RESEARCH ARTICLE SUMMARY

REGENERATION

Changes in regeneration-responsive enhancers shape regenerative capacities in vertebrates

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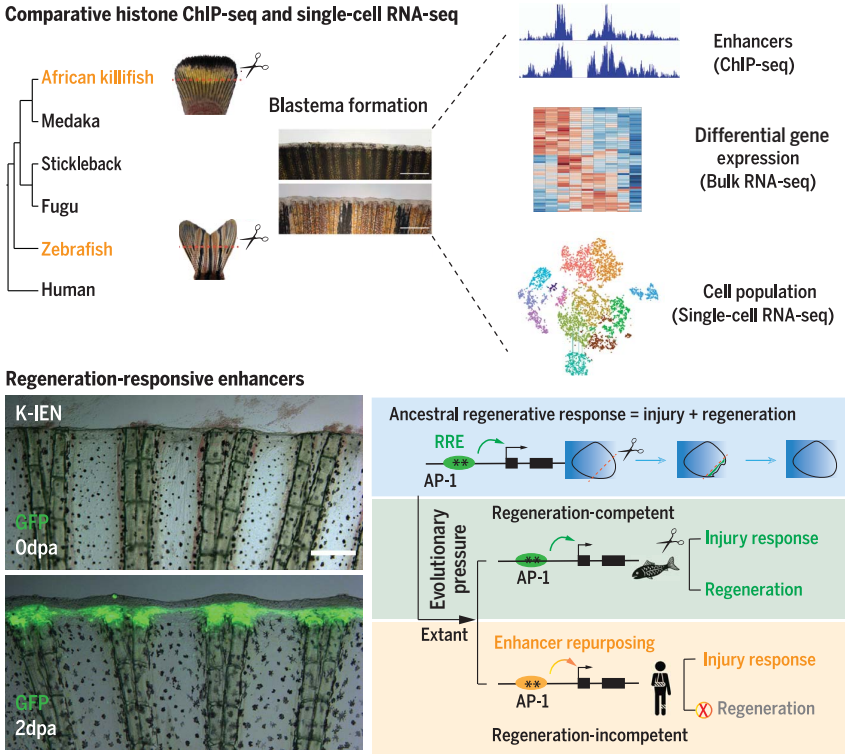
INTRODUCTION: The ability to regenerate tissues lost to damage or disease is widely but nonuniformly distributed in vertebrates. Some animals such as teleost fishes can regenerate a variety of organs, including amputated appendages, heart ventricles, and the spinal cord, whereas others such as mammals cannot. Even though regeneration has been the subject of extensive phylogenetic, developmental, cellular, and molecular studies, the mechanisms underlying the broad disparity of regenerative capacities in animals remain elusive. Changes in cis-regulatory elements have been shown to be a major source of morphological diversity. Emerging evidence indicates that injury-dependent gene expression may be controlled by injury-responsive enhancer elements. However, ablations of these previously characterized elements from the zebrafish (*Danio rerio*) and *Drosophila* have shown that they are generally dispensable for regeneration. Therefore, whether conserved regeneration-responsive, rather than injury-responsive, elements exist in vertebrate genomes and how they evolved remain to be conclusively demonstrated.

RATIONALE: Identification of conserved regeneration-responsive enhancers (RREs) requires two related but evolutionarily distant species that are capable of regeneration. The dramatic differences in life history and the ~230 million years of evolutionary distance between the zebrafish and the African killifish *Nothobranchius furzeri* provide a unique biological context in which to distinguish between species-specific and conserved RREs. We reasoned that applying histone H3K27ac chromatin immunoprecipitation sequencing (ChIP-seq, a marker for active enhancers), bulk RNA sequencing (RNA-seq), and single-cell RNA-seq (scRNA-seq) would identify RREs activated by amputation and help to determine their target gene expression at the single-cell level. Furthermore, we took advantage of the fast sexual maturation of African killifish to rapidly generate transgenic reporter assays to validate predicted RREs and to facilitate their functional testing in adult regeneration.

RESULTS: We uncovered both large differences in the genomic responses to amputation in killifish and zebrafish and an evolutionarily conserved teleost regeneration response program (RRP), which is mainly deployed by regeneration-specific blastema cells. Bioinformatic analyses revealed that activation of the RRP, which includes known effectors of regeneration in zebrafish such as *inhibin beta A* (*inhba*), was differentially activated in mammals that are robust (*Acomys cahirinus*) and weak regenerators (*Mus musculus*). Functional testing by systematic transgenic reporter assays of the conserved *inhba* RRE from killifish, zebrafish, and humans identified species-specific variations. Deletion of the killifish *inhba* RRE significantly perturbed caudal fin regeneration and abrogated cardiac regeneration. Furthermore, *inhba* RRE activity required the presence of predicted binding motifs for the activator protein 1 (AP-1) complex. Lastly, AP-1–binding motifs can be identified in the conserved and nonconserved teleost RREs reported in this study, indicating that AP-1 may be required for both injury and regeneration responses.

CONCLUSION: We propose an RRE-based model for the loss of regenerative capacities during evolution. In our model, the ancestral function for AP-1–enriched RREs was to activate a regenerative response that included both injury and regeneration. Through the course of evolution and speciation, regeneration and injury responses became dissociated from each other in some but not all enhancers. In extant species, regeneration-competent animals maintain the ancestral enhancer activities to activate both injury and regeneration responses, whereas in regeneration-incompetent animals, repurposing of ancestral enhancers may have led to the retention of injury response activities but to the loss of the regeneration response. ■



RREs and vertebrate regeneration. Comparative H3K27ac ChIP-seq, bulk RNA-seq, and scRNA-seq of two distantly related teleost species (African killifish and zebrafish) during the early stages of regeneration helped to identify evolutionarily conserved RREs active in blastemal cells. Systematic transgenic reporter assays validated the putative RREs and helped to identify species-specific variations of an RRE essential for killifish regeneration. Our study provides a testable hypothesis based on enhancer repurposing to explain the uneven distribution of regenerative capacities in vertebrates.

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RESEARCH ARTICLE

REGENERATION

Changes in regeneration-responsive enhancers shape regenerative capacities in vertebrates

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Vertebrates vary in their ability to regenerate, and the genetic mechanisms underlying such disparity remain elusive. Comparative epigenomic profiling and single-cell sequencing of two related teleost fish uncovered species-specific and evolutionarily conserved genomic responses to regeneration. The conserved response revealed several regeneration-responsive enhancers (RREs), including an element upstream to *inhibin beta A* (*inhba*), a known effector of vertebrate regeneration. This element activated expression in regenerating transgenic fish, and its genomic deletion perturbed caudal fin regeneration and abrogated cardiac regeneration altogether. The enhancer is present in mammals, shares functionally essential activator protein 1 (AP-1)–binding motifs, and responds to injury, but it cannot rescue regeneration in fish. This work suggests that changes in AP-1–enriched RREs are likely a crucial source of loss of regenerative capacities in vertebrates.

Regeneration in response to tissue damage is not uniformly distributed in vertebrates (*1*). For instance, teleost fishes and salamanders can regenerate a variety of organs, including amputated appendages, heart ventricle, and spinal cord, whereas mammals have relatively little regenerative capability (*2*, *3*). Moreover, the ability to regenerate is generally limited to only early developmental stages in certain species (*4*, *5*). Changes in cis-regulatory elements or enhancers are a major source of morphological diversity (*6*, *7*). Emerging evidence suggests that the activation of injury-dependent gene expression may be directed by injury-responsive enhancer elements (*8*). Two such elements, the *leptin-b* (*lepb*) enhancer in the zebrafish (*Danio rerio*) and the *WNT* gene cluster *BRV118* enhancer in the fruit fly *Drosophila melanogaster*, modulate gene expression after injury. However, ablation of *lepb* in zebrafish or the fly *WNT* enhancer has shown these injury-responsive components to be generally dispensable for regeneration (*8*, *9*). Therefore, whether conserved regeneration-responsive, rather than injury-responsive, elements exist in vertebrate genomes and how they evolve have not been conclusively demonstrated.

The identification of enhancers across species is complicated by the fact that these elements change rapidly during evolution (*10*). A recent study showed that fin and limb regeneration share a deep evolutionary origin (*11*). Therefore, we hypothesized that if the genetic mechanisms driving regeneration are evolutionarily conserved in distantly related species subjected to different selective pressures, then it should be possible to distinguish between species-specific and conserved regeneration-responsive enhancers (RREs). The vivid differences in life history and the ~230 million years of evolutionary distance between the zebrafish and the African killifish *Nothobranchius furzeri* (fig. S1, A and B) provide an exclusive biological context in which to test this hypothesis. Both species can regenerate missing body parts after amputation. However, whereas zebrafish are found in moderately flowing freshwater habitats in Southern Asia, killifish inhabit temporal ponds subjected to annual desiccation in the southeast of Africa (*12*). The strong selective pressure of seasonal desiccation has driven killifish to evolve interesting features, including rapid sexual maturation (as short as 2 weeks) (*13*), diapause embryos (*14*), and an extremely short life span (4 to 6 months) (*12*). Here, we report that a systematic comparison of the epigenetic and transcriptional changes during the early stages of regeneration uncovered an evolutionarily conserved regeneration program. We also provide evidence that elements of this program are subjected to evolutionary changes in vertebrate species with limited or no regenerative capacities.

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Amputation-responsive enhancers evolved in teleosts

Despite the drastic differences in fin shapes and lifestyles, the early morphology of regenerating tail tissues in killifish and zebrafish appear indistinguishable from each other (Fig. 1A and fig. S1, C and D). A tail blastema formed by 1 day postamputation (dpa) in both species, as indicated by the presence of E-cadherin–negative mesenchymal cells above the amputation line (Fig. 1B). Blastema cells proliferated and expanded rapidly after 1 dpa in both killifish (fig. S2) and zebrafish (*15*). Because the cells driving the formation of a specialized regenerative blastema are recruited to the wound site at this stage, we chose this time point for comparison.

Active enhancers and promoters are characterized by histone H3K27ac and H3K4me3 marks (*16*, *17*). We assayed both killifish and zebrafish genomes (~1.5 gigabases) for H3K27ac and H3K4me3 enrichment using chromatin immunoprecipitation sequencing (ChIP-seq) in samples of uninjured (0 dpa) and regenerating (1 dpa) caudal fin. Our results revealed a marked difference in the total number of H3K27ac-marked putative RREs that did not overlap with promoter regions defined by H3K4me3 peaks at transcriptional start sites and available gene models between the two species: There were 1877 peaks (5% of total detected peaks) in killifish and 4162 peaks (7%) in zebrafish (Fig. 1C, fig. S3, and table S1). Whole-genome alignment revealed a low level of sequence conservation of these putative RREs compared with gene exons among multiple fish species (fig. S4). Furthermore, a relatively small portion of the RREs were linked to the same genomic loci with H3K4me3-marked active promoters in both species (310 genes), whereas most peaks were only detected in one species or the other (Fig. 1D, fig. S5, and table S2). Likewise, there were approximately twice as many regeneration-responsive genes detected by RNA sequencing (RNA-seq) in zebrafish (2829 up-regulated and 3363 down-regulated genes) than in killifish (1172 up-regulated and 1368 down-regulated genes) (Fig. 1E and table S3). Less than half of the detected regeneration-responsive genes were conserved, including 528 up-regulated and 546 down-regulated genes [>1.5-fold or <–1.5-fold, false discovery rate (FDR) < 0.01; Fig. 1, E and F, and table S3]. Similar RNA-seq mapping rates and BUSCO scores (an assessment of the completeness of genome assembly) were obtained for both species (fig. S6), indicating that the substantial differences observed were unlikely to be caused by the differential quality of genome assembly. Although some identified H3K27ac peaks might derive from differences

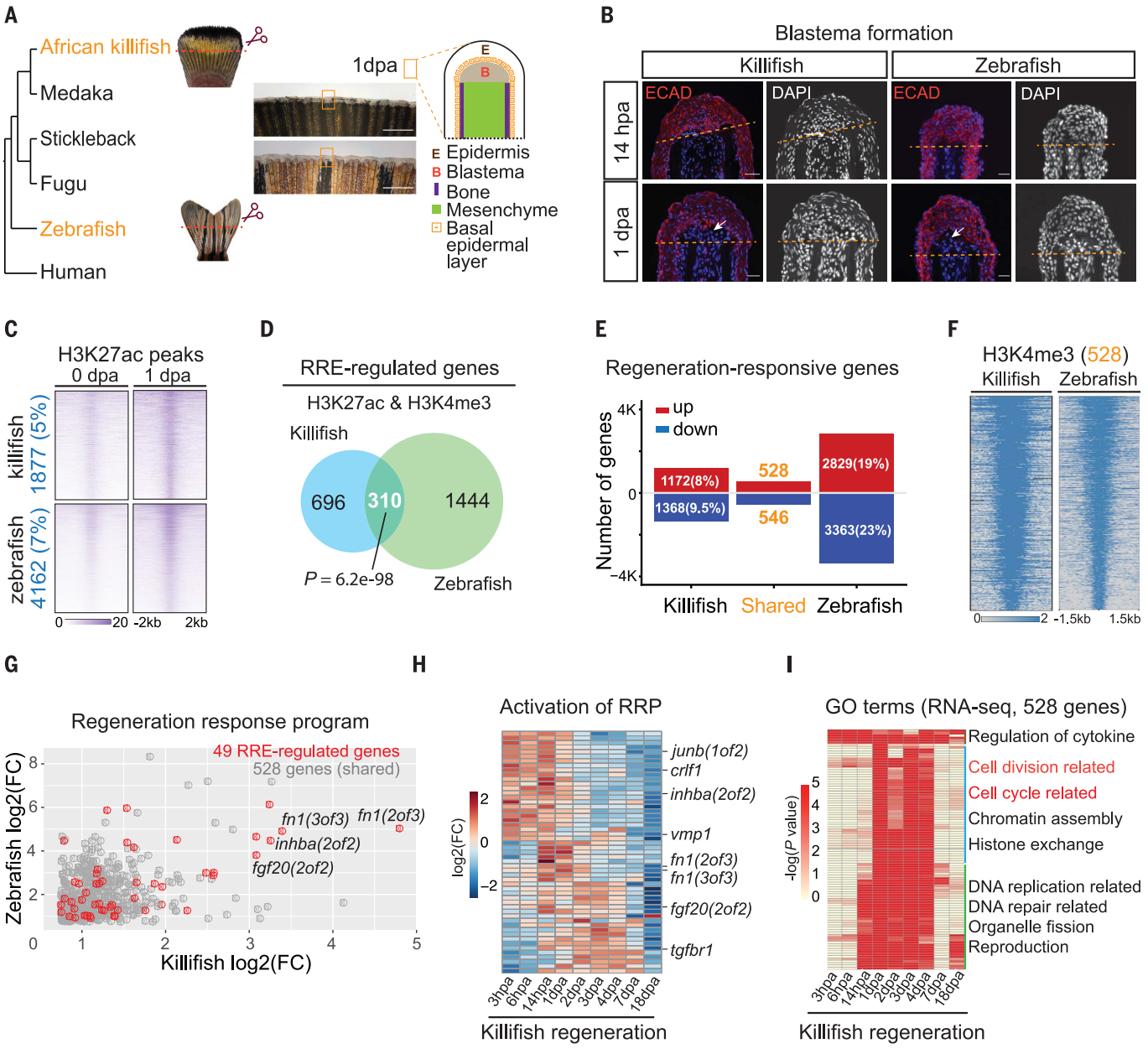

Fig. 1. Evolutionary changes and maintenance of the cis-regulome of regeneration in teleosts. (**A**) Regenerating caudal fins at 1 dpa and a phylogenetic tree showing the evolutionary relationships between the African killifish and the zebrafish. Scale bar, 200 μm. (**B**) A tail blastema, indicated by the white arrow, forms by 1 dpa in both killifish and zebrafish. E-cadherin (ECAD) labels epithelial cells (red). Scale bar, 50 μm. (**C**) Heatmaps of regeneration-responsive H3K27ac peaks (nonpromoter regions) in killifish and zebrafish. (**D**) Venn diagram showing 310 overlapping genes regulated by RREs between killifish and zebrafish. All genes have H3K4me3-marked active promoters that do not overlap with H3K27ac-defined RREs. P = 6.2e-98 (hypergeometric test). (**E**) Large variations in the total number of regeneration-responsive genes (>1.5-fold or <–1.5-fold, FDR < 0.01) between killifish and zebrafish. (**F**) Heatmaps of H3K4me3 peaks linked to the 528 shared genes at 1 dpa. (**G**) A conserved RRP is composed of 49 RRE-regulated genes (red) with H3K4me3-marked active promoters and elevated gene expression. Three genes with known functions in zebrafish regeneration are highlighted. (**H**) Heatmap showing the dynamic expression of 49 RRP genes during killifish fin regeneration. (**I**) Heatmap showing the dynamic changes of GO terms enriched by 528 shared up-regulated genes during killifish regeneration. The top GO terms are highlighted in red.

in the cell type composition of the uninjured and regenerating tissues, the consistency of our results indicates that, compared with zebrafish, a relatively less complex genetic response to regeneration appears to be invoked by fin amputation in killifish.

Next, we reasoned that if regeneration in killifish and zebrafish is driven by similar mechanisms, then an evolutionarily maintained genetic program activated by RREs is likely to be present. Comparing the 528 shared up-regulated genes with the 310 shared RRE-regulated genes, we identified 49 genes (P = 1.1e-24, hypergeometric test) with H3K27ac-defined RREs, H3K4me3-marked active promoters, and elevated gene expression (Fig. 1, G and H, and table S4). This shared cohort encompasses several known and essential regulators of zebrafish regeneration, including *fgf20a*, *inhbaa*, *junbb*, and *fn1* (*18*–*21*), as well as putative new regulators such as *crlf1*, *vmp1*, and *tgfbr1*. Gene ontology (GO) term analyses of common RRE-regulated genes (n = 310) and up-regulated genes (n = 528) revealed

top GO terms associated with cell migration and cell motility (fig. S7A, Fig. 1D, and table S5), and cell division and cell cycle, respectively (Fig. 1, I and E, and table S5). Similar analyses of species-specific genes showed a species-dependent regulation of distinct biological processes during regeneration (fig. S7, B to E, and table S6). Our data uncovered not only large-scale differences in the activation of RREs and gene expression during early stages of regeneration but also an evolutionarily conserved regeneration response program (RRP) activated by RREs in fish subjected to markedly different selective pressures.

Blastema cells are the primary source of RRP gene expression

To identify the cells deploying the identified RRP, we performed single-cell RNA-seq (scRNA-seq) of early killifish and zebrafish regeneration (KR and ZR, respectively). Unsupervised analyses uncovered 13 clusters (KR0 to KR12) from 7208 cells in killifish (Fig. 2A and fig. S8) and 16 clusters (ZR0 to ZR15) from 8605 cells in zebrafish (Fig. 2B and fig. S9). Macrophages (KR0 and KR1; ZR0, ZR3, ZR9, and ZR11), blastema cells (KR2, KR3, KR4, KR5, KR6, and KR11; ZR1, ZR2, ZR7, and ZR10), epidermal cells (KR7, KR9, and KR10; ZR4, ZR5, ZR6, ZR12, and ZR14), and neuronal cells (KR12 and ZR13) were the shared cell types identified (Fig. 2, C and D), whereas red blood cells (KR8), neutrophils (ZR8), and endothelial cells (ZR15) were only detected in one species, probably because of low abundance (Fig. 2D and fig. S9K). The blastema cell clusters were defined by the known blastema markers *msx* homeobox genes (figs. S8F and S9F) (*22*). A new early blastema marker, *fstl1*, was also identified and confirmed by in situ hybridization (Fig. 2C). Using four different markers, *cyclin A2*, *mki67*, *cyclin B1*, and *pcna*, we found that the cycling cells were mainly enriched in blastema cells and in subsets of epidermal cells and macrophages (fig. S10, A to D). Additionally, the blastema clusters identified can be categorized by the expression of two *wnt* genes (*wnt5a* and *wnt10a*) into two major groups with partial overlap in both species (fig. S10, E to J).

The integrated single-cell analysis identified both conserved (630 genes) and species-specific blastema marker genes (such as the previously identified zebrafish *lepb* gene) (Fig. 2E, fig. S11, and table S7). Additionally, we observed some cell-type discrepancies of gene expression between killifish and zebrafish regeneration. For instance, *complement factor d* (*cfd*) was specifically expressed in killifish blastema cells, yet the expression of *cfd* was shifted to epidermal cells in zebrafish (fig. S11C). Consistent with GO term enrichment analysis (Fig. 1I), the expression of shared up-regulated genes (n = 528) was enriched (P < 0.01) in cycling cells (KR2, ZR1, and ZR14) (figs. S12, A and B, and S13, A and B). Among these genes, 80 were specifically expressed in the blastema populations (Fig. 2F). The 49 RRP genes displayed significant enrichment (P < 0.05) in blastemal clusters (KR2, KR3, KR4, KR5, KR6, and KR11; ZR1, ZR2, and ZR7) and basal epidermal cells (KR10 and ZR5) in both species (figs. S12, C and D, and S13, C and D; Fig. 2G; and fig. S14A). Our scRNA-seq data support the hypothesis that the identified RRP genes were mainly expressed in regeneration-specific cells, i.e. blastema cells.

Dysregulation of the RRP in animals with limited regeneration

Next, we investigated whether changes in the regulation of RRP genes correlated with a variation of regenerative capacities in other vertebrates. We compared the RRP gene expression in published RNA-seq datasets for mouse species that respond to injury with either regeneration (*Acomys cahirinus*) or scarring (*Mus musculus*) (*23*, *24*). Twenty of 49 teleost-defined RRP genes were significantly up-regulated (>1.5-fold, FDR < 0.01) during ear pinna regeneration in *A. cahirinus* (fig. S14, B and C). By contrast, their expression in the nonregenerating ear pinna of *M. musculus* was dysregulated (Fig. 2H; fig. S14, B and C; and table S8). For example, *crlf1*, *itga4*, and *tha1* were significantly up-regulated in *A. cahirinus* during regeneration but not in *M. musculus* during scarring. Moreover, the transforming growth factor–β (TGF-β) ligand *inhba* (i.e., activin A or activin) was highly and continuously activated during scarring in *M. musculus* but was only up-regulated during early stages of regeneration in *A. cahirinus* (Fig. 2H and fig. S14, B and C). This is consistent with reports that overexpression of *inhba* in mouse skin accelerates wound healing but enhances scar formation (*25*, *26*). Similarly, dysregulation of RRP genes was also observed between skin regeneration and scarring (fig. S14, D to F, and table S8). The failed or altered activation of certain RRP genes during scarring suggests that teleost-defined RRP has likely been subjected to evolutionary changes in regeneration-competent and -incompetent animals.

The RRE *K-IEN* directs gene activation after amputation and is essential for regeneration

To test whether the identified enhancers play a role in regeneration, we validated five ChIP-identified RREs regulating *inhba(2of2)*, *fgf20 (2of2)*, *junb(1of2)*, *vmp1*, and *mbd2* in killifish (Fig. 3, A and B; fig. S15; and Fig. 1H). We focused on the gene *inhba* because it is required in both tail and heart regeneration in zebrafish (*19*, *27*) and is differentially regulated between regenerating and nonregenerating tissue (Fig. 2H). Two copies of *inhba* exist in the genome of killifish, but only *inhba(2of2)* responded to amputation (fig. S16A). To characterize the killifish *inhba(2of2)* enhancer, we cloned a 1159-bp DNA sequence (referred to as K1159) marked by a H3K27ac peak upstream of the gene promoter into a transgenic vector with a green fluorescent protein (GFP) reporter and produced stable transgenic killifish (Fig. 3C). Robust reporter expression was detected in the blastema region after fin amputation in *K1159:GFP*-transgenic fish (fig. S16B). Similarly, we also observed amputation-activated GFP expression for four additional enhancers (fig. S15), supporting the validity of our approach for identifying regeneration-activated enhancers.

By generating four additional constructs with different truncations (Fig. 3C), we identified a minimal sequence for the killifish *inhba(2of2)* enhancer (*K-IEN*), which recapitulated the original *K1159:GFP* expression and the endogenous *inhba(2of2)* expression (Fig. 3, A and D, and fig. S16C). We found that not all types of injury activated the identified enhancer similarly. The most robust response was observed when the damage involved the regeneration of multiple tissues (e.g., bone and interray tissues) compared with only interray tissue removal, and noticeably less robust expression was detected after performing a small incision without tissue loss (Fig. 3E). We also observed a stronger response in proximal amputations compared with distal amputations (Fig. 3F). We conclude from these data that *K-IEN* directs gene expression in response to different types of injuries and positional cues.

Because *inhba* is activated and required during zebrafish heart regeneration (*27*), we next investigated whether *K-IEN* also exhibited enhancer activity in killifish hearts. Similar to zebrafish heart regeneration (*28*), we observed a minor fibrotic scar at 7 days post-injury (dpi) and regression of the scar at 18 dpi through acid fuchsin orange G (AFOG) staining (Fig. 4, A to C). Moreover, killifish cardiomyocytes maintained the ability to proliferate in response to injury (Fig. 4D). These results confirm that the killifish heart is regeneration competent. Upon heart resection of *K-IEN:GFP* killifish, we observed robust GFP activation in the regenerating heart tissue, which had yet to form fully differentiated cardiac myofibers as defined by the lack of differentiated cardiac muscle marker tropomyosin (Fig. 4E). By contrast, the uninjured region (tropomyosin positive) was devoid of detectable GFP expression. Additionally, the expression of GFP was not detected in the developing fins and hearts of *K-IEN:GFP* killifish (fig. S16, D to F). We conclude that, as in caudal fin regeneration, the activation of *K-IEN* is regeneration dependent in the heart.

To determine whether *K-IEN* is required for regeneration, we designed two guide RNAs to target *K-IEN* in killifish using the CRISPR-Cas9 approach (Fig. 4F). Disruption of *K-IEN*

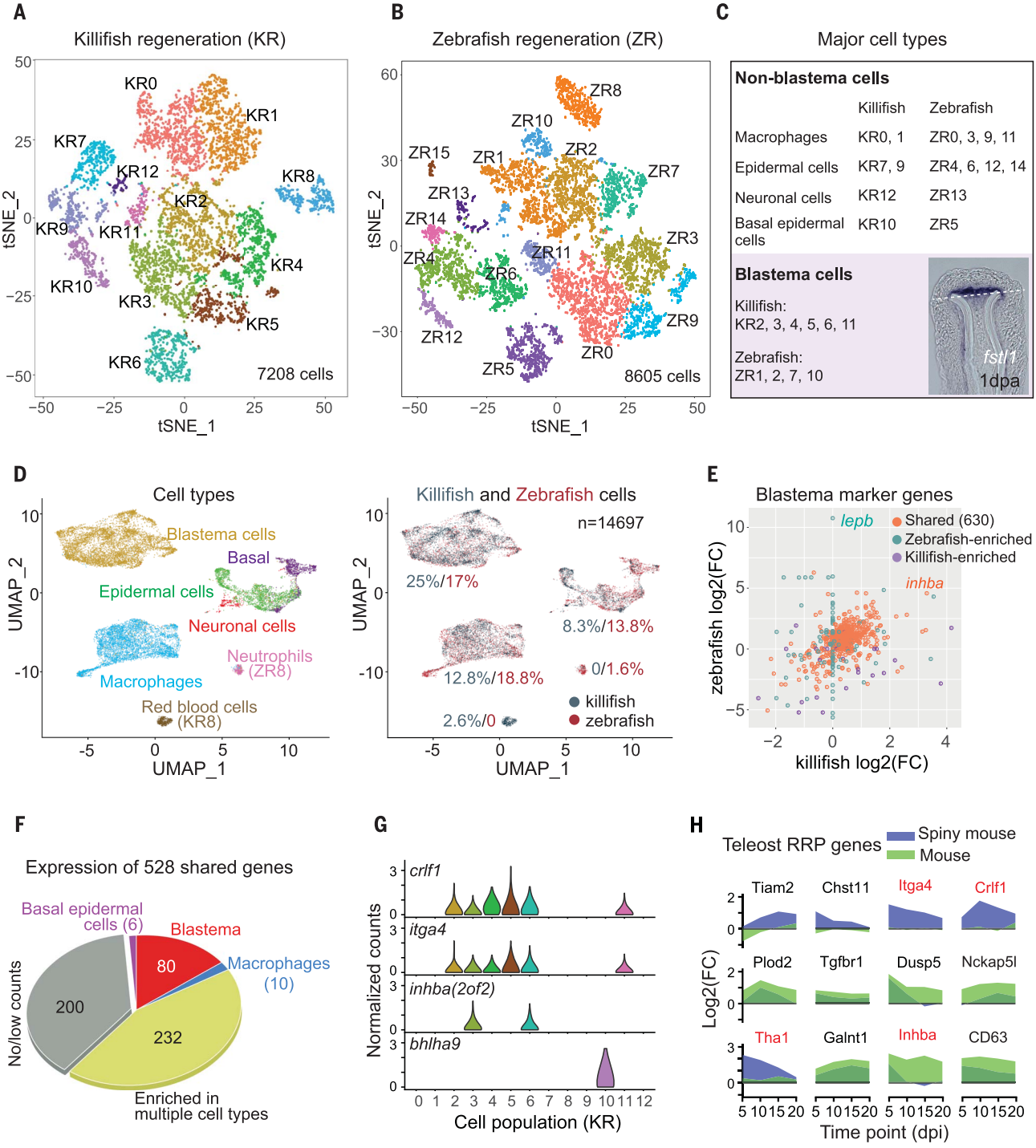

Fig. 2. The RRP deployed by regeneration-specific cells is dysregulated in regeneration-incompetent animals. (**A**) T-distributed stochastic neighbor embedding (t-SNE) plot showing 13 different cell clusters identified in early KR; 7208 cells were included in the analyses. (**B**) t-SNE plot showing 16 different cell clusters identified in early ZR; 8605 cells were included in the analyses. (**C**) Annotation of killifish and zebrafish cell clusters. The expression of *fstl1* in the early killifish blastema cells was confirmed by in situ hybridization. White dashed line indicates the amputation site. (**D**) Integrated single-cell analysis between killifish and zebrafish. Left, annotation of major cell types. Right, percentage of cells contributed by killifish and zebrafish. (**E**) Expression of shared and species-specific blastema marker genes identified in the integrated analysis. (**F**) Expression of 528 shared genes in different cell types identified by scRNA-seq; 80 genes were specifically detected in the blastema cells, and 232 genes were detected in two or more cell types. (**G**) Examples of the expression of RRP genes in t-SNE–clustered killifish cells. Only the enriched clusters are displayed for each gene. (**H**) Differential regulation of 12 teleost-defined RRP genes between regenerating ear pinna in the African spiny mouse *A. cahirinus* (blue) and nonregenerating ear pinna in the house mouse *M. musculus* (green). Four representative genes are highlighted in red.

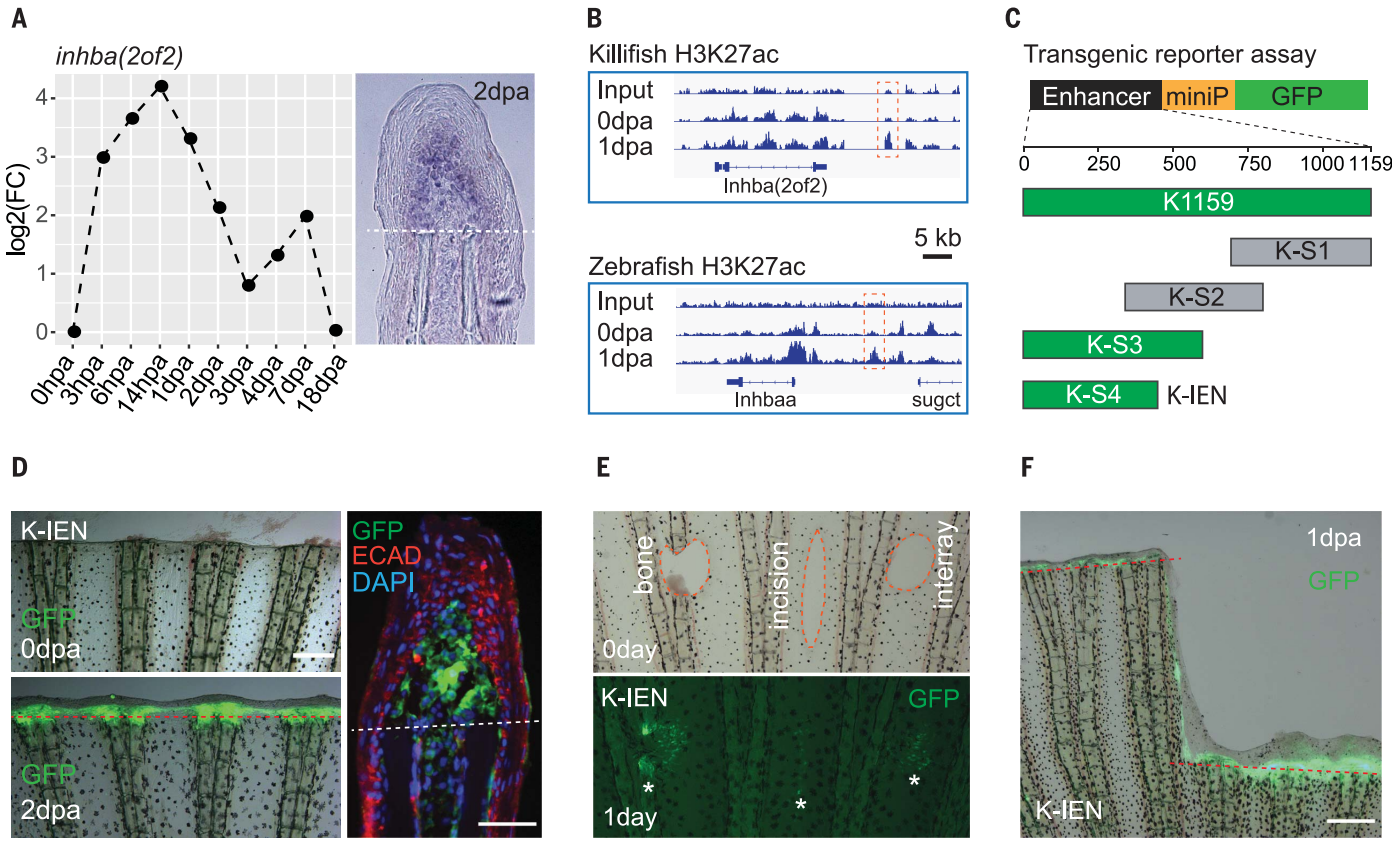

Fig. 3. Regeneration-activated *inhba* expression is mediated through the RRE *K-IEN*. (**A**) The dynamic expression of *inhba(2of2)* in killifish caudal fin regeneration. Right, the expression of *inhba(2of2)* in blastema cells at 2 dpa. (**B**) An RRE marked by H3K27ac peaks (red box) at the *inhba* locus in killifish and zebrafish. (**C**) Transgenic constructs examined for regeneration-dependent expression in killifish caudal fin. Top, design of a Tol2 transgenic vector. Constructs marked with green (K1159, K-S3, and K-S4) display enhancer activity in fin tissue. K-IEN (K-S4) is the minimal enhancer. (**D**) Images from the transgenic reporter line *K-IEN:GFP*. Left, expression of GFP at 0 and 2 dpa. Right, costaining of GFP (green) and E-cadherin (red) on 2 dpa cryosections. Scale bar, 50 μm. (**E**) Expression of *K-IEN:GFP* in different types of injury. Tissues were removed by a 1-mm-diameter biopsy punch. Top, the damaged regions at 0 dpa are outlined (red). Bottom, GFP expression in the damaged regions (star) at 1 dpa. (**F**) Expression of *K-IEN:GFP* in response to proximal and distal amputation. The orientation of all caudal fin images is proximal to the bottom and distal to the top. Dashed line indicates the amputation site.

significantly delayed tail regeneration in homozygous mutants compared with wild-type animals (Fig. 4G and fig. S17, A and B). Furthermore, heart regeneration was also impaired, leading to a failure of scar resolution at the injury site (Fig. 4H and fig. S17, C and D). However, cardiomyocyte proliferation was not affected in the mutants (Fig. 4I). Our data reveal that *K-IEN* is an RRE with pleiotropic function and is required for tissue regeneration in killifish.

Detecting evolutionary changes of an essential RRE in vertebrates

To determine whether RREs with essential regeneration roles are evolvable, we sought to identify orthologs of *K-IEN* in different vertebrate lineages using mVISTA (*29*). Multiple sequence alignments detected a relatively conserved noncoding block near the *inhba* loci in killifish, zebrafish, and human (Fig. 5A). The overlap between the predicted zebrafish sequence and the H3K27ac-marked region suggests that the predicted enhancers are likely to be biologically relevant. We cloned DNA fragments containing the predicted zebrafish and human elements and generated stable transgenic reporter lines in killifish for each of them. The zebrafish *inhba* enhancer (*Z-IEN*) drove regeneration-dependent GFP expression in a manner indistinguishable from that of *K-IEN* (Fig. 5, B and C). By contrast, the expression of GFP directed by the predicted human *inhba* enhancer (*H-IEN*) was barely detectable before 2 dpa but was robustly observed by 3 dpa and persisted to 5 dpa (Fig. 5D and fig. S16B). Unlike *K-IEN:GFP* and *Z-IEN:GFP*, the expression of *H-IEN:GFP* was restricted to the basal epidermal cells rather than to the mesenchymal cells (Fig. 5D), reminiscent of the activation of *inhba* in human and mouse skin upon injury (*26*, *30*). We also observed amputation-dependent activation of GFP expression for *Z-IEN:GFP*, but not *H-IEN:GFP*, during killifish heart regeneration (Fig. 5E). Instead, *H-IEN* directed GFP expression during homeostasis in the endocardium cells and some epicardium cells (Fig. 5F). The ability to rescue the fin regeneration phenotype in *K-IEN*$^{-/-}$ mutants through reexpression of killifish *inhba* driven by *Z-IEN*, but not *H-IEN*, implies a functional change of the enhancer (fig. S18). These results suggest that an ancestral, evolutionarily conserved teleost RRE with an indispensable role in regeneration has diversified its functions, implicating RRE evolutionary turnover as a potential mechanism underlying variation in the regenerative capacities of vertebrates.

Genomic occupancy of AP-1 motifs is essential for RRE activities

To investigate what determines the injury responsiveness in the identified RREs, we performed motif enrichment analyses on both sets of conserved and species-specific elements. We found that a consensus 12-O-tetradecanoylphorbol-13-acetate responsive element (TRE), TGA[G/C]TCA, which was recognized by the AP-1 transcription factor complex, was the

most enriched motif in all analyses performed (Fig. 6, A and B, and fig. S19, A to C). Similarly, AP-1 motifs were enriched in the open chromatin regions involved in *Drosophila* imaginal disc regeneration and regeneration in the acoel worms (fig. S19, D and E) (*31*, *32*). The AP-1 complex is a heterodimer composed of members from different families of DNA-binding proteins, including the Jun, Fos, ATF, JDP, and Maf families (*33*). AP-1 binds both TRE and the cAMP response element (CRE; TGACGTCA) (*34*). Cell-type–biased expression of AP-1 components was detected in both killifish (fig. S20, A to E) and zebrafish (fig. S21) scRNA-seq of blastema formation, indicating

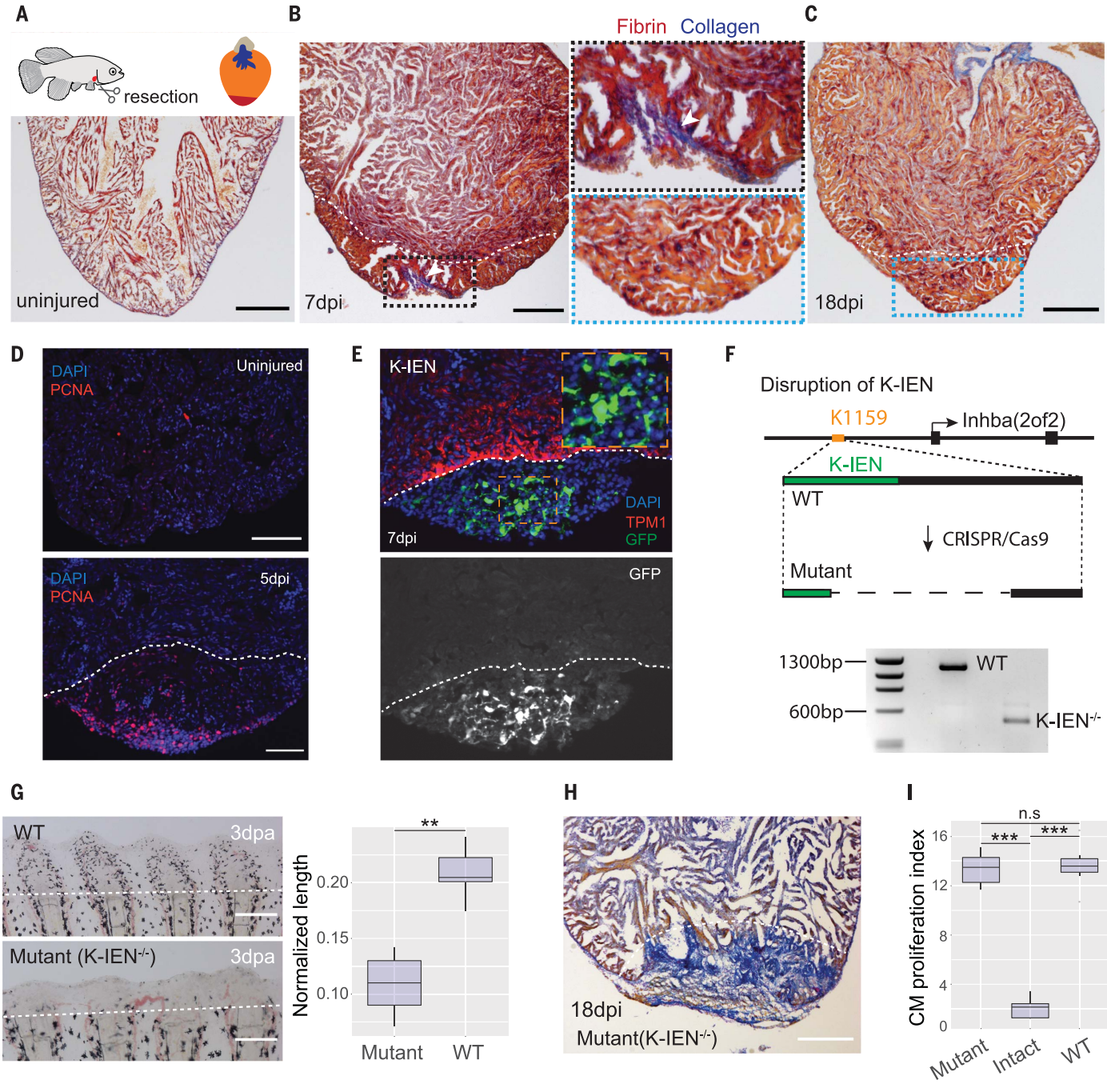

Fig. 4. The RRE *K-IEN* is required for regeneration. (**A** to **C**) AFOG staining on cryosections of uninjured (A), 7 dpi (B), and 18 dpi (C) killifish hearts to detect fibrin (red) and collagen (blue). Diagram in (A) shows the resection of killifish heart ventricle. Magnified views of collagen deposition (white arrowhead) in the injured region are outlined with a dashed box. $n = 10$. (**D**) PCNA (red) and DAPI (blue) staining on cryosections of uninjured (top) and 5 dpi (bottom) killifish hearts. $n = 10$. (**E**) Expression of *K-IEN:GFP* in 7 dpi killifish hearts. Top, merge of GFP, TPM1, and DAPI images. The uninjured region is marked by tropomyosin (TPM1). A magnified view of GFP is outlined with a dashed box. $n = 5$. (**F**) Generation of homozygous *K-IEN*$^{-/-}$ mutants. Top, schematic diagram showing the disruption of *K-IEN* through CRISPR-Cas9. Bottom, PCR genotyping of a homozygous *K-IEN*$^{-/-}$ mutant. (**G**) Fin regeneration is significantly delayed in *K-IEN*$^{-/-}$ mutants. Right, quantification of the regenerated tissue at 3 dpa. $n = 10$. $^{**}P < 0.01$. (**H**) AFOG staining on cryosections of *K-IEN*$^{-/-}$ mutant hearts at 18 dpi. $n = 10$. (**I**) Injury-triggered cardiomyocyte proliferation was not altered in the *K-IEN*$^{-/-}$ mutant at 5 dpi. The percentages of myocardial nuclei undergoing DNA replication (PCNA staining) at the injury site were quantified. $n = 10$. $^{***}P < 0.001$. n.s., not significant ($P > 0.05$). Student's *t* test was performed in the results shown in (G) and (I). Dashed line indicates the injury site.

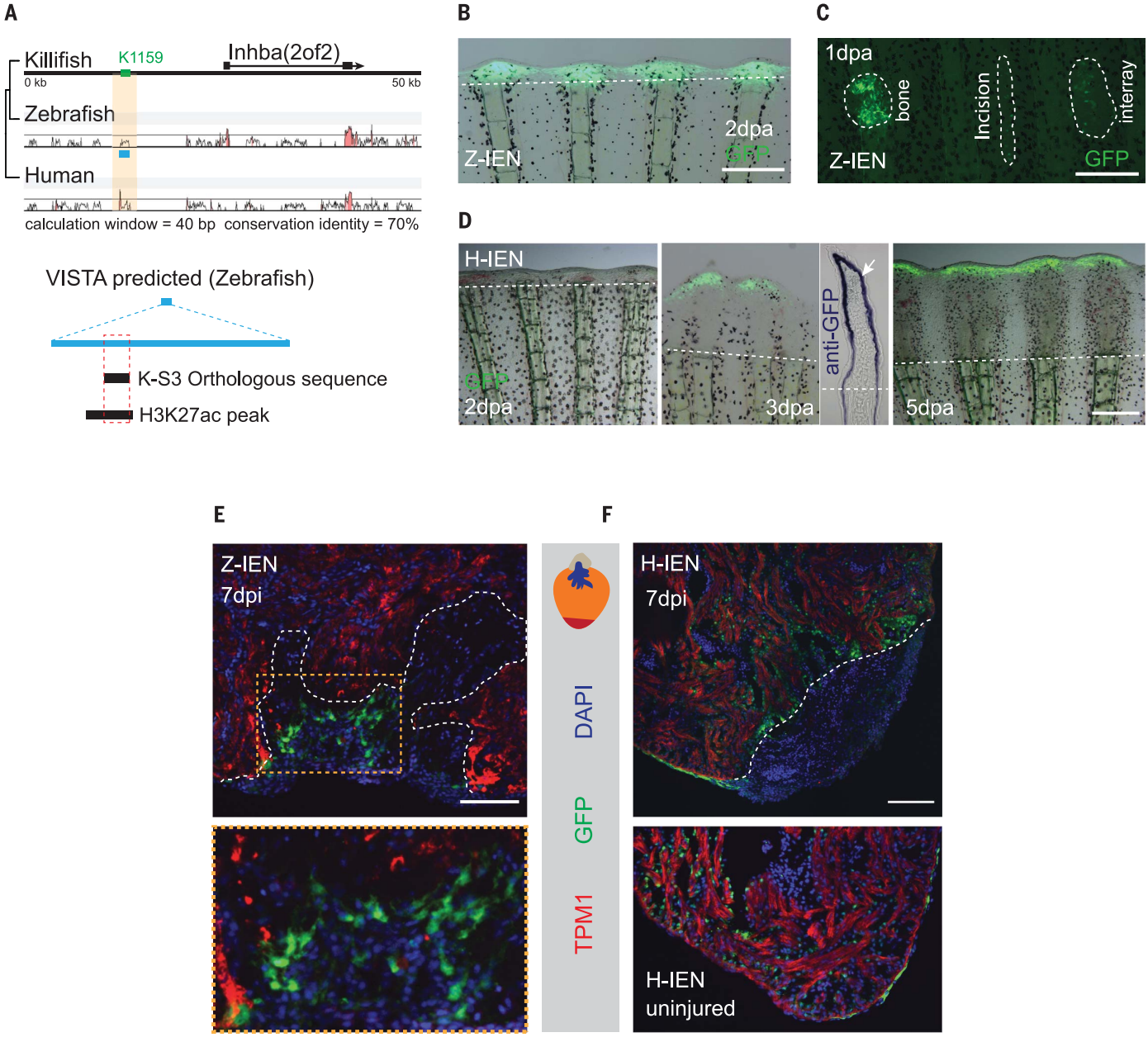

Fig. 5. Evolutionary changes of *K-IEN* activities in vertebrates. (**A**) VISTA alignment of *inhba* loci among killifish, zebrafish, and human. Red peaks represent high levels of sequence conservation, and the absence of peaks indicates no significant conservation. The killifish RRE is marked in green. Bottom, schematic diagram showing the overlap between the zebrafish H3K27ac peak and the predicated enhancer (blue). (**B**) GFP expression driven by the zebrafish enhancer *Z-IEN* at 2 dpa in killifish caudal fin. (**C**) Expression of *Z-IEN:GFP* under different types of injury in killifish caudal fin. (**D**) GFP expression driven by the human enhancer *H-IEN* initially detected at 3 dpa (middle) in killifish caudal fin. GFP was detected in the basal epidermal cells (arrow). (**E**) Regeneration-dependent expression of *Z-IEN:GFP* at 7 dpi in killifish hearts. Magnified view is outlined with a dashed box. (**F**) The expression of *H-IEN:GFP* is present during homeostasis and is not regeneration dependent. Dashed line indicates the injury or amputation site. Scale bar, 50 μm.

that different cell populations may form distinct AP-1 complexes. Further, genome-wide prediction of AP-1 motifs among different species showed that CRE motifs recognized by the Jun family proteins (Jun, JunB, and JunD) exist at a much higher frequency in regeneration-competent fish genomes than in human and mouse genomes (fig. S22). Taken together, these results identify AP-1–binding sites as a shared characteristic of all RREs identified in this study and uncover differences in the frequency of predicted AP-1–binding motifs between regeneration-competent and -incompetent animals.

To determine whether AP-1 motifs are essential for the activity of RREs, we identified predicted AP-1–binding sites in both *K-IEN* (GCTGACTCAGA and GCTGACTCACTG) and *Z-IEN* (GCTGACTCAT and GCTGACTCTA) and subjected them to site-specific mutagenesis (Fig. 6C). All motifs were mutated into GCAAAAAAAAA or GCAAAAAAAAA (Fig. 6, C to E). Stable transgenic reporter assays revealed that the expression of GFP driven by either the *K-IEN*^{MI2}– or *Z-IEN*^{MI2}–mutated enhancers was completely abolished compared with the original enhancers (Fig. 6, C to E). Furthermore, blocking the activation of the AP-1 complex through the JNK pathway inhibitor SP600125 diminished the activity of *K-IEN* and inhibited

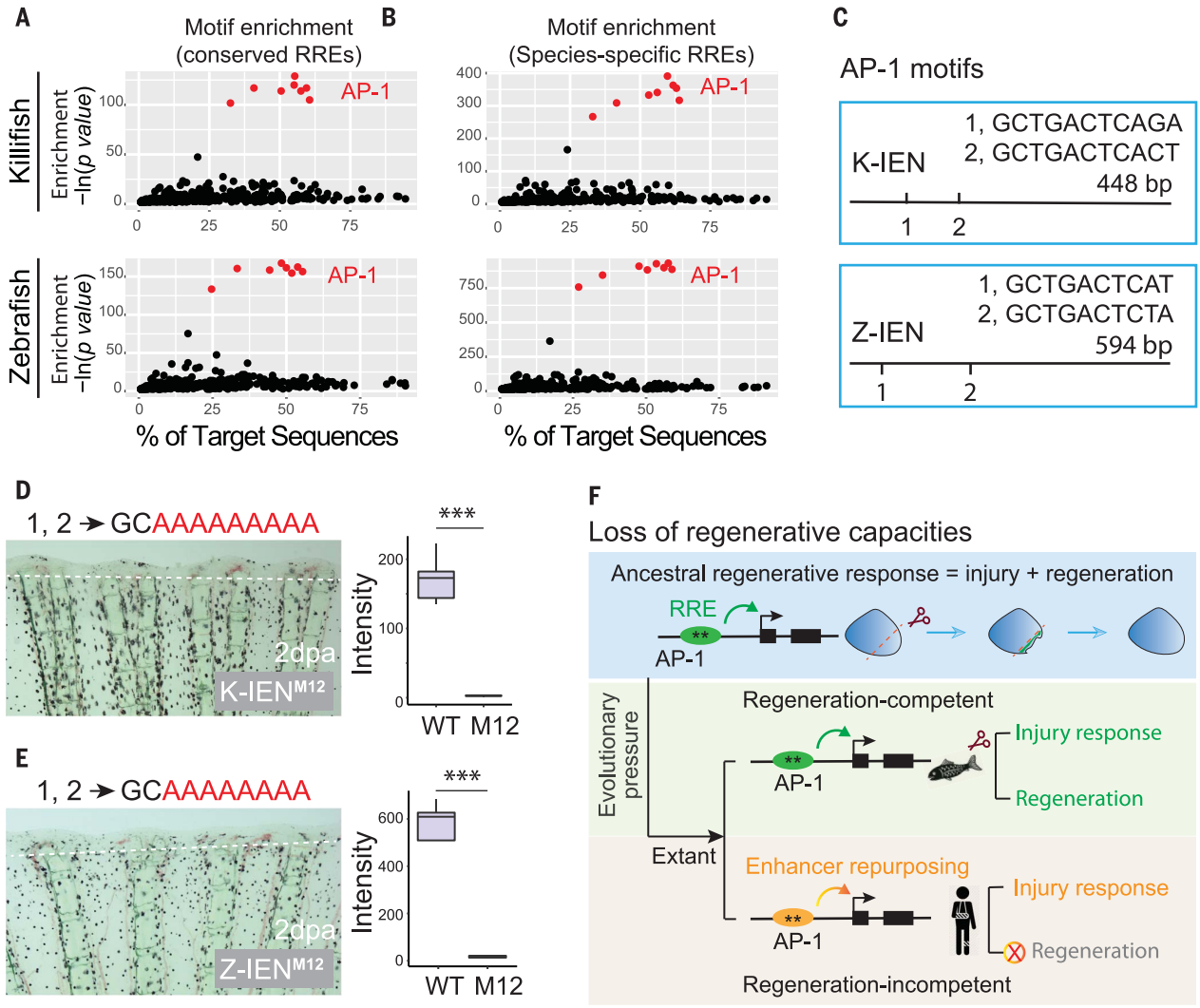

Fig. 6. Occupancy of AP-1–binding motifs is essential for RRE activities. (**A** and **B**) Motifs enriched in the conserved (A) and species-specific (B) RREs identified in killifish (top) and zebrafish (bottom) caudal fin regeneration. AP-1 motifs are highlighted in red. Each dot in the graph represents a single binding motif. The sequence of AP-1 motifs is shown in fig. S19. (**C**) Identification of AP-1 motifs in the RREs *K-IEN* and *Z-IEN*. (**D** and **E**) Expression of GFP driven by *K-IEN*^{M12} and *Z-IEN*^{M12} is abolished at 2 dpa in transgenic reporter lines. Right, quantification of the fluorescence intensity between wild-type and mutant enhancers. $P < 0.001$ (Student's t test). $n = 10$. Dashed lines indicate the amputation site. (**F**) RRE-based model for the loss of regenerative capacities during evolution. We propose a regenerative response to injury as the ancestral function of AP-1 motif–enriched enhancers. In the course of evolution and speciation, regeneration and injury responses became dissociated from each other in some, but not all, enhancers. In extant species, regeneration-competent animals maintain the ancestral enhancer activities to activate both injury response and regeneration, whereas repurposing of ancestral enhancers in regeneration-incompetent animals led to loss of regenerative capacities.

tail regeneration (fig. S20, F and G). We conclude that AP-1 motifs are required for the activation of RREs in response to amputation.

Discussion

Activation or inactivation of genes is suspected to underlie changes in regenerative capacities, yet how these genetic activities are regulated remains poorly understood. AP-1 transcription factors are essential for many biological processes, and their diverse functions are part of complex dynamic networks of signaling pathways known to depend on subunit composition and interactions with other nuclear factors, which in turn are in part determined by both cell type and cellular environment (*33*). In this study, AP-1 components were not ubiquitously expressed in all cell types and were present in both mesenchymal and epithelial cells, suggesting that specific subunit compositions may be required to restrict expression of the enhancers to either the mesenchyme (*K-IEN*, *Z-IEN*) or epithelial cells (*H-IEN*). Additionally, the presence of a predicted p53/p63–binding motif in the human enhancer, which was absent in *K-IEN* and *Z-IEN* (fig. S23A), and the abundance of *p63* expression in basal epidermal cells (fig. S23B) suggest that interactions of AP-1 with other nuclear factors may also play a role in regulating enhancer activity.

Given the ancient evolutionary origin of the AP-1 complex (*35*, *36*), we hypothesize that the ancestral function of AP-1 motif–enriched enhancers was to activate a regenerative response, and that through the course of evolution and speciation, regeneration and injury responses became dissociated from each other in some, but not all, enhancers (Fig. 6F). Repurposing of ancestral regulatory sequences to generate new regulatory functions is not without precedent (*37*) and has been well documented in both vertebrates (*38*) and invertebrates (*39*). For instance, frequent regulatory element repurposing was revealed by DNase I–hypersensitive sites in the mouse and human genomes (*40*).

These studies demonstrated that regulatory elements in orthologous loci were functionally active in distinct tissues, indicating that cis-regulatory plasticity may be a key facilitator of vertebrate evolution (*40*). Future experiments aimed at determining the in vivo composition of the AP-1 complexes associated with both the evolutionarily conserved RREs and the species-specific injury response enhancers may not only help to identify mechanisms underpinning enhancer repurposing but also help to resolve the long-standing problem of why some species can regenerate missing body parts after amputation whereas others cannot.

Material and methods summary

Bulk RNA-seq and ChIP-seq (H3K27ac and H3K4me3) data were obtained from amputation sites at 0 dpa (control) and 1 dpa for transcriptomic and epigenomic analyses of blastema formation in African killifish and zebrafish. Regeneration time-course RNA-seq was performed at 3, 6, and 14 hours postamputation and at 1, 2, 3, 4, 7, and 18 dpa in African killifish. These data were used to define the RREs and genes and to identify an evolutionarily maintained RRP. scRNA-seq data were obtained from regenerating blastema at 1 dpa and used to determine cell types deploying the identified RRP. To characterize RREs, transgenic reporter assays were performed in African killifish. The function of the killifish *inhba* enhancer was determined using CRISPR-Cas9–mediated genome engineering. The human *inhba* enhancer was identified using the mVISTA tool. Motif analysis was used to identify key transcription factor–binding sites enriched by ChIP-identified RREs. The function of these binding sites was validated using site-specific mutagenesis followed by transgenic reporter assays.

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SUPPLEMENTARY MATERIALS

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MDAR Reproducibility Checklist

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Science

Changes in regeneration-responsive enhancers shape regenerative capacities in vertebrates

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Regulatory elements of fish regeneration

Some animals regenerate extensively, whereas others, such as mammals, do not. The reason behind this difference is not clear. If the genetic mechanisms driving regeneration are evolutionarily conserved, the study of distantly related species that are subjected to different selective pressures could identify distinguishing species-specific and conserved regeneration-responsive mechanisms. Zebrafish and the short-lived African killifish are separated by ~230 million years of evolutionary distance and, as such, provide a biological context to elucidate molecular mechanisms. Wang *et al.* identify both species-specific and evolutionarily conserved regeneration programs in these fish. They also provide evidence that elements of this program are subjected to evolutionary changes in vertebrate species with limited or no regenerative capacities.

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