Transcriptional components of anteroposterior positional information during zebrafish fin regeneration

Gregory Nachtrab¹, Kazu Kikuchi¹,*, Valerie A. Tornini¹ and Kenneth D. Poss¹,²,‡

SUMMARY

Many fish and salamander species regenerate amputated fins or limbs, restoring the size and shape of the original appendage. Regeneration requires that spared cells retain or recall information encoding pattern, a phenomenon termed positional memory. Few factors have been implicated in positional memory during vertebrate appendage regeneration. Here, we investigated potential regulators of anteroposterior (AP) pattern during fin regeneration in adult zebrafish. Sequence-based profiling from tissues along the AP axis of uninjured pectoral fins identified many genes with region-specific expression, several of which encoded transcription factors with known AP-specific expression or function in developing embryonic pectoral appendages. Transgenic reporter strains revealed that regulatory sequences of the transcription factor gene alx4a activated expression in fibroblasts and osteoblasts within anterior fin rays, whereas hand2 regulatory sequences activated expression in these same cell types within posterior rays. Transgenic overexpression of hand2 in all pectoral fin rays did not affect formation of the proliferative regeneration blastema, yet modified the lengths and widths of regenerating bones. Hand2 influenced the character of regenerated rays in part by elevation of the vitamin D-inactivating enzyme encoded by cyp24a1, contributing to region-specific regulation of bone metabolism. Systemic administration of vitamin D during regeneration partially rescued bone defects resulting from hand2 overexpression. Thus, bone-forming cells in a regenerating appendage maintain expression throughout life of transcription factor genes that can influence AP pattern, and differ across the AP axis in their expression signatures of these and other genes. These findings have implications for mechanisms of positional memory in vertebrate tissues.

KEY WORDS: Regeneration, Anteroposterior patterning, Zebrafish, Fin, Blastema, Hand2, Vitamin D, Positional memory

INTRODUCTION

Many recent studies have employed cell transplantation or genetic fate-mapping approaches to identify the cellular sources of tissues that arise during injury-induced regeneration (Buckingham and Meilhac, 2011; Tanaka and Reddien, 2011). Upon defining sources of regeneration through these experiments, a key priority is then to understand how these stem/progenitor cells and differentiated cell types successfully restore complex tissues of the correct size, pattern and function after organ damage.

Limbs and fins are complex three-dimensional structures composed of numerous tissue types. Remarkably, many fish and salamanders retain the ability to regenerate amputated appendages throughout their adult lives. To do this, cells within the appendage stump must retain and recall detailed patterning information, which is commonly referred to as positional memory. A regulator of regenerative positional memory is expected to possess two main characters: (1) presence in a gradient or restricted pattern within the intact and regenerating adult appendage; and (2) its overexpression or blockade impacts the regenerative pattern. In planarians, which are invertebrates that undergo vigorous cellular turnover and regenerate through a stem cell population known as neoblasts, recent evidence indicates that the adult pattern is actively maintained by the regionalized expression of developmental pathway regulators. Upon injury, these same factors help restore tissue and reinitate pattern (Reddien et al., 2007; Gurley et al., 2008; Petersen and Reddien, 2011; Roberts-Galbraith and Newmark, 2013).

During amphibian limb regeneration, retinoic acid (RA) has been implicated in positional memory, as RA treatment causes wrist-level amputations to sprout shoulder-level regenerates (Maden, 1982). Although this finding is remarkable, the role of endogenous RA in positional memory is unclear as there is no discernible proximodistal (PD) gradient of RA in the intact limb. A second candidate factor is Prod1, a proposed receptor for the newt blastemal D-inactivating enzyme encoded by cyp24a1, contributing to region-specific regulation of bone metabolism. Systemic administration of vitamin D during regeneration partially rescued bone defects resulting from hand2 overexpression. Thus, bone-forming cells in a regenerating appendage maintain expression throughout life of transcription factor genes that can influence AP pattern, and differ across the AP axis in their expression signatures of these and other genes. These findings have implications for mechanisms of positional memory in vertebrate tissues.

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the robust effects of their gain- or loss-of-function on limb patterning (Riddle et al., 1993; Qu et al., 1997; Charité et al., 2000; Zákány et al., 2004). For instance, the transcription factor Hand2 is localized in the posterior region of developing pectoral appendages, and its overexpression causes developmental transformations along the AP axis. Despite abundant experimental data on AP patterning in embryonic limbs, positional memory of regenerating appendages has focused instead on PD regulation.

Adult zebrafish regenerate amputated fins with speed and precision. Recent work has produced cellular and molecular models for blastema formation and regenerative outgrowth during fin regeneration, but positional memory remains largely unexplained (Knopf et al., 2011; Blum and Begemann, 2012). Here, we used RNA sequencing to identify factors with potential roles in positional memory along the AP axis in zebrafish pectoral fins. We found many genes with AP region-specific expression in uninjured fins, several of which are transcription factor genes with known roles in AP patterning of embryonic pectoral appendages. Transgenic overexpression of one of these genes, hand2, modified bone patterning during regeneration, in part through regulation of vitamin D metabolism and signaling. These experiments identify a factor with the characteristics of a positional memory component, and provide evidence that adult zebrafish fins maintain positional information via the sustained regional restriction of key embryonic patterning genes.

MATERIALS AND METHODS
Zebrafish
Males of several zebrafish strains show defects in pectoral fin regeneration (Nachtrab et al., 2011), necessitating the use of females for these experiments. All animals were between 4 and 12 months of age and in an outbred Ekkwill (EK) strain background. Pectoral fins were amputated proximal to the first bifurcation point at approximately one-third of their average 47% longer and 65% wider than the eighth (posterior) ray. Fin ray regeneration proceeds by material Fig. S1). Fin regeneration in zebrafish is initiated by the formation of a blastema at the healed distal tip of each ray by 2-4 days post-amputation (dpa). Fin ray regeneration proceeds by maintenance of proliferative blastemal tissue just distal to a patterning zone, in which osteoblasts align and mineralize bone (Akimenko et al., 2003; Poss et al., 2003). We analyzed ray morphology and osteoblast differentiation events during regeneration, aided by a transgenic reporter strain visualizing expression of the osteoblast transcription factor osterix (osx or sp7). Differences in the patterns of aligned osteoblasts among rays on the AP axis began to manifest during a period from 5-7 dpa, and the AP ray pattern was restored by 10 dpa (Fig. 1A,B).

To identify genes that might be responsible for AP differences in ray pattern, we sequenced RNAs collected from the most anterior (AP1) and most posterior (AP5) regions of pectoral fins (Fig. 1C;
sequencing data are deposited in the NCBI SRA database, accession SRP027598). We found 235 predicted genes with significantly different expression in these two regions, representing ~0.8% of the transcriptome (supplementary material Fig. S2A). These 235 predicted genes comprised 195 annotated genes and 40 putative genes. Of the 195 AP genes, 105 were elevated in anterior rays and 90 in posterior rays. To assess whether certain categories or classes of genes were enriched in our AP dataset, we performed a gene ontology (GO) search. Using the GO category ‘biological process’, we found our AP genes to be enriched for general terms such as ‘developmental process’ and ‘biological regulation’. However, we also saw enrichment for the more specific terms ‘fin development’, ‘nervous system development’ and ‘regulation of transcription, DNA-dependent’ (supplementary material Fig. S2B).

For several reasons, we focused our initial analysis on differentially expressed transcription factor genes. First, transcription factors are central to many developmental programs; thus, their regionalized expression has the potential to impact multiple downstream genes. Second, as mentioned above, a rich field of embryonic limb development has detailed the relationship between transcription factors and AP skeletal patterning (Qu et al., 1997; Charité et al., 2000; Fernandez-Teran et al., 2000; Zákány et al., 2004; Tzchori et al., 2009; Galli et al., 2010; Sheth et al., 2012). Third, our dataset revealed many examples of developmental transcription factors with expression differences along the AP axis. In particular, the transcription factor hand2, which is crucial for pectoral fin development and expressed in the posterior region of developing pectoral fin and forelimb buds (Charité et al., 2000; Fernandez-Teran et al., 2000; Yelon et al., 2000), showed the most polarized expression of all genes enriched in posterior fin rays (Table 1). Additionally, the embryonic anterior patterning factors alx4a and lhx9 were fifth and eighth on the list of genes enriched in anterior rays (Table 1).

To define transcription factor expression signatures in pectoral fins, we performed quantitative RT-PCR (qPCR) using tissues across five different regions of the AP axis. This approach...
confirmed restriction of *hand2* to posterior fin rays, and also verified region-specific expression for eight other transcription factor genes: *alx4a, hox9, id4, pax9, tbx2a, hoxc8a, hoxd11a* and *hoxd13a*. Six of these eight transcription factors have roles in AP patterning during limb or fin development (Qu et al., 1997; Harrelson et al., 2004; Zákány et al., 2004; McGlenn et al., 2005; Tzchori et al., 2009), whereas *id4* has been implicated in bone homeostasis (Tokiwa et al., 2010). The anterior expression of the Hoxc genes has been described during pectoral fin development (Molven et al., 1990), and *HOXC8* has specifically been shown to be expressed in the anterior region of developing chick wings (Nelson et al., 1996). Gauged by the expression of these nine factors, each set of two rays across the AP axis displayed a unique gene signature (Fig. 1D).

Notably, *hand2, hoxd13a, alx4a* and *hox9* displayed regional expression in adult fins similar to their reported expression in embryonic forelimb or pectoral fin buds (Qu et al., 1997; Charité et al., 2000; Ahn and Ho, 2008; Wang et al., 2011). If important for patterning regenerating structures, these transcription factors would be expected to retain their regionalized character during regeneration. Indeed, at 4 dpa, all nine factors had a profile similar to that in uninjured fins (Fig. 1E).

In summary, adult zebrafish pectoral fins retain region-specific signatures of transcription factor genes important for AP patterning of embryonic pectoral appendages, consistent with potential roles in patterning and positional memory.

### Table 1. The top 20 genes enriched in the fin anterior or fin posterior

<table>
<thead>
<tr>
<th>Gene</th>
<th>Anterior (FPKM)</th>
<th>Posterior (FPKM)</th>
<th>log2 fold-change</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>rspo4</em></td>
<td>1.478</td>
<td>0.000</td>
<td>−1.79769±308</td>
<td>1.24E-04</td>
</tr>
<tr>
<td><em>zgc:165423</em></td>
<td>1.353</td>
<td>0.018</td>
<td>−6.259</td>
<td>9.9E-05</td>
</tr>
<tr>
<td><em>mpz</em></td>
<td>20.003</td>
<td>0.757</td>
<td>−4.724</td>
<td>3.8E-08</td>
</tr>
<tr>
<td><em>rspo1</em></td>
<td>2.937</td>
<td>0.113</td>
<td>−4.705</td>
<td>1.0E-13</td>
</tr>
<tr>
<td><em>alx4a</em></td>
<td>5.257</td>
<td>0.221</td>
<td>−4.575</td>
<td>0.00E+00</td>
</tr>
<tr>
<td><em>CR925813.1</em></td>
<td>3.400</td>
<td>0.163</td>
<td>−3.808</td>
<td>1.5E-07</td>
</tr>
<tr>
<td><em>Bx927244.2</em></td>
<td>40.493</td>
<td>1.993</td>
<td>−3.345</td>
<td>3.9E-13</td>
</tr>
<tr>
<td><em>lhx9</em></td>
<td>8.475</td>
<td>0.424</td>
<td>−4.320</td>
<td>3.2E-09</td>
</tr>
<tr>
<td><em>chd1l</em></td>
<td>0.940</td>
<td>0.059</td>
<td>−3.985</td>
<td>1.2E-04</td>
</tr>
<tr>
<td><em>fcbp</em></td>
<td>1.193</td>
<td>0.077</td>
<td>−3.947</td>
<td>2.8E-08</td>
</tr>
<tr>
<td><em>si:ch211-36i19.1</em></td>
<td>1.297</td>
<td>0.087</td>
<td>−3.900</td>
<td>1.1E-04</td>
</tr>
<tr>
<td><em>tyr1b</em></td>
<td>23.081</td>
<td>1.598</td>
<td>−3.852</td>
<td>0.00E+00</td>
</tr>
<tr>
<td><em>scl7a8 (2 of 2)</em></td>
<td>2.980</td>
<td>0.222</td>
<td>−3.749</td>
<td>9.3E-08</td>
</tr>
<tr>
<td><em>plich3</em></td>
<td>0.101</td>
<td>0.085</td>
<td>−3.572</td>
<td>4.1E-06</td>
</tr>
<tr>
<td><em>scinla</em></td>
<td>55.695</td>
<td>4.687</td>
<td>−3.571</td>
<td>0.00E+00</td>
</tr>
<tr>
<td><strong>Anterior-enriched genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>hand2</em></td>
<td>0.097</td>
<td>18.979</td>
<td>7.614</td>
<td>0.00E+00</td>
</tr>
<tr>
<td><em>scpp7</em></td>
<td>0.287</td>
<td>50.526</td>
<td>7.462</td>
<td>0.00E+00</td>
</tr>
<tr>
<td><em>ucp1</em></td>
<td>0.105</td>
<td>16.550</td>
<td>7.294</td>
<td>2.3E-04</td>
</tr>
<tr>
<td><em>si:dkey-22i16.3</em></td>
<td>0.520</td>
<td>35.554</td>
<td>6.095</td>
<td>0.00E+00</td>
</tr>
<tr>
<td><em>and2</em></td>
<td>0.410</td>
<td>12.387</td>
<td>4.918</td>
<td>0.00E+00</td>
</tr>
<tr>
<td><em>tbx4</em></td>
<td>0.026</td>
<td>0.780</td>
<td>4.901</td>
<td>6.9E-06</td>
</tr>
<tr>
<td><em>asip</em></td>
<td>2.140</td>
<td>36.870</td>
<td>4.107</td>
<td>8.0E-11</td>
</tr>
<tr>
<td><em>and3</em></td>
<td>0.572</td>
<td>8.420</td>
<td>3.880</td>
<td>1.8E-12</td>
</tr>
<tr>
<td><em>si:dkey-189n19.4</em></td>
<td>5.103</td>
<td>66.136</td>
<td>3.696</td>
<td>4.8E-05</td>
</tr>
<tr>
<td><em>hoxd12a</em></td>
<td>0.983</td>
<td>12.602</td>
<td>3.680</td>
<td>8.7E-12</td>
</tr>
<tr>
<td><em>and1</em></td>
<td>4.981</td>
<td>60.058</td>
<td>3.572</td>
<td>0.00E+00</td>
</tr>
<tr>
<td><em>hoxd11a</em></td>
<td>1.125</td>
<td>12.779</td>
<td>3.506</td>
<td>0.00E+00</td>
</tr>
<tr>
<td><em>frem2a</em></td>
<td>0.143</td>
<td>1.575</td>
<td>3.459</td>
<td>0.00E+00</td>
</tr>
<tr>
<td><em>hoxd13a</em></td>
<td>0.674</td>
<td>5.491</td>
<td>3.026</td>
<td>1.0E-12</td>
</tr>
<tr>
<td><em>cyph24a1</em></td>
<td>6.780</td>
<td>52.182</td>
<td>2.944</td>
<td>1.0E+00</td>
</tr>
</tbody>
</table>

*Among the posterior-enriched genes, hand2 displays the greatest differential expression. P-values by Student’s t-test. FPKM, fragments per kb of transcript per million map reads.*

### Fin fibroblasts and osteoblasts differ across the AP axis in the expression of patterning transcription factor genes

To visualize the differential expression of anterior and posterior factors, we generated a BAC transgenic reporter line *Tg(alx4a:DsRed2)p272* and also assessed reporter expression in the *Tg(hand2:EGFP)* BAC transgenic line (Kikuchi et al., 2011). Using these lines, we found that the *alx4a* region extended from the most anterior to the third rays of the pectoral fin. Conversely, the *hand2* domain extended from the sixth ray to the posterior edge of the fin (Fig. 2A). In uninjured fins, both regulatory sequences activated expression along the entire PD axis of the fin, and similarly during regeneration to the near distal tips (Fig. 2A-C).

These adult expression domains were reminiscent of their profiles in developing embryonic fin buds (Fig. 2D). We visualized in the same way the expression of a third transcription factor, *tbx18*, which is known to be expressed along the entire AP axis of developing zebrafish pectoral fins (Liu and Stainier, 2010). The BAC transgenic reporter line *Tg(tbx18:EGFP)p272* revealed fin-wide expression of *tbx18* in adult zebrafish pectoral fins, a result verified by qPCR (supplementary material Fig. S3A,B).

To assess expression differences among fins, we also examined *alx4a*, *hand2* and *tbx18*-driven transgenic reporter expression in other adult zebrafish fins. This revealed a similar pattern of *alx4a*, *hand2* and *tbx18* expression in pelvic fins as in pectoral fins. In the anal and dorsal fins, *alx4a* was expressed in the most anterior marginal ray, whereas *hand2* was expressed weakly in the most caudal ray. In the caudal fin, *alx4a* was expressed only in the most ventral ray, and *hand2* expression was not detectable. *tbx18* was expressed weakly in these unpaired fin types (supplementary material Fig. S3C,D). These observations indicated that all adult zebrafish fins maintain the expression of transcription factors associated with their initial development, with different expression domains in differentially patterned appendages.

To determine which cells contained AP regionalized expression of *hand2* and *alx4a*, we histologically assessed pectoral fins from the reporter strains. Longitudinal and transverse sections of regenerates revealed that the expression of *hand2* and *alx4a* was limited to the mesenchymal compartment of fins (Fig. 2C,E). Although the majority of cells in the mesenchyme are fibroblasts, we also found that osteoblasts lining the bone rays were distinctly positive for either of these transcription factors (Fig. 2F). Thus, AP patterning transcription factors are maintained in unique expression domains in the bone-forming cells of uninjured and regenerating fins. Although differentiated osteoblasts from different fin ray regions appear identical and have been considered as such in models of regeneration, it is now clear that they have different gene expression signatures.

### hand2 overexpression alters bone lengths and widths in regenerating fins

Next, we examined whether the regional restriction of transcription factors is required for normal patterning during fin regeneration. We generated transgenic zebrafish permitting heat-inducible expression of the anterior genes *alx4a, hox9* or *id4* or of the posterior gene *hand2*. This approach enables inducible expression of a candidate factor across the entire AP axis of the adult pectoral fin. We reasoned that the overexpression of potential positional memory components would alter regenerative patterning and be manifested in changes in the lengths and widths of fin rays. This approach is analogous to the overexpression techniques that defined key regions and factors for embryonic limb patterning in mouse and chick, prior
to the development of many conditional knockout models (Maccabe et al., 1973; Riddle et al., 1993; Charité et al., 2000).

We identified inducible transgenic lines for the anterior genes alx4a, id4 and lhx9 [Tg(hsp70l:alx4a)pd3, Tg(hsp70l:id4)pd4 and Tg(hsp70l:lhx9)pd5] that enabled ~13-, 10- and 13-fold increases, respectively, in the expression of these genes in posterior rays after a single heat shock at 4 dpa (supplementary material Fig. S4A). We then examined whether this overexpression could alter regenerative pattern by amputating pectoral fins in these lines and administering a daily heat shock. At 7 dpa, there was no gross change in the appearance of the fins (supplementary material Fig. S4B). We quantified the lengths and widths of regenerated rays to ascertain whether there were any subtle alterations. Heightened id4 levels during regeneration produced an 11% increase in posterior fin ray lengths (supplementary material Fig. S4C). Elevated alx4a increased relative medial and posterior ray widths by 4% and 6%, respectively, and there was a 6% increase in the anterior and medial regions caused by id4 overexpression (supplementary material Fig. S4D). These modest changes in patterning suggested that the anterior factors we examined are not individually sufficient to influence patterning in regenerating pectoral fin rays.

We next assessed the effects of hand2 overexpression [Tg(hsp70l:hand2)pd56], which was increased 58-fold in anterior fin regions after a single heat shock at 4 dpa (Fig. 3A), a change that reflects more the negligible endogenous levels of hand2 mRNA in anterior rays, rather than the sheer magnitude of hand2 induction (which is similar to that of alx4a, lhx9 or id4 after heat-shock induction of the respective transgenes) (Fig. 1D,E; supplementary material Fig. S5A). Seven days of hand2 induction initiated after amputation was sufficient to produce moderate to severe defects in the patterns of regenerating fin rays (Fig. 3B). Overall, there was a 42% decrease in the lengths of regenerated anterior rays at 7 dpa and a 56% decrease in lengths of posterior rays (Fig. 3C). We also found reductions in width ratios across pectoral fins ranging from 14 to 22% (Fig. 3D). To assess whether the hand2 overexpression phenotype was the result of a transient delay in regeneration, we extended the experiment to 30 dpa, approximately twice the time within which regeneration is normally completed. We observed
shortened regenerates with smaller rays and fewer segments in the \textit{hsp70l:hand2} transgenic fish at 30 dpa, similar to phenotypes at 7 dpa (Fig. 3E-G), indicating that the patterning phenotype was not the result of a transient regenerative delay.

Overexpression of \textit{hand2} in developing mammalian limbs results in striking phenotypes, including polydactyly and anterior-to-posterior digit conversions (Charité et al., 2000). However, overexpression of \textit{hand2} during zebrafish pectoral fin regeneration caused a less striking phenotype – an increase in the posterior character of all fin rays. There are several reasons that might explain this result. First, unlike an embryonic fin bud, mammalian limb bud or newt limb blastema, in which a single primordium is patterned by signaling molecules, an adult fin regenerate is a composite of individual ray primordia. The overall fin shape is a cumulative readout of the patterning of the individual ray blastemas to give each ray a length and width, and it is unlikely that the overexpression of a single factor such as \textit{hand2} would produce a phenotype similar to that seen in other contexts. Second, \textit{hand2} is only one of several transcription factors with differential AP expression in adult pectoral fins, and overexpression of \textit{hand2} did not cause similar significant alternations in the expression of other AP transcription factors (supplementary material Fig. S5B). Third, heat shock raises expression without altering the underlying endogenous asymmetry, leading to a shallower but still significant AP gradient of \textit{hand2} across the AP axis (supplementary material Fig. S5C). Finally, as many adult fin rays have no detectable \textit{hand2} expression, there is unlikely to be a striking change in length and width caused by changing only Hand2 levels.

To identify a critical window for the effects of \textit{hand2} overexpression, we controlled the timing and number of heat shocks with respect to amputation. When \textit{hand2} was induced only during the first 3 days after amputation and prior to significant bone

Fig. 3. Overexpression of \textit{hand2} during fin regeneration alters ray patterning. (A) \textit{hand2} expression is induced 58-fold in anterior regions of \textit{hsp70l:hand2} pectoral fins 4 hours after a single heat shock at 4 dpa. Values are normalized to \textit{actb1} levels and relative to wild-type controls. n=3; mean ± s.e.m. (B) Appearance of \textit{hsp70l:hand2} and wild-type clutchmate fins at 7 dpa after a series of daily heat shocks. \textit{hand2} overexpression generates shorter rays with a reduced number of bone segments. Phenotypes range from moderate (upper right) to severe (lower right). Representative regenerative growths of wild-type rays 3 and 8 are denoted by dotted lines. (C) Overexpression of \textit{hand2} reduces the lengths of regenerating rays across the AP axis of pectoral fins. n=16 (wild type) and n=15 (\textit{hsp70l:hand2}). (D) Overexpression of \textit{hand2} during regeneration reduces the widths of regenerating fin rays across the AP axis. Transgenic fish that displayed a moderate phenotype were quantified. n=13 (wild type) and n=6 (\textit{hsp70l:hand2}). (E) Appearance of \textit{hsp70l:hand2} and wild-type clutchmate fins at 30 dpa after daily heat shocks. \textit{hsp70l:hand2} regenerates remain stunted with shorter and thinner fin rays. Representative regenerative growths of wild-type rays 3 and 8 are denoted by dotted lines. (F) Quantification of \textit{hsp70l:hand2} ray lengths at 30 dpa. n=12. (G) Quantification of \textit{hsp70l:hand2} segment numbers at 30 dpa. n=12. (H) Quantification of \textit{hsp70l:hand2} ray widths at 30 dpa. n=12. *P<0.05, **P<0.005, Student’s t-test; bar indicates mean. Arrowheads (B,E) indicate amputation plane. Scale bars: 0.5 mm.
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**Hand2 effects on vitamin D metabolism in zebrafish fins**

hand2 regulates the localization of sonic hedgehog (shh) in the posterior region of developing embryonic forelimbs, wings or pectoral fins (Charité et al., 2000; Fernandez-Teran et al., 2000; Yelon et al., 2000; Galli et al., 2010). However, shha is expressed in epidermal cells at the distal tip of each uninjured and regenerating adult fin ray, regardless of whether that ray expresses hand2 (Quint et al., 2002; Lee et al., 2009). We examined our RNA-Seq dataset for possible alternative targets of hand2 during regeneration, with a focus on genes known to be involved in bone formation or homeostasis. A significant AP asymmetry was observed for cyp24a1, which encodes a vitamin D-inactivating enzyme (Knutson and DeLuca, 1974), which was expressed at higher levels in posterior regions of uninjured pectoral fins (supplementary material Fig. S6D). The expression of each of these genes was significantly higher in the cyp24a1-low anterior regions of uninjured pectoral fins (supplementary material Fig. S6E), suggesting differential vitamin D signaling across the fin AP axis. Additionally, hand2 overexpression in regenerating pectoral fins reduced calb2a, bglap and spar levels by 64%, 57%

Deposition, there was no significant change in the lengths of regenerating fins (Fig. 4A,B). In agreement with this, daily hand2 induction had no significant effect on indices of blastemal cell proliferation at 3 dpa, as compared with heat-shocked wild-type zebrafish (Fig. 4C,D). However, when daily hand2 induction was initiated at 3 dpa, there was a 20% decrease in the lengths of regenerated anterior rays and an 8% decrease in ray widths by 7 dpa (Fig. 4E-G). These results indicated that elevating the levels of the normally posterior-restricted hand2 throughout the fin does not affect early regenerative growth, but alters ray patterning by reducing ray lengths and widths.

Together, our data indicate that the maintenance of hand2 in the mesenchyme of posterior rays contributes to the restoration of posterior ray characteristics during regeneration.

**Fig. 4. hand2 overexpression exerts its effects during later stages of regeneration.**

(A,B) No difference in the appearance of hsp70l:hand2 and wild-type clutchmate fins at 3 dpa after a series of daily heat shocks. n=16. (C,D) Blastemal proliferation as measured by BrdU incorporation is also unaffected by hand2 overexpression. n=13. (E) Stunted regeneration in an hsp70l:hand2 pectoral fin compared with a wild-type clutchmate fin at 7 dpa after a series of daily heat shocks beginning at 3 dpa. Representative regenerative growth of wild-type ray 3 is denoted by a dotted line. (F,G) Significant reductions in anterior ray lengths and widths manifest when hand2 is overexpressed during later stages of regeneration. n=8. *P<0.05, Student’s t-test; bar indicates mean. Arrowheads (A,C,E) indicate amputation plane.
and 22%, respectively, consistent with a model in which Hand2 regulates vitamin D signaling in pectoral fin rays (supplementary material Fig. S6F).

We also examined whether Hand2 influences a second class of posterior genes involved in bone formation and fin ray pattern. The actinodin genes are structural components of actinotrichia, which are unmineralized fibrils found in developing pectoral fins (Zhang et al., 2010). Unexpectedly, the entire gene family, and1-4, was elevated in the posterior regions of uninjured adult pectoral fins, as detected by RNA-Seq and qPCR (Table 1; supplementary material Fig. S7A). In contrast to cyp24a1, daily induction of hand2 during regeneration did not significantly increase the levels of actinodin family genes in the anterior regions of pectoral fins at 4 dpa (supplementary material Fig. S7B). This result indicates that Hand2 controls a subset of genes that show AP regionalized expression and potentially contribute to posterior ray characteristics.

To define the significance of vitamin D signaling as a target of Hand2, we supplemented zebrafish with vitamin D and assessed pectoral fin regeneration. Daily vitamin D injection had no effects on pectoral fin regeneration in wild-type fish. By contrast, this systemic regimen led to 24% and 20% increases in the lengths of regenerating anterior and posterior rays, respectively, during hand2 overexpression (Fig. 5E,F). Accompanying the length increase was an ~11% increase in the relative widths of the rays across the AP axis (Fig. 5G).

**DISCUSSION**

A defining feature of appendage regeneration is the maintenance and recall of positional information, evident in the restoration of...
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Fig. 6. Model for Hand2 influences on positional memory. Adult zebrafish pectoral fins maintain region-specific expression of transcription factor genes. Levels of the posterior transcription factor gene hand2 can help control bone patterning during pectoral fin regeneration. Hand2 levels regulate bone formation during regeneration by direct or indirect regulation of the vitamin D-inactivating enzyme encoded by cyp24a1, restricting bone formation in the posterior rays of pectoral fins.

Correctly patterned structures. Here we discovered a transcription factor gene expression signature and a downstream signaling pathway that might underlie AP positional memory in zebrafish pectoral fins. Many genes displayed region-specific expression across the AP axis of uninjured adult pectoral fins, and we examined the impact of four of them on patterning during regeneration. One of these transcription factor genes, hand2, displayed the expected aspects of a regulator of positional memory. First, hand2 expression is maintained in an AP region-specific manner in bone-forming cells of uninjured and regenerating fins. That is, anterior ray osteoblasts have a different transcription factor signature to posterior ray osteoblasts, as defined minimally for hand2 and alx4 expression. Second, hand2 overexpression changes the AP patterning of bone during fin regeneration. Furthermore, our data indicate that hand2 regulates posterior bone formation in part through local control of the activity of a systemic signal — vitamin D.

Our findings reveal that zebrafish pectoral fins maintain a basal level of transcription factors in preferential domains throughout all life stages, as opposed to turning off expression after patterning and differentiation. We suggest that this concept is likely to be central to positional memory in zebrafish fins, and potentially other regenerative vertebrate tissues. The expression of embryonic signaling factors in uninjured adult structures has been indicated in other examples of appendage regeneration (Nicolas et al., 2003; Schnapp et al., 2005; Wills et al., 2008; Poss, 2010), and mammalian fibroblasts are known to express region-specific Hox gene codes (Chang et al., 2002; Rinn et al., 2006).

Although positional memory factors are likely to recapitulate aspects of the embryonic developmental program, they are also likely to regulate pattern by mechanisms that are distinct from embryogenesis. Relevant to this second idea, our data do not indicate that Hand2 influences pattern via Shh signaling in adult pectoral fins as it does in embryonic pectoral appendages. Rather, they implicate the control of vitamin D signaling as an important regulatory mechanism, an interaction that to our knowledge has not previously been reported. This mechanism might be relevant to other tissues in which Hand2 is of influence, such as the developing craniofacial structures. Previous work has demonstrated the complex role of vitamin D in mammalian bone homeostasis (Lieben and Carmeliet, 2013). Although implanting vitamin D-soaked beads was shown to have somewhat minor effects on axolotl limb regeneration (Washabaugh and Tsonis, 1995), to our knowledge there have been no reports that vitamin D signaling helps define AP patterning in regenerating appendages. This lack of evidence can be explained in part by the fact that only when signaling was compromised by hand2 overexpression was a vitamin D-related phenotype observed (Fig. 5E-G). Aside from appendage regeneration, vitamin D has been shown to influence liver, axon and skeletal muscle regeneration (Ether et al., 1990; Chabas et al., 2008; Stratos et al., 2013). It will be interesting to examine whether the regulation of vitamin D signaling is important in additional regenerative contexts.

By what mechanism(s) do adult cells maintain the regionalized expression of patterning transcription factors? There are at least two important gene regulatory components of regeneration that might on the surface appear contradictory. First, cells must be capable of rapid gene expression flux to enact major changes such as dedifferentiation in response to injury. Second, as shown here, cells also lock in the expression of key developmental regulators in a region-specific manner throughout life. Novel epigenetic regulation, possibly at the chromatin level, is likely to underlie this versatility.

Although we found differential AP expression of numerous transcription factors, functional manipulation of just one of these produced clear patterning effects during regeneration. This might reflect the limitations of the overexpression technologies currently available for use in adult zebrafish fins. Alternatively, it is possible that multiple transcription factors act in concert as a code to specify positional information (Grobelein et al., 2004; Dassen et al., 2005; Tümpel et al., 2009). Consistent with this notion, we also found posterior-enriched genes that were not influenced by the overexpression of hand2 in anterior rays. Moreover, complete reprogramming of positional memory is likely to require simultaneous increases and decreases in gene expression affecting multiple genes. Emerging genetic toolsets for zebrafish and other highly regenerative vertebrates should enable the discovery of core modules of positional memory (Meng et al., 2008; Bedell et al., 2012; Dahlem et al., 2012), bringing us closer to an understanding of how regeneration recalls and replicates complex tissues.

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The authors declare no competing financial interests.

Author contributions
G.N. conducted the experiments and analyzed the data, G.N. and K.D.P. designed the experiments and wrote the paper, K.K. generated the hand2:EGFP and tbx18:EGFP reporter lines, and V.A.T. performed RT-PCR experiments.

Supplementary material
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