  $P = 0.000$  (data not shown). The statistical significance is considerably stronger when using only the last three periods, providing increased support for our derived equation as representative of the pattern of cell proliferation during segment growth.

As mentioned, more medial regions of the caudal fin tend to have shorter segments than in the lobes. The cellular basis for the differences in segment length are likely reflected by the data presented here. Of interest, the ultimate segment in the shortest fin ray displays only three periods of

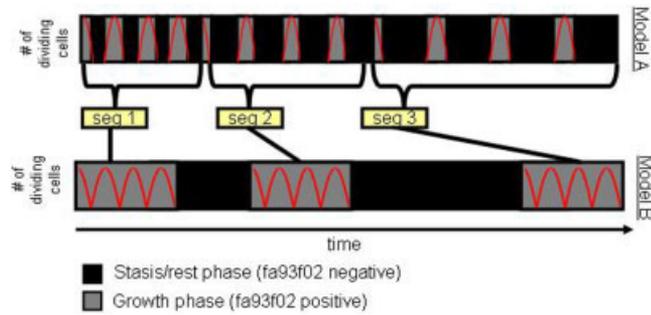
growth, whereas the ultimate segment in the longest ray displays four pulses (Fig. 4). In contrast, the mean number of dividing cells in the longest rays ( $n = 26.7 \pm 12.6$ ) was not significantly different from the mean number of dividing cells in the shortest rays ( $n = 21.8 \pm 12.0$ ), suggesting that the overall amplitude of the periods are similar across the fin. Thus, the frequency of pulses is likely the primary contributor for the regulation of segment length across the fin rays of the entire fin.

As a final test of our data, we completed an additional statistical analysis to provide confidence that the area over which BrdU-positive cells were counted did not influence the overall pattern or mathematical model for both the lateral and medial fin rays. Because it is apparent already that increasing the area containing BrdU-positive cells also results in an increase in the number of BrdU-positive cells, it did not make sense to compare the amplitudes of the curves. Rather, it was important to demonstrate that the overall shape of the curves is similar.

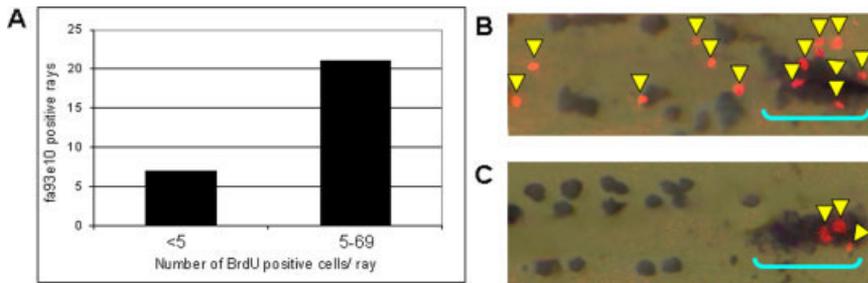
We limited our analyses to the 150- $\mu\text{m}$  and 250- $\mu\text{m}$  data sets because it is apparent that the number of cells measured from 60  $\mu\text{m}$  was insufficient for identifying the number of dividing cells that reasonably contribute to new segment growth. A  $z$ -test for equal slope parameters was completed. When comparing the differences in slope across the entire curve, we found that the slopes were not different (i.e., we did not reject the null hypothesis, that 150  $\mu\text{m} = 250 \mu\text{m}$ ) between the 150  $\mu\text{m}$  and 250  $\mu\text{m}$  data sets for either the lateral ( $P = 0.88$ ) or the medial ( $P = 0.78$ ) fin rays. Therefore, the patterns of cell proliferation are similar among the 150  $\mu\text{m}$  and 250  $\mu\text{m}$  data sets.

### Updated Model of Segment Addition During Fin Growth

Previous genetic and molecular analyses revealed the saltatory nature of fin growth. This cellular analysis of cell division confirms that growth occurs in pulses and more clearly defines a single saltation as the proliferation of  $\sim 40$  cells (i.e., approximate number of BrdU-positive cells during



**Fig. 5.** Updated models for how regulation of growth (cell proliferation) and stasis contributes to segment growth. **Model A:** Valleys during pulsatile growth represent the stasis period throughout fin growth. **Model B:** Valleys during pulsatile growth are distinguishable from true stasis periods.



**Fig. 6.** Double labeling of young fins with the growth marker *fa93e10*, and with bromodeoxyuridine (BrdU). **A:** Fins positive for *fa93e10* may have a low number of BrdU-positive cells (<5; n = 7) or a high number of BrdU-positive cells ( $\geq 5$ ; n = 21). **B:** An example of a *fa93e10*-positive fin (bracket) with a high number of BrdU-positive cells (arrows, n = 13). **C:** An example of a *fa93e10*-positive fin (bracket) with a low number of BrdU-positive cells (arrows, n = 3). Brown cells are melanocytes. N = 28 fins.

the peak of the pulses). If the length of the cell cycle is less than 6 hours (the time of exposure to BrdU), the number of proliferative progenitors could be lower than the estimated 40 cells described here (i.e., if the initial progenitors divided more than once during the labeling period). Still, that does not preclude the conclusion that saltations occur as part of segment growth. Unexpected insights from this report revealed that multiple pulses of cell proliferation are required for segment size rather than a single saltation, as previously predicted (Iovine and Johnson, 2000; Goldsmith et al., 2003). How do these saltations fit into earlier models for fin growth?

Prior models of fin growth predicted that a single growth phase/saltation would account for the growth of a single segment and that rest phases flank each segment addition. Molecular evidence supporting this model revealed that expression of a gene-based growth marker, *fa93e10*, occurred 100% of the time in young, rapidly growing fins, and less frequently with aging, slowly growing fins. One possi-

bility is that the saltations that we define here represent the saltations identified using *fa93e10*, but the assumption that one saltation was equal to one segment is false (Fig. 5, Model A). If correct, then growth and rest simply alternate as predicted previously, and rapidly enough that it is not possible to identify young fins in the rest phase (i.e., *fa93e10*-negative). This is the simplest interpretation of our data. Alternatively, the saltations we define may represent minisaltations within a growth phase (Fig. 5, Model B). In this model, *fa93e10* would be positive throughout growth of a single segment. For example, fins would be *fa93e10*-positive at all times—in the “valleys” of less than five BrdU-positive cells, during the “peaks” of cell proliferation, and at all points in between. This may explain why it has not been possible to identify young fins that are *fa93e10*-negative even though we readily identify young fins with a small number of BrdU-positive cells. Indeed, fins would be *fa93e10*-negative only during a “true rest” phase occurring upon

segment completion. If correct, then a rapidly growing fin is in growth phase (i.e., *fa93e10*-positive) until a segment is complete and regardless of the number of proliferating cells (i.e., ~5–40 cells for the longest fin rays). This interpretation suggests there are layers of control during segment growth, and predicts that one can distinguish between “valleys” in the growth phase and a hypothetical “true rest” phase by following both *fa93e10* expression and BrdU labeling.

To test this prediction, we labeled 30 young fish with BrdU as before, and processed the caudal fins for both *fa93e10* expression and BrdU. Indeed, it was possible to identify *fa93e10*-positive fin rays that contained only one to five BrdU-positive cells (Fig. 6), indicating that fins with a small number of BrdU-positive cells are in fact in the growth phase. This finding occurred ~25% of the time (7/28), which is consistent with the frequency of “BrdU valleys” from the initial profile of proliferating cells (i.e., ~23% of fin rays in valleys in Fig. 2), indicating that a similar population was sampled in both experiments. These data provide strong evidence favoring Model B (Fig. 5) and indicate that the valleys identified in our cell proliferation profiles are part of the normal segment growth cycle and are distinct from a “true rest” phase (i.e., *fa93e10*-negative).

## Conclusions

In this report, we identify saltations during fin growth, define a saltation as the proliferation of up to 40 cells, and reveal that fin shape is partially regulated by the number of saltations occurring during new segment growth. The conclusion that the growth of a single segment requires multiple saltations is supported by two independent methods to identify dividing cells. Furthermore, mathematical modeling not only provides an equation representing the pattern of cell proliferation, but also provides statistical support for the existence of saltations.

What is the purpose of saltations? In humans, it has been suggested that growth and stasis reflect synchronized cell cycle activity at the organismal level and that long episodes of stasis are required for normal development to proceed (Lampl et al., 1992; Lampl

and Johnson, 1993). However, the coordination of cellular processes has not been examined during bone growth in humans. In zebrafish, one possibility is that regulation of saltations contributes to the maintenance of fin shape in adult fins (recall that the establishment of fin shape occurs during juvenile fin growth; Goldsmith et al., 2006). Circulating systemic factor(s) likely induce cell proliferation in the distal population of undifferentiated mesenchymal cells during fin growth, and individual fin rays may respond differentially, resulting in slight variations in segment length across the fin. Environmental conditions, such as nutrition, may provide the systemic factor regulating cell division. Indeed, it has recently been demonstrated that inhibition of insulin/insulin-like growth factor signaling by means of rapamycin treatment significantly reduces cell proliferation during fin growth (Goldsmith et al., 2006). When nutrients are limiting, the fin may remain in stasis or initiate a single saltation as nutrients become available; when nutrients are plentiful saltations may occur more frequently. "Valleys" identified here may provide additional opportunities to enter stasis in the event that nutrients are depleted during the growth of a segment (~5–7 days, Iovine and Johnson, 2000). Similarly, as body growth and fin growth are coordinated (Iovine and Johnson, 2000), saltations may allow for finer regulation of the coupling of growth processes and ensure adequate nutrient distribution. Such possibilities are not mutually exclusive, and in fact likely describe different aspects of the same problem, i.e., regulation of the growth and morphogenesis of the adult fin. Future aspects of this research will elucidate the molecular pathways regulating the dynamics of vertebrate bone growth.

## EXPERIMENTAL PROCEDURES

### Stocks and Fish Rearing

The wild-type fish stocks were obtained from the C32 strain. Fish were maintained at a constant temperature of 28°C and exposed to a 14-hr light: 10-hr dark photoperiod (Westerfield, 1993).

### Detection of BrdU-Labeled Cells

Fish were allowed to swim in BrdU (50 mg/ml) in fish water for 6 hr (Nechiporuk and Keating, 2002). The caudal fins were amputated asymmetrically to preserve dorsal and ventral identity and fixed in 4% paraformaldehyde at 4°C overnight. After fixation, fins were rinsed twice in methanol and stored at –20°C. They were rehydrated into PBTx (phosphate buffered saline [PBS] with 0.3% Triton X-100) and incubated in DnaseI (50 units/ml) for 45 min. Tissue was incubated with PBTxB (PBTx with 0.25% bovine serum albumin [BSA]) for 45 min and then oscillated overnight at 4°C in mouse anti-BrdU antibody (1:50). After extensive washing, fins were incubated in anti-mouse Alexa-546 at 1:200 overnight at 4°C. Individual fins were examined for BrdU-positive cells by confocal microscopy.

### Confocal Microscopy

The results of the BrdU staining were observed using the Zeiss LSM 510 Meta confocal microscope. To optimize the visible range, optical z-sections were collected through the distal most tips of the fin rays. Individual pictures were transferred to Microsoft Office PowerPoint and recompiled in a z-stack. The longest and shortest fin rays were examined from each fin. The longest fin ray in the ventral lobe was typically the third fin ray from the lateral side of the ventral lobe (identified by harvesting fin with an asymmetric cut), and is also referred to as the "lateral" ray. The shortest fin ray was identified as the most medial fin ray in the fin, and is also referred to as the "medial" ray.

### ZNS5 Detection

After confocal microscopy, fins were transferred to individual wells in a 48-well dish. Fins were treated with collagenase (1 mg/ml) in PBS for 45 min at room temperature, washed in a blocking reagent (2% BSA in PBS) and incubated at 4°C in mouse ZNS5 antibody at 1:200 (Zebrafish international resource center, <http://zebrafish.org/zirc/home/guide.php>). After removal of the primary antibody, fins were

washed and exposed to an unlabeled goat anti-mouse secondary antibody for 2 hr at room temperature. Subsequently, samples were washed and incubated in mouse PAP (peroxidase antiperoxidase) overnight at 4°C. Lastly, fins were washed in PBS and rinsed in diaminobenzidine (DAB; 0.03% DAB in 0.1 M PBS); 0.01% hydrogen peroxide was added. Fins were mounted in glycerol and visualized using a Nikon SMZ1500 microscope, and segment length was measured using ImagePro software.

### Detection of Mitotic Cells

Phosphorylation of Ser10 on histone H3 occurs specifically during mitosis (Hendzel et al., 1997). We examined fins for both mitotic cells using an anti-H3-phospho histidine antibody (H3P, Upstate Biotechnology) and for ZNS5 to follow the number of mitotic cells during segment growth. The H3P antibody was used at 1:100 and was detected by using anti-rabbit Alexa-546 following the ZNS5 detection protocol (i.e., double labeling along with ZNS5/anti-mouse Alexa-488). Fins were analyzed by confocal microscopy.

### Mathematical Modeling

To statistically confirm the periodic nature of cell division, a mathematical model of the form  $y = a|\cos(bx-c)| + d + \epsilon$  was proposed, where the number of dividing cells was a function of distal-most segment length. The period  $b$  and shift  $c$  were approximated so we could treat  $|\cos(bx-c)|$  as a new predictor variable, say  $x'$ , and the remaining fitting parameters  $a$  and  $d$  were found using simple linear regression method and using the Minitab Statistical Package 14. We tested the normality, equal variance, and independence assumptions of the linear regression models. We did not find significant violation of these assumptions. This alternative hypothesis was tested against the null hypothesis  $y = k$  (mean number of dividing cells), using the  $F$ -ratio test. Equations were linearly transformed, and corresponding  $P$  values were also found using Minitab. Final equations were plotted using Maple 9.5.

To determine whether the mean number of dividing cells was statis-

tically different from the lateral and medial fin rays, we completed a two-sample *t*-test. To begin, we found the data passed the three normality tests in Minitab (i.e., *P* values greater than significance level  $\alpha = 0.05$  for each test), and the data passed the equal variance test for a normal distribution (*P* = 0.818). Completion of the two-sample equal variance *t*-test revealed a *P* value of 0.162, indicating that there was no statistical difference in the mean number of dividing cells from the two data sets.

### Double-Labeling With *fa93e10* and BrdU

Fish (14 weeks and 12 lateral fin ray segments) were treated with BrdU for 6 hr, immediately harvested, fixed, and stored in methanol as described above. In situ hybridization using antisense RNA probe (UTP-digoxigenin) was completed as described (Poss et al., 2000).

After manual color development, fins were processed for BrdU as described above. BrdU-positive, mesenchymal cells were identified by confocal microscopy.

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