Identification of Novel Genes Involved in the Development of the Sword and Gonopodium in Swordtail Fish

Nils Offen, Axel Meyer, and Gerrit Begemann*

Male swordtail fish of the genus *Xiphophorus* develop a sword, a colourful extension of the caudal fin, that evolved by sexual selection through female choice. Swords and gonopodia, an intromittent organ developing from the male anal fin, can be prematurely induced by exogenous testosterone, offering the opportunity to examine the identity and expression profiles of genes required during various stages of fin metamorphosis. Here, we employed suppression subtractive hybridisation to identify genes specifically up-regulated during two early stages of sword and gonopodium development. We identified 128 different sequences with significant similarity to known genes and characterized the *rack1*, *dusp1*, *klf2*, and *tmsβ*-like genes as specifically up-regulated in developing as well as regenerating fin rays of the sword and gonopodium. We show that some of these genes follow distinct expression profiles in swords and gonopodia, suggesting differences in the genetic networks underlying the development of anal and caudal fin modifications. Developmental Dynamics 238:1674–1687, 2009. © 2009 Wiley-Liss, Inc.

Key words: sword development; sword; gonopodium; SSH; *rack1*; *dusp1*; *klf2*; *tmsβ*; *Xiphophorus*; swordtail; fin metamorphosis

INTRODUCTION

Swordtail fish are a suitable and popular model for the study of sexual selection in a vertebrate organism (reviewed in Rosenthal and García de León, 2006). Male fish of the Green Swordtail, *Xiphophorus helleri*, possess a sword, a prominent extension of the caudal fin with contrasting pigmentation, that was first introduced by Charles Darwin as an example of a sexually selected trait in fishes (Darwin, 1871). The sword is composed of approximately four elongated ventral fin rays covered by pigment cells that form a distinctive pattern of a yellowish stripe flanked by black borders (Fig. 1A,B) (Basolo and Trainor, 2002). Both traits, sword length and coloration, have been shown to be important for mating success (Rosenthal and Evans, 1998; Basolo and Trainor, 2002). In contrast to swordtails, male platyfish, a monophyletic clade within the genus *Xiphophorus*, lack a sword, even though females of some platy species show a bias for sworded males (Basolo, 1990, 1995). For this reason, the evolutionary history of the sword has been studied intensively to discover the origin of sword gains and losses. Molecular data support the hypothesis that all extant *Xiphophorus* species descend from a sworded ancestor. In this scenario, the sword was secondarily lost in the lineage leading to the platyfish (Meyer et al., 1994, 2006; Meyer, 1997). Even though the sword is an important trait that is evaluated by females during courtship, its evolutionary loss in platyfish might have been caused by high predation pressure associated with its maintenance. It has been shown that...
Fig. 1. Efficiency of subtractive hybridisation and overview of obtained genes. A, B: Caudal fins of Xiphophorus helleri males before (A) and after (B) sexual maturation; four ventral caudal fin rays, V7–10, contribute to the sword. C, D: Anal fins of juvenile (C) and adult (D) male X. helleri; three fin rays, R3–5, form the gonopodium, an intromittent organ for internal fertilisation. E: A fragment of the gapdh transcript was amplified from subtracted and unsubtracted cDNA, derived from testosterone-treated fins. PCR was performed for 15, 20, and 25 cycles using species-specific oligonucleotide primers. Amplification of the gapdh fragment is detected five cycles later in the subtracted rather than in the unsubtracted cDNA, suggesting successful reduction of common transcripts in the subtracted pool. F: A total of 406 size-selected clones from the SSH-library were sequenced and analysed. Sequences fell into 201 independent contigs and singletons, of which 128 showed similarity to known genes from other species. These 128 positive hits were grouped into five categories according to their predicted functions. A contig/sequence was scored as independent, when no significant overlap with other contigs/sequences was found; **a sequence was considered to be similar to a sequence in the database if the e-value (obtained by Blast) was e^{-15} or smaller. Scale bars = 1 mm (A,B), 500 μm (C,D).
males with longer swords experience higher metabolic costs during swimming, that predators are attracted by the conspicuous ornamentation of the sword, and that exposure to predators can reverse the female bias for swords (Rosenthal et al., 2001; Basolo and Alcaraz, 2003; Johnson and Basolo, 2003). Therefore, the opposing effects of sexual and natural selection could almost certainly also lead to sword loss.

On the molecular level, however, the evolutionary changes that caused the gain or loss of the sword have not yet been revealed. To dissect the molecular pathways involved in these processes, it will be important to identify the genes that regulate caudal fin metamorphosis into the sword and to resolve the evolutionary modifications that led to the loss or gain of the sword. This can be achieved within a phylogenetic framework of the entire genus that includes swordtails and platyfish. Hybridisation experiments between X. helleri and X. cortezi suggested that multiple genes, collectively termed “sword genes” (“Schwertgene”), contribute to sword formation (Zander and Dzwillo, 1969). In male fish, the endogenous synthesis of androgens during sexual maturation, or the artificial exposure to testosterone, trigger the signalling events that induce the activation of sword genes (Dzwillo, 1962, 1964). Moreover, testosterone also controls the metamorphosis of the male anal fin into an intromittent organ, the gonopodium (Fig. 1C,D) (Gordon et al., 1964). Since Xiphophorus-specific microarrays are not yet available, we have employed suppression subtractive hybridisation (SSH) (Diatchenko et al., 1996) as an unbiased alternative to identify genes involved in sword and gonopodium development. SSH allows one to detect differences in the abundance of individual transcripts between two transcriptomes, e.g., those of different tissues or developmental stages. This technique has been successfully applied to identify genes differentially regulated in caudal fin regeneration (Padhi et al., 2004). We have constructed an SSH library with the aim to detect genes that are differentially expressed in testosterone-induced, developing swords and gonopodia, as compared to juvenile fins prior to induction, and have identified more than 100 of these by sequencing. Gene expression analysis for a subset of genes confirmed their up-regulation during fin metamorphosis and caudal fin regeneration.

RESULTS

Construction of a Suppression Subtractive Hybridisation Library From X. helleri

To identify genes that are differentially expressed upon the initiation of sword and gonopodium development from juvenile fins without ab initio assumptions regarding gene function or identity, we employed a suppression subtractive hybridisation (SSH) scheme. We induced sword and gonopodium development in caudal and anal fins, respectively, of immature X. helleri with 17α-methyltestosterone with the objective (1) to allow both for the simultaneous generation of large numbers of experimental animals required for tissue isolation and (2) for a precisely timed induction of both processes. Because sword and gonopodium development are induced by increasing testosterone levels in both sexes and fin morphology after metamorphosis is very similar in the two sexes (Groebstein, 1942; Dzwillo, 1964; Zander and Dzwillo, 1969; Ogino et al., 2004), we reasoned that testosterone treatment induces the same genes that act during normal metamorphosis of these fins and independent of the individual animal’s sex. To produce the SSH library, we pooled cDNAs from caudal and anal fins that had been treated with testosterone for 1, 2, 4, or 5 days, and used untreated fins to collect transcripts before testosterone treatment. Based upon the time-course of changes in fin morphology, we reasoned that direct targets of testosterone-induced signalling should be activated within the first two days, whereas at 4 and 5 days of treatment genes should be induced that are more downstream and controlled indirectly by testosterone signalling. Because developing swords and gonopodia are thought to modify the activity of parts of the same gene regulatory networks (Zauner et al., 2003; Offen et al., 2008), both tissues from both fins were pooled to increase the starting material. We employed a PCR-based SSH approach (Diatchenko et al., 1996) to enrich for cDNAs that are more abundant in the cDNA pool derived from testosterone-induced swords and gonopodia. These cDNAs are likely to represent genes specifically up-regulated in developing swords and go-
# Table 1. Genes With Known Functions That Show Similarity to Identified *X. helleri* Sequences

<table>
<thead>
<tr>
<th>Category</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Housekeeping genes</strong></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>16S ribosomal RNA (2×)</td>
</tr>
<tr>
<td>2.</td>
<td>28S ribosomal RNA</td>
</tr>
<tr>
<td>3.</td>
<td>Abhydrolase domain containing 12</td>
</tr>
<tr>
<td>4.</td>
<td>ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C3</td>
</tr>
<tr>
<td>5.</td>
<td>ATPase synthase protein 9</td>
</tr>
<tr>
<td>6.</td>
<td>ATPase, H+ transporting, lysosomal accessory protein 2</td>
</tr>
<tr>
<td>7.</td>
<td>Cytochrome c oxidase subunit I (2×)</td>
</tr>
<tr>
<td>8.</td>
<td>Cytochrome c oxidase subunit II</td>
</tr>
<tr>
<td>9.</td>
<td>Deoxyhypusine hydroxylase/monooxygenase</td>
</tr>
<tr>
<td>10.</td>
<td>Elongation factor 1a</td>
</tr>
<tr>
<td>11.</td>
<td>Eukaryotic translation elongation factor 2</td>
</tr>
<tr>
<td>12.</td>
<td>Histone 3B</td>
</tr>
<tr>
<td>13.</td>
<td>Integral membrane protein 2B</td>
</tr>
<tr>
<td>14.</td>
<td>kaa190 solute carrier family 25 member 5</td>
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<tr>
<td>15.</td>
<td>Mitochondrial ATP synthase H+ transporting complex 1 delta subunit</td>
</tr>
<tr>
<td>16.</td>
<td>40S Ribosomal protein S2 (3×)</td>
</tr>
<tr>
<td>17.</td>
<td>40S Ribosomal protein S3a</td>
</tr>
<tr>
<td>18.</td>
<td>40S Ribosomal protein S5</td>
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<tr>
<td>19.</td>
<td>40S Ribosomal protein S7</td>
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<td>40S Ribosomal protein S9</td>
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<td>24.</td>
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<td>25.</td>
<td>40S Ribosomal protein S16 (2×)</td>
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<td>40S Ribosomal protein S19</td>
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<td>30.</td>
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<td>31.</td>
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<td>32.</td>
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<td>60S Ribosomal protein L13</td>
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<td>48.</td>
<td>60S Ribosomal protein L31</td>
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<td>49.</td>
<td>60S Ribosomal protein L35a</td>
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<tr>
<td>50.</td>
<td>60S Ribosomal protein L35b</td>
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<tr>
<td>51.</td>
<td>60S Ribosomal protein L36</td>
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<tr>
<td>52.</td>
<td>60S Ribosomal protein L37a</td>
</tr>
<tr>
<td>53.</td>
<td>60S Ribosomal protein L38</td>
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<tr>
<td>54.</td>
<td>Succinate-CoA ligase, alpha subunit</td>
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<td>55.</td>
<td>Tomm40</td>
</tr>
<tr>
<td>56.</td>
<td>Transglutaminase 2</td>
</tr>
<tr>
<td>57.</td>
<td>Translation factor sui1-like</td>
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<tr>
<td>58.</td>
<td>Translation initiation factor 4E transporter</td>
</tr>
<tr>
<td>59.</td>
<td>Ubiquitin</td>
</tr>
</tbody>
</table>

**Structure and cytoskeleton**
- 60. Actin-related protein 3
- 61. Alpha-tubulin
- 62. Beta actin (6×)
- 63. Tubulin beta-1 chain
- 64. BslR19 keratin
- 65. type 1 collagen alpha 1 (3×)
- 66. type 1 collagen alpha 2
- 67. Collagen, type X, alpha 1
- 68. Cytokeratin
- 69. Keratin 15 (2×)
- 70. Keratin 5 protein, transcript variant 1
- 71. Keratin 5 protein, transcript variant 2
- 72. Keratin K10
- 73. MID1 interacting protein 1
- 74. Osteonectin
- 75. type V/XI collagen pro-alpha 1
- 76. type I Keratin isoform 1
- 77. type II keratin
- 78. type II keratin E3

**Stress and immune response**
- 79. Amet protein
- 80. Ferritin heavy chain subunit
- 81. B2-microglobulin
- 82. Peroxiredoxin 4 (2×)

**Signalling and transcription factors**
- 83. C-fos
- 84. 14-3-3, a protein
- 85. Calmodulin (2×)
- 86. Cystatin B
- 87. Dual specificity phosphatase 1
- 88. Kruppel-like factor 2a
- 89. Kruppel-like factor 2b (2×)
- 90. M-Calpain
- 91. Protein phosphatase 1, catalytic subunit, beta isoform
- 92. Receptor for activated protein kinase C (RACK1) (2×)
- 93. S100-like
- 94. Thymosin beta a-like

**Other**
- 95. Cysteine-rich protein 2
- 96. DEAD (Asp-Glu-Ala-Asp) box polypeptide 5
- 97. Hemoglobin beta-A chain
- 98. Human DNA sequence from clone RP5-1107C24 on chromosome 20
- 99. Protein LOC553453
- 100. Setenoprotein W2a
- 101. Sperm plasma glycoprotein 120
- 102. Splicing factor 3b, subunit 1
- 103. Translationally controlled tumor protein-like
- 104. Tetraodon nigroviridis full-length cDNA
- 105. Tetraodon nigroviridis full-length cDNA
- 106. Tetraodon nigroviridis full-length cDNA
nopodia and are putative candidates to function in sword and gonopodium development.

To test the efficiency of the subtractive hybridisation, a fragment of gapdh, a ubiquitously expressed gene, was amplified from the pool derived from testosterone-induced fins before and after subtractive hybridisation was performed. We examined the presence of gapdh-specific PCR-products after different numbers of PCR cycles. A gapdh PCR-product was obtained 5 cycles later after subtractive hybridisation was performed, demonstrating that the amount of gapdh transcript thought to be present in fins before and after treatment was successfully reduced by SSH (Fig. 1E). The subtracted cDNAs were used to construct an SSH library and 406 clones were chosen for sequencing (Fig. 1F). The average insert size of the chosen clones was ~400 base pairs (bp), with an individual insert size between 100 and 700 bp. A more detailed analysis of the 406 sequences reduced the sequence data to 201 contigs and independent sequences (Fig. 1F). A contig or sequence was considered to be independent if it showed no significant overlap with other contigs and sequences. One hundred twenty-eight out of these 201 contigs/sequences (~64%) showed reliable similarity (exhibiting E-values of e^{-15} or less) to known genes using the Blast algorithm (Altschul et al., 1990) (Fig. 1F).

The remaining 73 sequences consisted of repetitive elements (6/8%), vector or poly-A-sequences (5/7%), and sequences without reliable blast hits (62/85%), likely to be UTR sequences. From the 128 sequences with a significant blast hit, 27 (53%) showed reliable similarity to ribosomal or other housekeeping genes, 27 (21%) to components of the cytoskeleton and structural genes, 16 (13%) to transcription factors or genes involved in signal transduction, 5 (4%) to known stress and immune response genes, and 12 (9%) to genes with other or unknown function (Fig. 1F and Table 1). Some of these sequences, although independent from each other, showed similarity to the same gene and might just represent different parts of it (Table 1).

**SSH-Enriched Genes Are Expressed in Developing Swords and Gonopodia**

In trying to better understand the molecular mechanisms of sword and gonopodium development, we were strongly interested in transcription factors and signalling pathways that might control these processes. Therefore, out of the class of 16 genes that showed similarities to transcription factor genes or genes involved in signal transduction, we chose seven clones with similarity to 14.3.3a, c-fos, dual specific phosphatase1 (dusp1), receptor for activated protein kinase C (rack1), krueppel-like factor 2 (klf2), m-calpain, and thymosin β-like (tmsβ-like) for further analysis. As an assay independent of the SSH procedure, we confirmed the expression of the selected genes in developing gonopodia and swords by non-quantitative reverse-transcribed (RT)-PCR in fin tissue from fish treated with testosterone for 2 and 5 days. Two days of treatment represented the stage of primary response to testosterone (possible direct targets), whereas 5 days represented the stage of secondary response to testosterone (possible indirect targets). All genes were expressed in caudal and anal fins after 2 and 5 days of testosterone treatment (dt) as well as in untreated control fins (Fig. 2). The RT-PCR experiment confirmed that all genes are indeed expressed in developing swords and gonopodia and are not an artefact created by the method itself.

**Expression of SSH-Enriched Genes During Sword Development**

Next we determined whether these genes are differentially expressed (1) in developing sword rays, compared to control fin rays, and (2) in sword rays after testosterone treatment for a range of time periods. We induced sword development in juvenile fish with 17α-methyltestosterone and performed expression analysis on caudal fins by whole mount in situ hybridisation after 2 and 5 days. Given that some of the isolated cDNA clones were too short to yield antisense transcripts suitable for expression analysis by in situ hybridisation, we produced a full-length, non-subtracted cDNA library from induced swords and gonopodia after 1, 2, 4, and 5 dt, which we used to isolate cDNA fragments of sufficient length.

The sword is formed by the ventral caudal fin rays V7–V10, based on Dzwillo's nomenclature for the caudal fin rays of X. helleri (Dzwillo, 1964). After 2 dt, rack1 was expressed at similar levels throughout the whole caudal fin with no obvious differences between sword and non-sword rays and at the same overall levels as in control fins (Fig. 3A, C). In contrast, after 5 dt rack1 was strongly up-regulated in the distal tips of the sword forming rays V8–V9 compared to non-sword rays in the median or dorsal caudal fin (Fig. 3B, B'). rack1 expression levels in V7 were similar to non-sword rays and no outgrowth of V7 was yet visible (Fig. 3B).

klf2 was expressed similarly to rack1. After 2 dt, klf2 expression was detected at basal levels in all caudal fin rays (Fig. 3D), yet at 5 dt klf2 is more strongly expressed in ventral sword fin rays V7–V10 than in non-sword fin rays (Fig. 3E, E'). klf2 expression appears to overlap with that of rack1 (compare Figs. 3B' and E'). In control fins, klf2 is equally expressed at low levels in sword rays and the remaining fin rays (Fig. 3F).

tmsβ-like transcription did not differ between ventral, median, and dorsal caudal fin rays after 2 dt (Fig. 3G). In contrast, after 5 dt tmsβ-like is clearly up-regulated in the sword region and seems to enclose the distal tip of the sword rays (Fig. 3H, H').
Fig. 3. Regulation of SSH-enriched genes in developing swords. A–L: *rack1* is up-regulated in growing sword rays, compared to non-sword (nsw) rays after 5 days of testosterone treatment (dt)(B, B'). In caudal fins after 2 dt (A) and in control fins, *rack1* (C) is expressed at basal levels in all fin rays. Basal expression of *klf2* can also be detected in caudal fins after 2 dt (D) and control fins (F). After 5 dt, *klf2* is transcribed at higher levels in the sword rays compared to nsw rays (E, E'). *tms*/*H9252-like* is more strongly expressed in the developing sword compared to the rest of the caudal fin (H, H'), whereas in caudal fins after 2 dt (G) and in control fins (I), expression levels of *tms*/*H9252-like* are similar between sword and nsw rays. *dusp1* expression could not be detected in either testosterone treated fins after 2 dt (J) or 5 dt (K), nor in control fins (L). White arrowheads indicate gene expression. V, ventral ray. 5 dt: n = 5 for *rack1*, *klf2*, and *tms*/*H9252-like*; n = 10 for *dusp1*; 2 dt and control fins: n = 4 for every probe. Scale bars = 200 μm.
tmsβ-like is also expressed in non-sword rays, although at lower levels. In control fins, only weak expression of tmsβ-like could be detected in some fin rays (Fig. 3I).

dusp1 expression could not be detected after 2 and 5 dt even after prolonged staining (Fig. 3J, K, K') or in untreated fins (Fig. 3L). 14.3.3a showed ubiquitous expression in the whole caudal fin with no obvious differences between sword and non-sword rays. No distinct expression pattern could be obtained for c-fos and m-calpain (data not shown). In summary, expression analysis showed that rack1, klf2, and tmsβ-like are strongly up-regulated in sword rays during sword outgrowth at 5 dt. Phylogenetic data used for the annotation of rack1, dusp1, klf2, and tmsβ-like are available upon request.

Fig. 4. Regulation of SSH-derived genes in developing gonopodia. A–P: rack1, dusp1, klf2, and tmsβ-like are up-regulated in the 3-4-5 complex of developing gonopodia. The anal fin rays 3, 4, and 5 show higher levels of rack1 (B), dusp1 (F), klf2 (J), and tmsβ-like (N) transcripts after 5 dt than the remaining anal fin rays. A more detailed analysis of the expression pattern of the four genes in tissue sections revealed that dusp1 expression is localised to the distal mesenchyme (G), whereas klf2 is transcribed in the lateral mesenchyme (K). rack1 expression can be found in both mesenchymal compartments (C). tmsβ-like is expressed in the basal layer of the epidermis (O). After 2 dt, rack1 is also clearly up-regulated in the anal fin rays 3, 4, and 5 (A). tmsβ-like transcript levels appear slightly higher in the 3-4-5 complex after 2 dt (M). dusp1 (D) and klf2 (I) expression is not detectable by in situ hybridisation in anal fins after 2 dt. In control fins, rack1 (D) and tmsβ-like (P) are transcribed at basal levels in all fin rays, whereas dusp1 (H) and klf2 (L) expression cannot be detected. White arrowheads, gene expression. R, anal fin ray. 5 dt: n = 5 for every probe; 2 dt: n = 4 for every probe; control fins: n = 3 for every probe. Scale bars = (A, B, D, E, F, H, I, J, L, M, N, P) 200 µm, (C, G, K, O) 100 µm.
Specific Expression of SSH-Enriched Genes in Rays Forming the Gonopodium

In order to examine the idea that the molecular networks that are activated during sword development are also active in the gonopod, we analysed the expression pattern of these genes in the metamorphosing anal fin. The gonopodium develops from the anal fin rays 3, 4, and 5, the so-called 3-4-5 complex (Langer, 1913). rak1 is up-regulated in the 3-4-5 complex after 2 dt (Fig. 4A) and 5 dt (Fig. 4B) when compared to the remaining anal fin rays. Up-regulation of rak1, therefore, occurs earlier in the gonopodial rays than in rays of the developing sword. An analysis of rak1 expression on tissue section revealed that both the distal and the lateral compartment of the fin ray mesenchyme express rak1 (Fig. 4C). In untreated anal fins, rak1 is expressed at basal levels in all fin rays (Fig. 4D). Expression of dusp1 could not be detected in anal fins at 2 dt (Fig. 4E). After 5 dt, however, dusp1 is strongly expressed in the distal mesenchyme of the 3-4-5 complex (Fig. 4F, G), but not in any other anal fin rays or in control fins (Fig. 4F, H). Thus, differential expression of dusp1 could be detected in growing gonopodia, but not in swords (compare Figs. 3K and 4F). The spatio-temporal expression pattern of klf2 in developing gonopodia is comparable to that of swords. After 2 dt, the klf2 transcript could not be detected in the anal fin (Fig. 4I), but after 5 days of testosterone treatment klf2 was exclusively up-regulated in the 3-4-5 complex (Fig. 4J). klf2 expression was located to the lateral mesenchyme (Fig. 4K) and, therefore, partially overlapping with rak1 expression (Fig. 4C). No expression of klf2 could be detected in control fins (Fig. 4L). tmsβ-like showed comparable expression in the 3-4-5 complex and the remaining anal fin rays after 2 dt (Fig. 4M). As in induced swords, tmsβ-like is up-regulated in the 3-4-5 complex (Fig. 4N). Unlike the other genes, tmsβ-like is not expressed in the mesenchyme, but in the adjacent basal cell layer of the fin ray epidermis (Fig. 4O). In some samples, tmsβ-like showed slightly stronger expression in rays 3 and 4 (Fig. 4N). Control fins expressed tmsβ-like at a basal level in all fin rays (Fig. 4P). 14.3.3a was also ubiquitously expressed as in the sword, and no distinct expression pattern could be detected for c-fos and m-calpain (data not shown).

Together, these results show that while rak1, dusp1, klf2, and tmsβ-like are all ultimately up-regulated in the 3-4-5 complex during gonopodial outgrowth at 5 dt, clear differences in gene regulation are also obvious. Transcription rates of rak1 are up-regulated much faster in the anal than in the caudal fin upon testosterone induction, while strong dusp1 expression appears to be specific to the developing gonopodial rays, but is mainly absent from sword rays.

SSH-Derived Genes Are Expressed in Regenerating Swords

The repertoire of signalling pathways controlling fin development often is also used again during regeneration of amputated fin tissue (reviewed in Lovine, 2007), a process in which fin rays exhibit accelerated growth similar to that experienced during adult fin metamorphosis. To examine whether the set of genes characterised during sword and gonopodium development was expressed during regeneration, we amputated the caudal fins of male swordtails and allowed them to regenerate for 4 days. Gene expression was then analysed by in situ hybridisation both on whole fins and on longitudinal sections, which allows the analysis of gene expression at cellular resolution and overcomes the limitations with the sensitivity of the in situ hybridization technique on whole fins (Smith et al., 2008).

All four genes, rak1, dusp1, klf2, and tmsβ-like, were also expressed during fin regeneration. rak1 was up-regulated in non-sword (Fig. 5A) and sword rays (Fig. 5B). Due to the larger size of the sword ray blastemata, the expression domain of rak1 in sword rays is clearly wider. rak1-expressing cells are found in the lateral compartments of the blastema, such as differentiating scleroblasts, and the distal region of the median mesenchyme (Fig. 5C). dusp1 is expressed in a cap-like pattern in the distal tip of non-sword (Fig. 5D) and sword rays (Fig. 5E). Fin sections revealed dusp1 to be expressed in the distal region of the median blastema (Fig. 5F). Thus both dusp1 and rak1 are activated in the distal part of the blastema during fin regeneration (compare Fig. 5C and F). klf2 shows an expression pattern in non-sword (Fig. 5G) and sword rays (Fig. 5H). Unlike rak1, klf2 is only expressed in proximal lateral mesenchymal cells, likely to be scleroblasts, but not in the distal blastema (Fig. 5I).

Judging from these analyses, it is apparent that all four genes are expressed during fin development (Figs. 3 and 4) as well as sword regeneration (Fig. 5) and thus are likely to fulfill similar roles in promoting the growth and regeneration of fin rays.

DISCUSSION

The molecular mechanisms controlling the development of the sword, a sexually selected trait in the genus Xiphophorus, have been targeted in previous studies in which candidate gene approaches were employed that focused on genes with known expression and functions during zebrafish fin regeneration (Zauner et al., 2003; Offen et al., 2008). As candidate genes were selected on the basis of prior information about their expression or function in other contexts, genes with unexpected functions or novel genes were not identified by this approach. In this study, we employed suppression subtractive hybridisation (SSH) to bypass this problem (Diatchenko et al., 1996). We successfully identified genes that are differentially expressed in developing swords and gonopodia compared to juvenile fins before metamorphosis.

Genes Up-Regulated During Xiphophorus Fin Metamorphosis Can Be Identified by Subtractive Hybridisation

In total, we identified 201 independent sequences, or contigs, of which 128 showed significant similarities to sequences in public databases. To our knowledge, this study provides the first collection of expressed sequence tags (ESTs) from developing swords and gonopodia. A large fraction of sequences (73) showed no significant similarity to
Fig. 5. Expression of SSH-derived genes in regenerating caudal fins. A–L: Expression of rack1, dusp1, klf2, and tmsβ-like in regenerating caudal fins. All genes are expressed at similar levels in non-sword (A, D, G, J) and sword rays (B, E, H, K). rack1 expression is detected in the distal medial region of the blastema as well as in more proximal and lateral regions, where it might overlap with scleroblasts (C). dusp1 is expressed in the distal part of the blastema, where it overlaps with rack1 expression (F). klf2 expression overlaps with that of rack1 in the lateral mesenchymal compartment that contains newly formed scleroblasts (I). tmsβ-like is expressed in the epidermis, specifically in the basal epidermal layer (L). White arrowheads, gene expression. days post amputation. n = 4 for every probe, except klf2: n = 7. Scale bars = (A, B, D, E, G, H, J, K) 200 μm, (C, F, I, L) 100 μm.

Fig. 6. Summary of the expression patterns of rack1, dusp1, klf2, and tmsβ-like. Summary of expression patterns of rack1, dusp1, klf2, and tmsβ-like in growing gonopodial rays of X. helleri. rack1 expression partly overlaps with that of dusp1 in the distal mesenchyme and with that of klf2 in the lateral mesenchyme (e.g., newly formed scleroblasts). tmsβ-like is expressed in the basal layer of the epidermis. Expression is similar in regenerating fin rays (4 days post-amputation), in which the mesenchymal domains described above are part of a blastema. BL, basal epidermal layer; DM, distal mesenchyme; E, epidermis; L, lepidotrichia; LM, lateral mesenchyme; M, mesenchyme.
sequences in the database. These sequences could represent untranslated regions (UTRs) or weakly conserved parts of the coding region.

A subset of 15 transcripts was represented by multiple (2–6), independent sequences, as can be expected for abundant transcripts, given that the average insert length of SSH clones is 400 bp. Housekeeping genes, but also genes encoding structural components inside and outside the cell, make up 75% of our EST pool. In line with our findings and the observation that genes required for fin development are re-used during regeneration, these transcripts were also quite abundant in an SSH library of regenerating fins (Padhi et al., 2004). In theory, one would expect that genes required for cellular maintenance should be eliminated by this method. However, transcripts of housekeeping genes are probably more abundant in growing fin rays, due to a higher demand for energy or protein synthesis in growing tissue and will be only partly removed by SSH. Genes encoding for structural components, like keratins and collagens, have been shown to be more strongly expressed in regenerating fins of zebrafish and medaka than in uninjured fins (Katogi et al., 2004; Padhi et al., 2004; Schebesta et al., 2006; Nishidate et al., 2007). They are likely to participate in the re-structuring of new fin tissue, since keratins are the major structural proteins in epithelial tissues and collagens are part of the lepidotrichia and actinotrichia (Bechara et al., 2004).

Approximately half of the obtained genes that code for transcription factors or are involved in cell signalling were also found to be expressed in regenerating caudal fins of zebrafish and medaka than in uninjured fins (Katogi et al., 2004; Padhi et al., 2004; Schebesta et al., 2006). This is not surprising, since both sword development and fin regeneration are characterised by elevated outgrowth of fin rays, which seems to be controlled by a conserved genetic network (reviewed in Iovine, 2007). However, it will be highly interesting to evaluate those genes that are strongly expressed in sword or gonopodial rays but less so in regenerating caudal fins, as they may be involved in the downstream response to testosterone signalling.

Differential Expression of SSH Candidates in Developing Swords and Gonopodia and Regenerating Caudal Fins

Our gene expression analyses of seven clones from the transcription factor/cell signalling category showed that the genes rack1, dusp1, kl2, and tmsβ-like are differentially expressed in developing swords and/or gonopodia compared to juvenile fins before testosterone-induced metamorphosis. For two genes with similarity to c-fos and m-calpain, we failed to show any distinct expression in developing swords and gonopodia or regenerating caudal fins. It is likely that both genes are expressed at rather low levels that escape detection by in situ hybridisation, as RT-PCR clearly showed that the two genes are transcribed in developing swords and gonopodia.

Interestingly, all genes, except rack1, are exclusively expressed at later stages of testosterone treatment, when outgrowth of sword and gonopodial fin rays becomes morphologically apparent. In induced gonopodia, but not in induced swords, rack1 transcription, was activated before outgrowth started.

It remains elusive why the SSH approach mainly obtained genes that become active during the outgrowth phase rather than earlier and as an immediate response to androgen signalling. One possibility is that cDNAs derived from genes that are directly regulated by androgen receptors were only present in low copy number within the SSH pool. If true, direct targets might be detected by sequencing a larger number of clones. Another possibility would be that the levels of transcriptional activation of direct targets are below the detection limits of the in situ hybridization technique. In the future, this may be possible to test through more sensitive quantitative methods, such as reverse-transcribed (RT)-PCR of candidate genes.

Even though the number of identified genes is strongly correlated with the number of sequenced clones, the SSH remains a valuable technique, since the number of alternative methods is limited for a non-model organism like X. helleri. Species-specific microarrays are not yet available and an efficient detection of differentially expressed genes by a mass sequencing approach would require more advanced resources such as next generation DNA sequencing (Hornsbe et al., 2009). Another technique, differential display RT-PCR (DDRT-PCR), cannot be used efficiently in Xiphophorus. DDRT-PCR selectively amplifies small fragments from the 3′-UTR of ideally all cDNAs to produce a gene expression profile that can be compared between two samples (Liang et al., 1992). For X. helleri, however, the genomic and EST information currently is insufficient to allow a fast and reliable identification of these sequences.

After 2 days of testosterone (dt) treatment, rack1 transcription is already increased in the anal fin, before the 3-4-5 complex starts to grow out, and might therefore act in the induction of fin growth. Interestingly, the genes we identified are expressed in all of the compartments relevant for regeneration, i.e., the distal median blastema, basal layer of the epidermis, and scleroblast-forming lateral blastema. Similar expression patterns are present in the mesenchyme and basal epithelial layer of growing fins. We summarize gene expression in growing and regenerating fin rays in Figure 6. All three compartments have a distinct role in promoting fin ray growth. The lateral mesenchymal compartment of both developing swords and gonopodia after 5 dt and regenerating caudal fins expresses rack1 and kl2 (Fig. 6). Studies in regenerating fins showed that the lateral compartment of the mesenchyme contains newly formed scleroblasts that align along the basal epithelial layer to produce new hemiray segments (Laforest et al., 1998; Quint et al., 2002). Scleroblast expression of rack1 and kl2 might indicate a role for both genes in dermal bone formation.

rack1 may be linked to the gene-regulatory network controlling hemiray development or regeneration via Fgf or Bmp signalling. Two lines of evidence support a putative interaction between rack1 and Fgf signalling: First, fgfr receptor 1 (fgfr1) is up-regulated in developing swords and gonopodia as well as regenerating caudal fins of X. helleri. In addition, fgfr1 is co-expressed with rack1 in scleroblast cells (Offen et al., 2008). Second, rack1 expression is regulated by Fgf signalling in developing chick limb buds (Lu et al., 2001). Rack1 is thought to bind and stabilize activated Protein kinase C (PKC) and re-
cruits the kinase to its targets (reviewed in McCall et al., 2002). Therefore, Fgf signalling both activates PKC and increases Rack1 levels to enhance its activity in the chick limb bud (Lu et al., 2001). rack1 might also act on bone formation via the Bmp signalling pathway. Rack1 has been shown to be required for Bmp2-induced phosphorylation of Smads via the Bmp receptor 2 (Zakrzewicz et al., 2007). In regenerating caudal fins of zebrafish, bmp2b is expressed in scleroblasts, and both knockdown of Bmp signalling and over-expression of bmp2b impairs dermal bone formation (Laforest et al., 1998; Quint et al., 2002; Smith et al., 2006).

Whether dermal bone formation also requires klf2 remains uncertain. Klf2 is a C2/H2 zinc finger transcription factor that can either activate or repress transcription of target genes (reviewed in Atkins and Jain, 2007). Two klf2 paralogs have been described in zebrafish due to the fish-specific genome duplication (Oates et al., 2001). Klf2a seems to fulfill the ancestral function of klf2 in blood vessel development and control of blood pressure, while the function of the second paralog is not known (Oates et al., 2001; Bhattacharya et al., 2005; Lee et al., 2006). The isolated klf2 sequence seems to represent the Xiphophorus klf2 ortholog (data not shown). It will be interesting, therefore, to further analyze whether the expression in scleroblasts of growing or regenerating fin rays is a unique feature of klf2b or if it points towards an ancestral function of klf2 in appendage growth and regeneration. Functional analyses of klf2 and rack1 in a suitable experimental system, e.g., the regenerating zebrafish caudal fin, will be required to show whether both genes act within the same or in parallel pathways.

dusp1 (also known as MAP kinase phosphatase-1, mkp1) might promote endothelial cell migration (Kinney et al., 2008) in the distal medial mesenchyme. In endothelial cells (EC), dusp1 is activated by Vegf-A and Vegf-E via the Vegf receptor 2, and knockdown of dusp1 perturbs VEGF-induced EC migration. Vegf2 is expressed in the distal-most blastema of regenerating caudal fins of zebrafish and inhibition of Vegf signalling showed that angiogenesis is essential for regenerative outgrowth of fin rays (Bayliss et al., 2006). dusp1, therefore, might be involved in regulating the migration of ECs in growing and regenerating fin rays to promote the formation of new blood vessels. As a MAP kinase phosphatase, Dusp1 is likely to regulate the activity of MAP kinases (Teng et al., 2007; Caunt et al., 2008) that transmit the Vegf signal (Chakroborty et al., 2008; Kinney et al., 2008). Inhibition of Vegf signalling (Bayliss et al., 2006) or knockdown of dusp1 in the zebrafish system will be helpful to validate or reject this hypothesis. Furthermore, functional data will useful to show whether dusp1 and rack1 fulfill different roles during ray outgrowth or interact with each other. It is unlikely that dusp1 is not expressed in developing sword rays, since its expression was confirmed by RT-PCR and because both growing gonopodia and regenerating swords showed up-regulation of the gene. Rather, we believe that dusp1 may be expressed in sword rays at levels below the detection limit of the in situ hybridisation technique.

The application of the SSH technique to Xiphophorus fins in early stages of testosterone-induced metamorphosis resulted in the identification of four candidate genes that showed differential expression in induced swords and gonopodia compared to control fins and is the first of this kind to also investigate the expression patterns of these genes at the cellular level in regenerating fins. The subtractive library generated in this study will be important for uncovering additional genes that promote the growth of fin rays or are themselves controlled by testosterone signalling. We further anticipate isolating more candidates that exhibit different temporal profiles or levels of gene expression between swords and gonopodia, as exemplified for rack1 and dusp1. In the future, it will be necessary to dissect the molecular functions of these genes, preferably in zebrafish as a genetically accessible model, and to analyse putative interactions between co-expressed genes.

**EXPERIMENTAL PROCEDURES**

**Fish Stocks and Maintenance**

Juvenile and adult green swordtails (X. helleri) were taken from stocks kept at the "Tierforschungsanlage" at the University of Konstanz. Fish were maintained on a 12:12h light:dark cycle at 24°C in 110-litre densely planted aquaria and were fed TetraMin flakes and Artemia.
Testosterone Treatment and Fin Regeneration

For SSH and λ-phage cDNA libraries, 120 juvenile individuals of X. helleri each, aged between 3 and 6 months, were treated with 17β-methyltestosterone (1 mg/ml stock solution in ethanol; Sigma-Aldrich, Munich, Germany) that was added to the water twice a week to a final concentration of 10 µg/l. The 120 individuals were divided into 4 groups of 30 individuals each and were treated in 110-litre tanks. After 1, 2, 4, and 5 days of treatment, 1/3 of the caudal and anal fin was harvested from individuals of one group with a sterile razor blade. For fin amputations, fish were anesthetized by incubation in a solution of 80 µg/ml tricaine (3-aminobenzoic-acid-ethylster-methanesulfonate; Sigma-Aldrich, Munich, Germany). For the SSH library, an additional 120 individuals were mock-treated with ethanol and fin tissue was amputated as described above. Testosterone- and ethanol-treated tissue was pooled and used for RNA extraction.

For RT-PCR, 5 to 8 juvenile fish were treated for 2 or 5 days with testosterone, or 5 days with ethanol, followed by the amputation of 1/3 of the distal part of the caudal fin and approximately 2/3 of the anal fin. Caudal and anal fin tissue from the 3 treatment groups was pooled and used for RNA extraction.

For gene expression analysis, up to six juvenile individuals were placed in a 30-litre tank and treated with 17β-methyltestosterone to a final concentration of 10 µg/l. After 2 or 5 days of testosterone treatment, fish were anesthetized and approximately 1/3 of the distal part of the caudal fin and approximately 2/3 of the anal fin. Caudal and anal fin tissue from the 3 treatment groups was pooled and used for RNA extraction.

λ-Phage cDNA Library Construction

Total RNA was isolated from caudal and anal fin tissue as described (Zauner et al., 2003). PolyA⁺-RNA was purified using the Qiagen Oligotex mRNA Mini kit (Qiagen, Hilden, Germany). Five micrograms of PolyA⁺-RNA was used to construct a λ-phage cDNA library with the ZAP-cDNA® Library Construction Kit (Stratagene, Heidelberg, Germany) according to the manufacturer’s instructions. The amplified library was stored in SM buffer (100 mM NaCl, 8 mM MgSO₄, 50 mM Tris-HCl, pH 7.5) with 5% DMSO at −80°C.

Isolation of cDNA From Recombinant λ-Phages

Seven hundred and fifty microliters of the amplified cDNA library was treated with 10 µ RNaseA and DNasel (Fermentas, St. Leon-Rot, Germany) prior to phage particle lysis for 10 min at 37°C. Phage particles were lysed by adding 150 µl STEP buffer (0.4 M EDTA, 50 mM Tris-HCl, pH 8, 1% SDS) and 100 µg Proteinase K (Sigma-Aldrich, Munich, Germany) at 65°C for 30 min. DNA was purified by standard methods (Sambrook et al., 1989).

Suppression Subtractive Library Construction

The SSH library was constructed using the PCR-Select cDNA subtraction kit (Takara Bio/Clontech, Heidelberg, Germany) subtraction, according to the manufacturer’s instructions. Two micrograms PolyA⁺-RNA (purified as described above) from testosterone-treated fins were used as tester, and 2 µg PolyA⁺-RNA from ethanol-treated fins were used as driver fractions. The driver pool was subtracted from the tester pool and the subtracted cDNAs were cloned into the pCRII vector using the T/A cloning kit and propagated in E. coli INVaF⁺ (Invitrogen, Karlsruhe, Germany). DNA contamination was removed by incubating total RNA with DNasel (1 U/µl; Fermentas, St. Leon-Rot, Germany) for 30 min. cDNA fragments of the selected genes were amplified by PCR using gene-specific primers (Supp. Table S2, which is available online). Primers were designed from SSH clone sequences using “Generunner” (Hastings Software Inc.). X. helleri gapdh primers were used for the positive control.

RT-PCR

To detect expression patterns of selected genes, total RNA was isolated from caudal and anal fin tissue as described (Zauner et al., 2003). One microgram of total RNA was transcribed into single-stranded cDNA using the Superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany). DNA contamination was removed by incubating total RNA with DNasel (1 U/µl; Fermentas, St. Leon-Rot, Germany) for 30 min. cDNA fragments of the selected genes were amplified by PCR using gene-specific primers (Supp. Table S2, which is available online). Primers were designed from SSH clone sequences using “Generunner” (Hastings Software Inc.). X. helleri gapdh primers were used for the positive control.

RNA Probe Synthesis

To obtain fragments of SSH clones with sizes appropriate for generating
RNA antisense probes, the 3’ ends were amplified from the cDNA library clones using PCR with gene-specific primers (Supp. Table S2). PCR products were gel-purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into the pCRII-TOPO vector (Invitrogen, Karlsruhe, Germany) for sequencing. Antisense and sense RNA probes were generated using either the digoxigenin or labelling kit (Roche, Mannheim, Germany).

**Whole-Mount In Situ Hybridisation**

In situ hybridisation of *Xiphophorus* fins and blastemata were performed as described (Poss et al., 2000) with several modifications. Pre-hybridisation was done for 4 h at 68°C in formamide solution (50% formamide, 5× SSC, 0.1% Tween 20, pH to 6 with 1 M citric acid). Post-hybridisation washing steps were initiated at 68°C with formamide solution. To block non-specific binding sites, 0.5% blocking reagent (Roche, Mannheim, Germany) in PBT (PBS + 0.1% Tween-20, both from Sigma-Aldrich, Munich, Germany) was used. Antibody incubation was done at 4°C overnight. After fixation of stained fins/blastemata, the tissue was washed twice for 20 min in PBT, 20 min in ethanol/PBT (70:30), and 20 min in 100% ethanol and stored at 4°C.

**In Situ Hybridisation on Longitudinal Sections**

In situ hybridisation was performed on longitudinal sections of 16-μm thickness from fixed caudal fin blastemata as described (Kuraku et al., 2005) with one exception: For pre-hybridisation and hybridisation, the same solution was used as for whole mount in situ hybridisation. Sections were cut with a Reichert-Jung Autocut 2040 Micrometre.

**Microscopy and Image Editing**

Whole mount fins were analysed using a Zeiss Stemi SV11 Apo. Logitudinal sections were analysed using a Zeiss Axiophot 2. Pictures were taken using the AxioVision software v3.1 (Zeiss) and the digital camera Zeiss AxioCam MRc. Images were processed using Adobe Photoshop 7.0.

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