

# Transient Ectopic Overexpression of Agouti-Signalling Protein 1 (Asip1) Induces Pigment Anomalies in Flatfish

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## Abstract

While flatfish in the wild exhibit a pronounced countershading of the dorso-ventral pigment pattern, malpigmentation is commonly observed in reared animals. In fish, the dorso-ventral pigment polarity is achieved because a melanization inhibition factor (MIF) inhibits melanoblast differentiation and encourages iridophore proliferation in the ventrum. A previous work of our group suggested that *asip1* is the uncharacterized MIF concerned. In order to further support this hypothesis, we have characterized *asip1* mRNAs in both turbot and sole and used deduced peptide alignments to analyze the evolutionary history of the agouti-family of peptides. The putative *asip* precursors have the characteristics of a secreted protein, displaying a putative hydrophobic signal. Processing of the potential signal peptide produces mature proteins that include an N-terminal region, a basic central domain with a high proportion of lysine residues as well as a proline-rich region that immediately precedes the C-terminal poly-cysteine domain. The expression of *asip1* mRNA in the ventral area was significantly higher than in the dorsal region. Similarly, the expression of *asip1* within the unpigmented patches in the dorsal skin of pseudoalbino fish was higher than in the pigmented dorsal regions but similar to those levels observed in the ventral skin. In addition, the injection/electroporation of *asip1* capped mRNA in both species induced long term dorsal skin paling, suggesting the inhibition of the melanogenic pathways. The data suggest that fish *asip1* is involved in the dorso-ventral pigment patterning in adult fish, where it induces the regulatory asymmetry involved in precursor differentiation into mature chromatophore. Adult dorsal pseudoalbinism seems to be the consequence of the expression of normal developmental pathways in an inaccurate position that results in unbalanced *asip1* production levels. This, in turn, generates a ventral-like differentiation environment in dorsal regions.

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## Introduction

In teleosts fish, pigment cells are commonly found in the dermis and can be divided into light-absorbing (melanophores, xanthophores, erythrophores and cyanophores) and light-reflecting (leucophores and iridophores) chromatophores. Fish melanophores contain eumelanins (black-brown pigments), whereas xanthophores and erythrophores synthesize carotenoids and/or pteridines that contribute to the reddish and yellowish components of the skin coloration. Iridophores are commonly localized in whitish and silvery areas of the skin, predominantly on the belly surface. They contain crystalline platelets composed of purines, mainly of guanine, which are responsible for the reflection of light [1]. Fish countershading is achieved by a patterned distribution of the pigment cells, with the light-absorbing and light-reflecting chromatophores mostly distributed in the dorsal and ventral areas, respectively [2,3]. Although the pigment pattern is most evident in the adult animal, its cellular basis is established during embryo-

genesis [4]. Experimental data in fish and amphibian species suggest that this dorso-ventral pigment pattern is achieved because a putative diffusible melanization inhibition factor (MIF), locally produced by cells in the ventral skin, inhibits melanoblast differentiation and stimulates iridophore proliferation in the ventrum [2,5,6]. Our recent studies support agouti-signalling protein 1 (*asip1*) as the fish MIF [7]. *Asip1* encodes a 131 amino acid protein with structural characteristics of a secreted protein, which has a hydrophobic signal sequence and lacks a transmembrane domain. A highly basic domain with a high proportion of arginine and lysine residues forms the N-terminal region of the agouti protein. The latter region heads a proline-rich area that immediately precedes the cysteine-rich C-terminal domain. This cysteine domain resembles the conotoxins and plectoxins of snails and spiders, respectively [8].

In goldfish (*Carassius auratus*), *asip1* is expressed in the ventral skin but not in the dorsal skin. It inhibits melanocortin-induced melanin dispersion in melanocytes and selectively binds melano-

cortin receptor 1 (MC1R) [7]. This receptor shows high sensitivity to the melanocyte-stimulating hormone ( $\alpha$ -MSH) and is profusely expressed within both dorsal and ventral skin [7,9]. Interestingly, frameshift mutations introducing a premature stop codon in melanocortin MC1R or inactivating mutations in blind Mexican cave tetra (*Astyanax mexicanus*) are responsible for a decrease in the number of melanocytes and of the melanin content. This phenotype is recapitulated by MC1R knockdown in zebrafish [10]. Taken together, the data support that interaction between  $\alpha$ -MSH/asipl and MC1R is involved in the establishment of the dorsal-ventral pigment pattern, controlling chromatoblast survival, differentiation and/or proliferation as well as melanin synthesis.

Flatfish exhibit a pronounced countershading and are an excellent model to study the establishment of the dorso-ventral pigment pattern. These fish species undergo a metamorphosis from symmetrical free-swimming larvae to asymmetrical bottom-dwelling animals with both eyes on the same side. The dorsal-ocular side becomes dark pigmented whereas the ventral-blind side is white in color [11]. This pigment asymmetry appears in the adult stage and is hypothesized to depend upon the asymmetry of organizational environments that potentially regulate latent chromatophore precursor survival, proliferation and differentiation [11,12]. Such regulatory asymmetry may be due to differences in the expression and distribution of secretory proteins involved in the precursor differentiation into mature chromatophore [13]. Accordingly, the common malpigmentation observed in reared flatfish, including pseudoalbinism (partial or total unpigmented ocular side) and hypermelanism (partial or total pigmented blind side), could be due to abnormalities in the asymmetry of the regulatory system [12,14,15]. The aim of this paper was to gain evidence supporting the view that *asipl* is able to generate a regulatory asymmetry that leads to dorsal-ventral pigment asymmetry. To this aim, we characterized sole (*Solea senegalensis*) and turbot (*Scophthalmus maximus*) *asipl* gene and analyzed tissue and developmental expression. We demonstrate that *asipl* is significantly more expressed in the ventral skin than in the dorsal skin. Moreover, when *asipl* is ectopically overexpressed in the ocular side it induces skin paling probably via inhibition of the melanogenic processes, whereas pseudoalbino animals exhibit increased *asipl* expression within the anomalous pigment areas.

## Results

### Cloning flatfish *asipl* gene

Reverse transcription-polymerase chain reaction (RT-PCR) using degenerate primers designed by alignments of available fish *asipl* sequences; produced a partial cDNA fragment of 135 and 159 bp for sole and turbot, respectively. The putative translations exhibited high identity with the C-terminal cysteine domain of the published *asipl* sequences. To obtain the sequence of the complete peptide precursor RACE-PCR was performed in the 3' and 5' directions with specific primers. 3' RACE generated unique bands of 422 and 499 bp for sole and turbot, respectively and provided information about the coding region of the exon 4 and the 3' untranslated region. 5' RACE experiments generated unique bands of 379 and 498 bp and provided information about the first *asipl* exons as well as the 5' untranslated region.

The peptide precursors have the same organization as other species. The first 22 amino acids are estimated to constitute the signal peptide, which is followed by the 101 (turbot) or 110 (sole) amino acids of the mature peptide. One putative glycosylation sites were found within the highly basic N-terminal region of the sole but no glycosylation consensus sites were found in the turbot mature peptide. A proline-rich region and a poly-cysteine C-

terminal domain followed the basic N-terminal region in both sequences. The poly-cysteine domain contains 10 cysteine residues with identical spatial pattern to that of agouti-like proteins, and similar to mammalian *asipl* molecules it does not exhibit a short amino acid extension following the tenth cysteine residue (Fig. 1). Sole and turbot *asipl* precursors were 73% identical. Flatfish amino-acid *asipl* sequences are only 15–19% identical to *asipl* of fish tetradontiform but they share 57–67% identical amino acids with *asipl* precursor of the same species. The identity level of flatfish sequences with fish *asipl* or *asipl*2 was 18–20% and 15–19%, respectively. Phylogenetic analysis grouped flatfish *asipl* sequences with the *asipl* precursors of fish and tetrapod species. A different branch of the same cluster grouped *asipl*2 and *agrp*2 sequences, whereas *agrp* precursors were grouped in a different cluster (Fig. 2).

### Temporal and spatial expression of *asipl*

The RT-PCR analysis (Fig. 3 A,B) showed that *asipl* transcripts existed maternally at a relatively low level, whereas zygotic expression persisted at relatively constant levels until the end of the sampling period (45 days post-fertilization, dpf) for turbot (Fig. 3A) and (29 dpf) sole (Fig. 3B).

At tissue level, *asipl* was highly expressed in the brain eye, heart, muscle, gonads and pineal organ of turbot. Very low expression levels were found in the hypophysis and liver. Residual levels were found in the remaining tested tissues including skin (Fig. 4A). Similar to turbot, sole *asipl* was expressed in the brain, hypophysis, eye, liver muscle and gonads but not in the heart. Additionally, high expression levels were detected in the gill, dorsal and ventral skin and adipose tissue (Fig. 4B).

### Spatially controlled expression of *asipl* gene

To examine whether the expression of *asipl* gene is spatially regulated in turbot and sole skin, samples of dorsal and ventral skin were taken and *asipl* gene expression evaluated by absolute qRT-PCR. Consistent with the dorso-ventral expression pattern of *asipl* gene described in other fish species [7], the *asipl* transcripts were significantly more expressed in the ventral non-pigmented skin than in the dorsal pigmented skin of both fish species (Fig. 5A,B).

In pseudo-albino turbot (Fig. 6A) and sole (Fig. 6C), *asipl* gene expression was upregulated in dorsal non-pigmented regions compared with the dorsal pigmented regions in both turbot (Fig. 6B) and sole (Fig. 6D), suggesting a relationship of *asipl* gene expression levels and changes in skin pigmentation.

### Transient ectopic overexpression of *asipl* gene

To investigate whether ectopic *asipl* expression could lead to pigment alteration on flatfish dorsal skin, we transiently overexpressed the *asipl* gene in turbot and sole dorsal skin area by *asipl*-capped mRNA injection and electroporation. The transient ectopic overexpression of *asipl* in the dorsal skin of turbot and sole induced a powerful paling of the skin 4 days after *asipl* gene overexpression (Fig. 7B; 8B). No skin pigmentation alteration was found in the antisense *asipl*-capped mRNA injected and electroporated fish (Fig. 7D; 8D) or *eGFP* (Fig. 7H; 8H) using brightfield illumination but increased fluorescence was evident in animals injected with sense *eGFP* (Fig. 7F; 8F). It means that sense *eGFP* injection and electroporation caused the expected effect without alteration of skin pigmentation.

To confirm the effects of *asipl* injection on melanogenic synthesis pathways, we studied tyrosinase-like protein 1 (Tyrp1) expression in intact, *eGFP*- and sense capped mRNA *asipl*-injected turbot skin. As expected Tyrp1 expression levels were lower in the ventral skin when compared to dorsal skin (Fig. 9A).

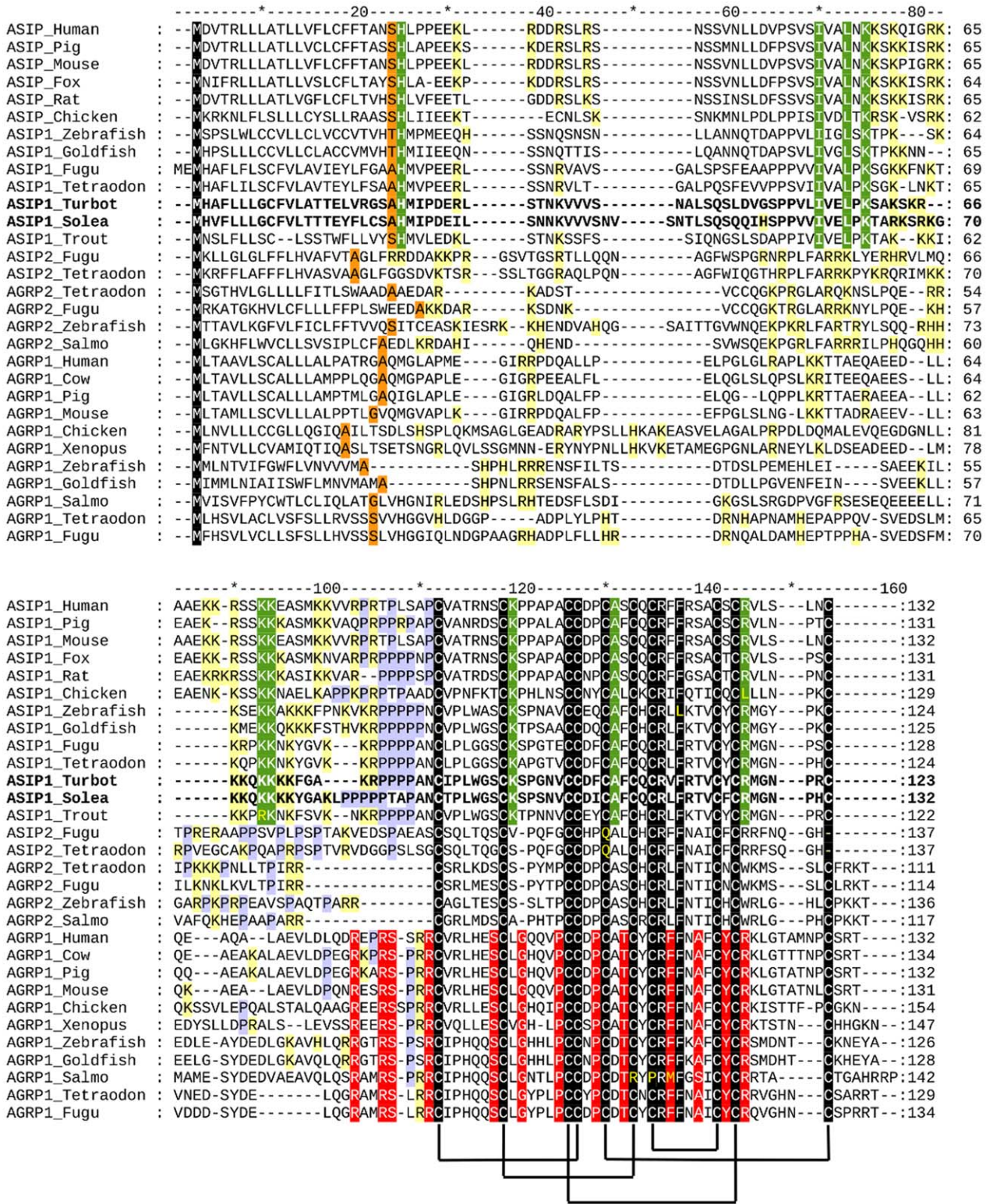
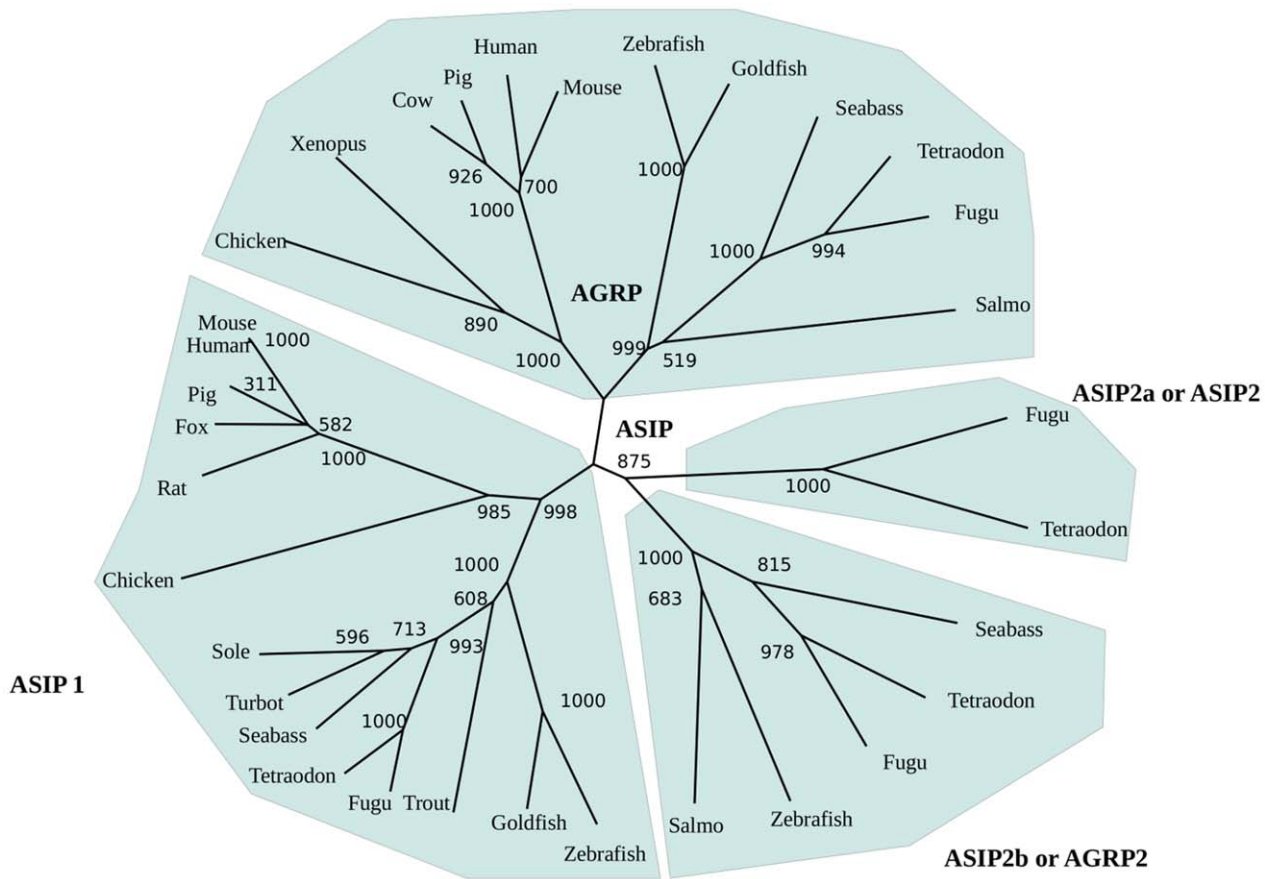


Figure 1. Alignment of agouti-signaling protein (asip) and agouti-related protein (agrp) amino acid sequences. Dashes were introduced to improve alignment. Orange boxes indicate the last residue of the predicted signal peptide. Black boxes show amino acid residues conserved in all sequences. Green boxes show residues only conserved in asip1 sequences. Red boxes indicate residues only conserved in agrp1 sequences. Yellow boxes indicate basic residues before cysteine. Blue boxes show residues of the short tail present in all agrp sequences. Purple boxes indicate putative glycosylation sites. Lines joining cysteine residues indicate putative disulfide bonds forming the cysteine domain. Arrow shows conserved motif for agrp post-transcriptional processing. doi:10.1371/journal.pone.0048526.g001



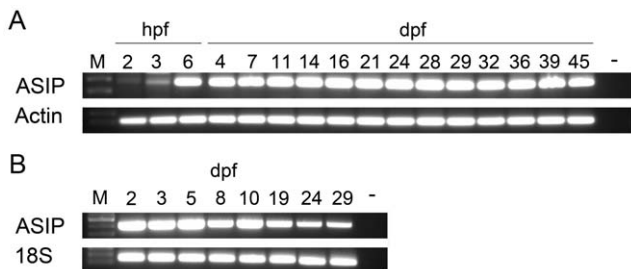
**Figure 2. Phylogenetic tree of *asip* and *agrp* amino acid sequences built using CulstalX, which uses the Neighbor-Joining method on a matrix of distances.** Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. Phylogenetic analysis were done also by maximum likelihood using Seaview free software and no considerable differences were found. doi:10.1371/journal.pone.0048526.g002

Similarly the injection of sense *asip1*-capped mRNA, but no eGFP mRNA, induced a severe decrease in the Tyrp1 expression levels (Fig. 9B).

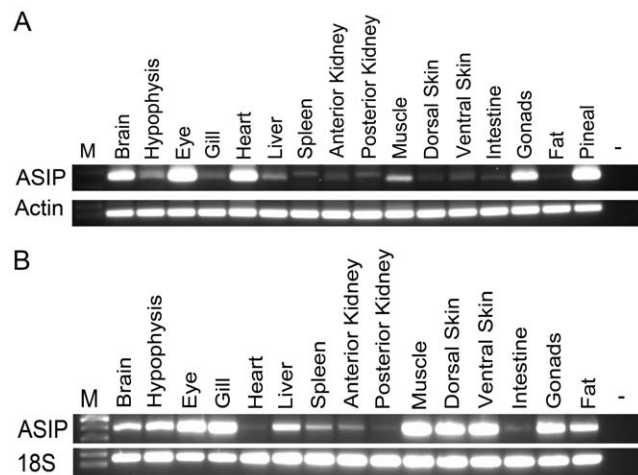
**Discussion**

In this paper, we characterize *asip1* mRNA sequences for sole and turbot. *Asip1* is expressed in the main pigment tissues, i.e. eye and skin, but also in the central nervous system, including the pineal complex of turbot. Transitory overexpression of *asip1* mRNA in the melanic-dorsal side induces skin paling in both

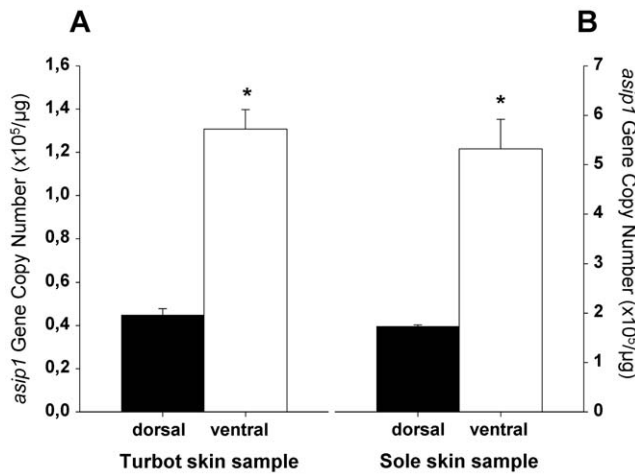
studied species and reduces the expression of key enzymes of the melanogenic pathway in turbot. Quantitative experiments demonstrated that *asip1* mRNA is overexpressed in non-pigmented regions of the dorsal skin in pseudoalbino turbot compared with



**Figure 3. Expression of *asip 1* gene during early development.** RT-PCR analysis of the temporal expression pattern of *asip1* in turbot (A) and sole (B). Hours post-fertilization, hpf; days post-fertilization, dpf. doi:10.1371/journal.pone.0048526.g003



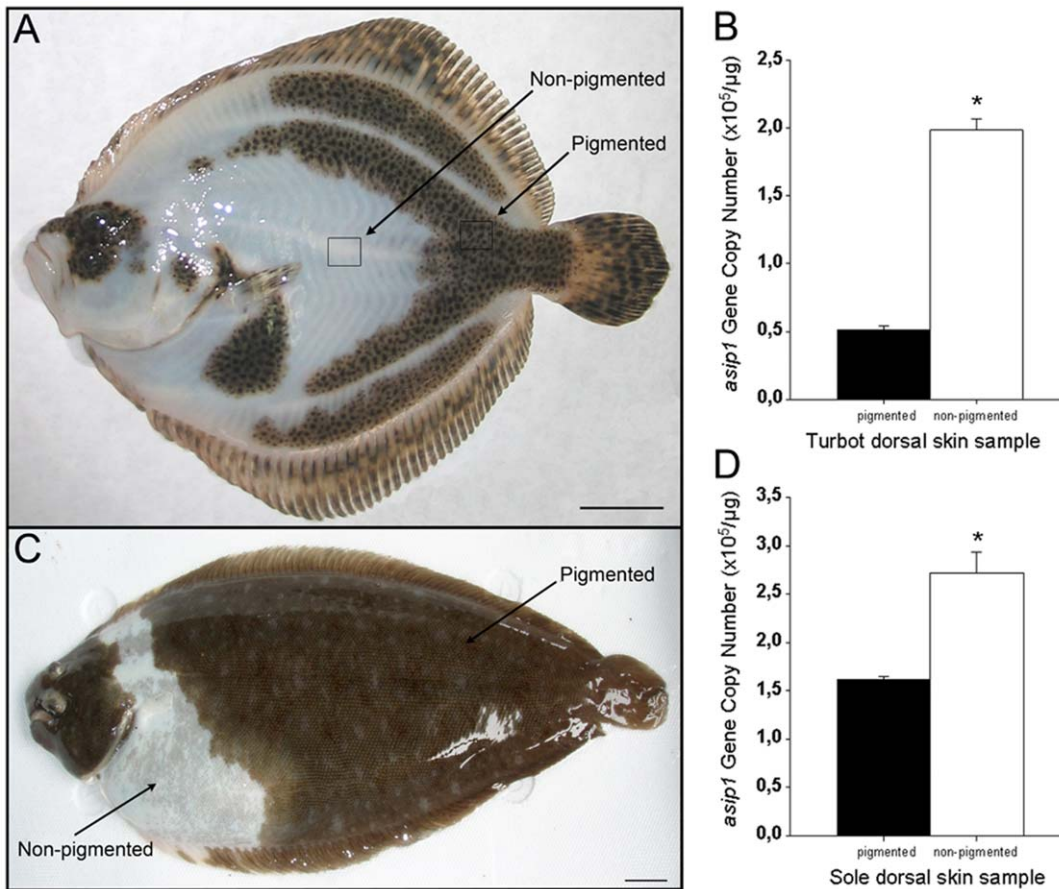
**Figure 4. RT-PCR analysis of the tissue specific expression pattern of *asip1*.** (A) Turbot and (B) sole. doi:10.1371/journal.pone.0048526.g004



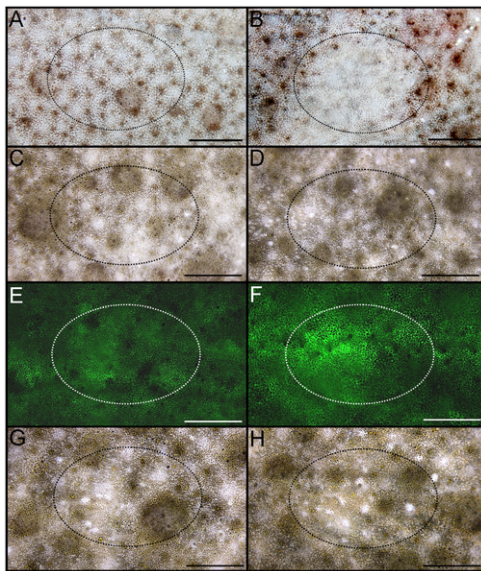
**Figure 5. Analysis of differential dorsal-ventral *asip1* gene expression.** *Asip1* was differentially expressed in ventral non-pigmented skin or dorsal pigmented skin in turbot (A) and sole (B). *Asip1* gene expression was quantified by absolute qRT-PCR. The average *asip1* gene copy number per μg of primed cDNA was calculated from 5 individuals analyzed each time in triplicate. Data are expressed as mean ± SEM. Comparisons of numerical data were made by paired Student t-tests. \*P<0.05. doi:10.1371/journal.pone.0048526.g005

melanic regions. The results demonstrate *asip1* participation in fish melanophore physiology and suggest its involvement in the organization of the dorsal-ventral pigment pattern.

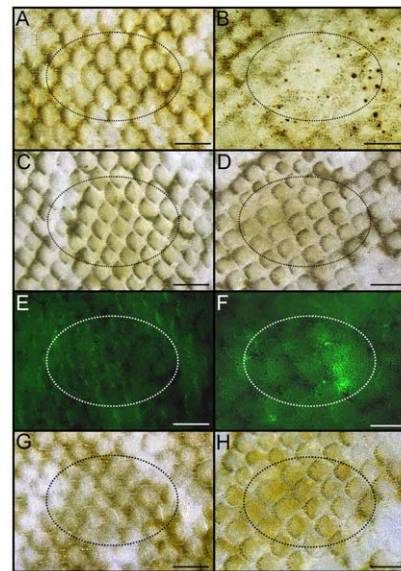
Flatfish *asip1* peptides keep the same structure exhibited by all agouti family of peptides. The putative *asip* precursors have the characteristics of a secreted protein, displaying a putative hydrophobic signal. Processing of the potential signal peptide produces 101 and 110-amino acid mature proteins in turbot and sole, respectively, including an N-terminal region, a basic central domain with a high proportion of lysine residues as well as a proline-rich region that immediately precedes the C-terminal polycysteine domain. Sole *asip1* exhibited one potential N-glycosylation site within the N-terminal region *asip* but no consensus glycosylation sites were found in the turbot sequence. In mice, glycosylation of *asip* is an important factor for protein functionality as disruption partially reduces peptide activity in transgenic mice [16]. Similar to mammalian species, the basic domain of the sole and turbot peptides exhibit 10 lysine (K) and 2 and 3 arginine (R) residues, respectively. The integrity of this basic domain is also key for the full activity of the *asip* protein [17,18]. The N-terminal region of mouse agouti has been shown to down-regulate melanocortin receptor signaling in *Xenopus* melanophore [19] and is also thought to mediate low affinity interactions with the product of the mahogany locus, i.e attractin [20]. Spacing of the 10 cysteines within the C-terminal poly-cysteine domain is totally



**Figure 6. Analysis of *asip1* gene expression in pseudoalbino flatfish.** Pseudo-albinism phenotype present in cultured turbot (A) and sole (C). *Asip1* was differentially expressed in non-pigmented white or pigmented brown dorsal skin areas in turbot (B) and sole (D). *Asip1* gene expression was quantified by absolute qRT-PCR. The average *asip1* gene copy number per μg of primed cDNA was calculated from 5 individuals analyzed each time in triplicate. Data are expressed as mean ± SEM. Comparisons of numerical data were made by paired Student t-tests. \*P<0.05. Scale bars: 1 cm. doi:10.1371/journal.pone.0048526.g006



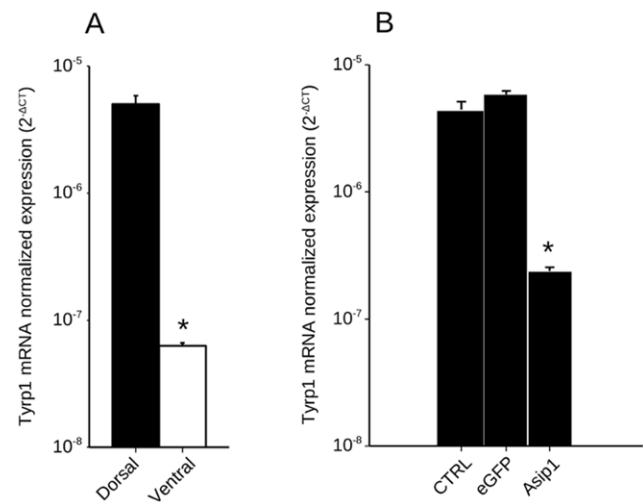
**Figure 7. Views of dorsal skin of turbot injected and eletroporated *in vivo* to evaluate the effect of asip 1 overexpression on skin paling.** Animals were injected with about 10 µg of capped sense (B) or antisense (D) mRNA per cm<sup>2</sup> of dorsal skin and the effect was evaluated 4 days post injection/electroporation. Dorsal skin of control non-treated turbots are shown in A, C, E, and G. E and F show dorsal skin of control (E) and injected/electroporated animals with capped sense eGFP-mRNA (F) animals under fluorescent incident light w. G and H display animals shown in E and F under brilliant incident light. Fluorescence was determined with a binocular Leica Stereoscope M165FC with digital camera (Leica Microsystem). Images were processed with Photoshop 7.0 (Adobe Systems) programs. Dorsal views, anterior to the right. Scale bars: 0.6 cm. doi:10.1371/journal.pone.0048526.g007



**Figure 8. Views of dorsal skin of sole injected and eletroporated *in vivo* to evaluate the effect of asip 1 overexpression on skin paling.** Animals were injected with about 10 µg of capped sense (B) or antisense (D) mRNA per cm<sup>2</sup> of dorsal skin and the effect was evaluated 4 days post injection/electroporation. Dorsal skin of control non-treated turbots are shown in A, C, E, and G. E and F show dorsal skin of control (E) and injected/electroporated animals with capped sense eGFP-mRNA (F) animals under fluorescent incident light. G and H display animals shown in E and F under brilliant incident light. See Figure 7 for more details. Dorsal views, anterior to the right. Scale bars: 0.2 cm. doi:10.1371/journal.pone.0048526.g008

conserved in *asip1* orthologues and the flatfish sequences are not an exception. This cysteine pattern resembles that of the conotoxins and agatoxins, suggesting that agouti-like proteins adopt an inhibitory cysteine knot (ICK) fold [21]. Structural studies have demonstrated that five disulfide bridges between cysteine residues, C87–C102, C94–C108, C101–119, C105–129 and C110–C117 stabilize the human *agrp* molecule [21–24]. Interestingly, *Asip2* proteins lack the 5<sup>th</sup> and 10<sup>th</sup> cysteine residues which form the last disulfide bridge of agouti-like molecules. How these structural differences affect the dimensional conformation and receptor binding is unknown but we do know that the C-terminal loop in *asip1* is required for MC1R binding [25].

Studies on the evolutionary history of the agouti family of peptides are controversial. Tetrapod species have two different melanocortin antagonists, i.e. *asip* and *agrp*, but teleost fish have four endogenous antagonists, *asip1*, *asip2*, *agrp1* and *agrp2*. Studies have suggested that *asip2* and *agrp2* are ohnologue genes of *asip1* and *agrp1*, respectively, which are generated during teleost-specific genome duplication (TSGD) [26]. Recent synteny data support the view that the *agrp2* chromosomal region does not share a synteny relationship with the fish *agrp1* or with the tetrapod *agrp*. The *agrp2* and *asip2* regions show conserved synteny with a region of human chromosome 8 that, in turns, shares paralogies with the *asip* region on chromosome 20. The model proposes that the *agrp/asip* precursor was duplicated twice during the two rounds of vertebrate genome duplication (R1, R2). *Agrp2* and *asip2* were missed in the tetrapod genome but *asip2* was retained in the teleost genome. After TSGD, the additional copy of *agrp* gene was missed again from the teleost genome but both copies of the *asip2* gene



**Figure 9. Normalized gene expression levels of tyrosinase-related protein 1 (Typr1) in turbot skin.** (A) Analysis of differential dorsal-ventral Typr1 gene expression in turbot. (B) Effect of the *in vivo* injection of capped mRNA on Typr1 expression. Shown are log<sub>10</sub> transformed  $\Delta$ Ct values of Typr1 relative to  $\beta$ -actin. Data are the mean  $\pm$  SD from four samples after triplicate PCR analysis. Comparisons of numerical data were made by paired Student t-tests. Asterisk indicate significant differences ( $P < 0.05$ ) between dorsal and ventral Typr1 expression (A) and between control non-treated (CTRL) and eGFP capped mRNA injected skin Typr1 expression (B). doi:10.1371/journal.pone.0048526.g009

were retained. These copies are named *asip2* and *agr2* [26] but the new model proposes naming them *asip2a* and *asip2b*, respectively [27]. Schiöth and collaborators rebuilt the phylogeny by introducing a sequence from elephant shark [28]. If *agr2* is used to root the tree, the results support Braasch and Postlethwait's hypothesis but if the tree is rooted by the ancient sequence, the *agr2* and *asip2* clusters group with the *agr2* cluster, supporting the previous nomenclature [26]. Flatfish sequences were grouped with *asip1* sequences, suggesting their orthology. The incorporation of flatfish *asip1* sequences does not modify the phylogeny reported by Braasch and Postlethwait's [27].

Structural and/or functional data could discern between both hypotheses. Human *agr2* is processed after the motif Arg<sup>79</sup>-Glu<sup>80</sup>-Pro<sup>81</sup>-Arg<sup>82</sup> to release the active peptide (*agr2* 83–132 [29]). Both arginine (R) residues are fully conserved in all *agr2* sequences but not in *asip1*, *asip2* and *agr2* sequences, which suggests that, unlike *agr1* but similar to *asip*-like peptides, *agr2* peptides are not processed. The N-terminal region of *asip* peptides is rich in basic residues, particularly lysine (K). Similar to *asip* peptides, *agr2* peptides also exhibit a high number of basic residues before the cysteine domain. *Asip1* sequences also present a proline domain immediately prior to the C-terminal cysteine domain. This domain is not present in *agr1* peptides and is not clearly defined in *agr2* or *asip2* peptides. Also noteworthy is the fact that all *asip2* and *agr2* sequences exhibit 5 residues between the second and third cysteine residue of the C-terminal domain, whereas *asip1* and *agr1* peptides exhibit 6 residues. Therefore, structural data seem to support Braasch and Postlethwait's hypothesis defending the *asip2/agr2* orthology and, by extension, the new nomenclature, *asip2a/asip2b*, respectively. However, one intriguing item of structural evidence disproves this reflexion. The alignment of peptide sequences show that all *agr2* sequences exhibit a short tail after the last cysteine residue and *agr2* sequences are not an exception. This short tail does not seem to confer any binding property to the molecule since the last twelve amino acids of the human *agr2* peptide can be eliminated without affecting MC3R or MC4R binding [21]. Therefore, the function of this conserved short C-terminal extension in all *agr1* and *agr2* sequences remains unknown. It is known that agouti peptides can interact with other molecules other than melanocortin receptors [20] suggesting a still undiscovered intermolecular interaction mediated by segments outside the cysteine-rich domain.

From the functional point of view, in mammalian species, *agr2* is expressed mainly in the hypothalamus where it regulates the energy balance. *Asip* is produced by dermal papillae cells in which it governs the switch between production of eumelanin and pheomelanin [30]. In fish all three *agr2*, i.e. *agr1*, *agr2* and *asip1* are expressed in the brain and skin [7,26,31]. In the brain, *agr1* is exclusively expressed within the lateral tuberal nucleus, the fish homologue of the arcuate nucleus [31], whereas *agr2* is only expressed in the pineal complex of zebrafish [32]. Our results demonstrate that, similar to *agr2*, *asip1* is expressed in the turbot pineal complex. Coincident expression in the pineal complex can be expected if both genes derive from a common ancestral pineal-expressed gene, once again supporting a relationship between *agr2* and *asip* genes. As in other fish species, *asip1* was also expressed in the brain of flatfish. Specific *asip1*-expressing brain areas or projections, as well as the *asip* function in the brain, are unknown. We have previously shown that both MC4R [33,34] and MC1R [9] are expressed in the fish brain. In addition, goldfish *asip1* can bind both receptors [7], as *agr1* does [9,34] thus significantly increasing the complexity of the central melanocortin signaling in fish.

In our previous studies, we proposed that *asip* could be the uncharacterized MIF in fish. We suggested that the ventral expression of *asip* induces an inhibitory effect on melanophore differentiation and/or proliferation but stimulates iridophore differentiation and/or proliferation via MC1R antagonism. Accordingly, the absence of *asip* expression in the dorsal skin allows melanoblasts to differentiate and/or proliferate, leading to dark coloration in the dorsal region [7]. In both sole and turbot, the expression of *asip1* in ventral skin was higher than in melanic dorsal skin. These findings are not so striking as those reported in goldfish, in which *asip1* expression in the dorsal skin was essentially absent. A possible reason for this discrepancy in dorsal/ventral relative expression levels could be the pigmental structure of the ocular side of flatfish. This side is normally patterned with dark patches and spots, as well as white and colored spots with a high number of iridophores, of all which are morphological entities (reviewed in [15]). Therefore, it is possible that *asip1* expression might also contribute to the dorsal heterogeneous pigment pattern in flatfish. In contrast, dorsal skin in goldfish is un-patched and much more homogeneous in dark pigmentation. We are currently testing the possibility that *asip1* might contribute to the pigment patterning outside the dorsal and ventral regions by comparing *asip1* expression in the lateral white and dark stripes of zebrafish.

We further demonstrated that transient *asip1* overexpression, following the injection of homologous capped mRNA, can induce skin paling in the dorsal melanic side of flatfish, *in vivo*. This result supports our hypothesis defending the involvement of *asip1* protein in the patterning of dorsal-ventral pigmentation in fish. Our experimental design cannot elucidate whether the observed paling in turbot and sole skin was induced by a transient melanosome reorganization, similar to that observed during short-term background adaptation or physiological color change, by a decrease in melanin synthesis or by a reduction in melanophore number, similar to that observed after long-term background adaptation or morphological color changes [15,35]. All three scenarios are possible and could concur concomitantly. Experiments using recombinant goldfish *asip1* demonstrated that this protein can inhibit melanin dispersion stimulated by melanocyte-stimulating hormone (MSH) in the melanophores of medaka scales in a reversible way [7]. However, our results show that treatment-induced effects persist even after 4 days post-administration, suggesting the presence of morphological color changes. *Asip 1* overexpression induced a significant reduction in the *Tyrp1* expression to reach similar levels to those exhibited in the ventral skin. *Tyrp1* promotes final steps of eumelanin synthesis supporting that *asip 1* overexpression inhibits melanogenesis and/or melanophore differentiation. Accordingly, *asip1* has been shown to inhibit MSH-induced *mitf* expression, melanogenic gene promoters including tyrosinase, *Tyrp1* and *Tyrp2*, melanoblast differentiation into melanocytes and induce melanocyte de-differentiation in mammals [36–38].

Capped mRNA administration experiments suggest a role for *asip1* in the adult pigment pattern of flatfish. Similar to *Danio* species, flatfish melanophores can be divided into larval, early or embryonic and adult or metamorphic-type melanophores [39]. During larval stages, pigment cell latent precursors are symmetrically located mainly along the dorsal and ventral margins of the flank and migrate continuously from these regions to the lateral sides. After late metamorphic stages, these precursors differentiate into adult-type chromatophore on the lateral asymmetrical sides. Pigment asymmetry in flatfish seems to depend upon an asymmetric organizational environment that may regulate survival, proliferation, distribution and differentiation of latent precursors into adult-type pigment cells since an asymmetric body plan,

including eye migration, precedes adult pigment pattern formation [13,40]. Recent studies in zebrafish have demonstrated that proliferative pigment cell precursors are associated with the peripheral nerve and ganglia and migrate to the hypodermis during pigment pattern metamorphosis, when they differentiate into melanophores or iridophores [41]. These precursors seem to be bipotential and thus capable of differentiating into melanophores or iridophores, depending on the interplay between forkhead transcription factor, *foxd3*, and microphthalmia subtype a, *mitfa*. Nacre zebrafish, a mutant for *mitfa*, exhibit an increased number of ectopic iridophores [42], while the loss of *foxd3*, a *mitfa* repressor, resulted in fewer iridophores [43,44]. We hypothesized that, after migration, these bipotent precursors reach different developmental environments patterned by *asip1* expression, which finally governs the differentiation into melanophores or iridophores. There is no information about whether *asip1* affects *foxd3* activity but we anticipate that *asip1* could stimulate the expression of this *mitf* repressor. This model would not be only true for the tandem melanophore/iridophore since xantic goldfish lack dermic melanophore but display striking differences in the dorsal-ventral expression of *asip1* mRNA [7]. Therefore, a more plausible scenario is that *asip1* could induce iridophore differentiation from bipotential melanophore/iridophore precursors which subsequently inhibit the differentiation of any type of chromatophore.

Pigment anomalies are common in reared flatfish including albinism of the ocular side and hypermelanism of the blind side. We demonstrated that *asip* expression in the albino regions of the ocular side in pseudoalbino turbot is similar to that observed in the ventral region but significantly higher than that seen in the dark areas of the ocular side. This suggests that ectopic expression of *asip 1* could be involved in flatfish pseudoalbinism. It has been reported that albino flatfish, including turbot, are able to feed more efficiently and grow faster than controls (reviewed in [12]). This phenotype is recurrent to that observed in agouti mice carrying the unusual allele *A<sup>y</sup>*. The associated phenotype is characterized by yellow fur and the ubiquitous expression of agouti gene, resulting in hyperphagia, hyperinsulinemia, increased linear growth, increased propensity for developing tumors, premature infertility and maturity-onset obesity [45,46]. This metabolic syndrome is mediated by antagonizing  $\alpha$ -MSH signaling at central MC4R that arbitrates the negative effects of melanocortin peptides on the energy balance [47]. We have previously demonstrated that central melanocortin system is involved in the regulation of the energy balance in fish via MC4R [31,33,48] and that *asip1* can antagonize MSH effects on the latter receptors [7]. However, we cannot discriminate whether the increased expression levels of *asip* in the anomalous dorsal pigmental regions are the consequence of the expression of a normal developmental pathway in an incorrect position as result of a patterning error. It means, albino areas expressing higher *asip1* mRNA levels within the melanic ocular side are indeed a portion of wrong-patterned ventral skin in dorsal position or, in other words, dorsal skin following the ventral skin developmental pathway. *Asip1* expression levels in ventral skin and albino areas of the dorsal skin of turbot were similar. In addition, preliminary experiments further demonstrated that injection of capped *Asip1* mRNA into the hypermelanic regions of the ventral skin of sole inhibited melanogenesis (unpublished data; Guillot R, Ceinos, R, Rotllant, J and Cerdá-Reverter, JM).

In summary, we have characterized *asip1* mRNAs in both turbot and sole and used deduced peptide alignments to study the evolutionary history of the agouti-family of peptides. Structural and functional data suggest that *agrp2* is more closely related to *asip* than *agrp1* sequences. Data suggest that fish *asip* is involved in

the dorsal-ventral pigment patterning in adult fish, where it induces the regulatory asymmetry involved in precursor differentiation into mature chromatophores. Adult dorsal pseudoalbinism seems to be the consequence of the expression of normal developmental pathways in an erroneous position, resulting in unbalanced *asip* production levels. These, in turn, generate a ventral-like differentiation environment in dorsal regions.

## Materials and Methods

### Experimental animals

Turbot (*Scophthalmus maximus*) and sole (*Solea senegalensis*) larvae reared under standard commercial conditions were provided by the Instituto Español de Oceanografía (IEO), Vigo, Spain. Control and pseudoalbino adult fish were also obtained from stocks of the IEO. Animals were anesthetized in 0.02% tricaine methanesulfonate (MS-222) before any manipulation and sacrificed by rapid decapitation when required. All experiments were carried out in accordance with the principles published in the European animal directive (86/609/EEC) for the protection of experimental animals and approved by the Consejo Superior de Investigaciones Científicas (CSIC) ethics committee (project numbers AGL2010-22247-C03-01 to JMC-R and ALG2011-23581 to JR). Unless otherwise indicated, all reagents were purchased from Sigma (St Louis MO, USA).

### Molecular cloning of flatfish *asip1* gene

Total RNA from ventral skin of sole and turbot was extracted with Tri-reagent and treated with RQ1-DNase I (Promega). Subsequently, mRNA was isolated with polyATrack mRNA isolation system III (Promega) following the manufacturer's manual. Synthesis of cDNA was primed with random hexameres (Invitrogen) and was used as template for PCR reactions with degenerate primers. These primers were designed based on *asip1* sequences from different species. The primers used to amplify sole *asip1* were Multi\_Agouti\_Fw 5' CCKCCTCCBSCBAACTGY 3' and Multi\_Agouti\_Rv 5' CCCATKCGRCARTARCASAC 3'. These primers did not work with turbot cDNA and new primers called Flatfish\_Agouti were designed based on cloned fish *asip1* sequences. The latter primers had the sequence: Flatfish\_Agouti\_Fw 5' CTCCTGCYAACTGCMYTYCCTT 3' and Flatfish\_Agouti\_Rv: 5' GGGTTGCCCATTCGRCAGWAACA 3'. Fragments of 135 bp and 159 bp for sole and turbot *asip*, respectively, were cloned into pGEM-T easy vector (Promega), sequenced and found to show a high similarity with fish *asip1* sequences. To resolve 5' and 3' ends of sole and turbot cDNAs, 5' and 3' rapid amplification of cDNA ends (RACE) were performed using the Smart-RACE PCR cDNA amplification system (Clontech) following the manufacturer's manual and specific primers designed according to the previously obtained sequences. Purified fragments were treated as above. To corroborate that 5' and 3' ends correspond to the same transcript, full *asip1* sequences were amplified using specific primers targeting the cDNA extremes. Full cDNAs were cloned and sequenced as before. The nucleotide sequences of turbot and sole *asip1* have been deposited with EMBL Nucleotide Sequence Database under accession numbers HE598752 and HE598753, respectively.

### Tissue and larvae RNA isolation and RT-PCR

Total RNA was purified as before. Superscript II reverse transcriptase (Invitrogen) was used for cDNA synthesis by priming total RNA from brain, hypophysis, pineal, eye, gill, spleen, anterior and posterior kidney, heart, liver, muscle, dorsal skin, ventral skin, intestine, gonads and fat with random hexameres



(Invitrogen). PCR amplification was carried out with the primers specific primers amplifying the full coding region. As internal control of the reverse transcription step, PCR for  $\beta$ -actin (turbot) or 18S (sole) cDNA amplification was carried out. The following primer sequences were used; for sole, 18S forward primer had the sequence 5' GAATTGACGGAAGGGCACCACCAG 3' and 18S-reverse primer had the sequence 5' ACTAAGAACGGC-CATGCACCACCAC 3'. Turbot  $\beta$ -actin primers were  $\beta$ -actin-forward 5' TGAACCCCAAAGCCAACAGG 3' and  $\beta$ -actin-reverse 5' CAGAGGCATACAGGGACAGCAC 3'. Similarly, RNA from embryos collected at 2, 3 and 6 hours post fertilization (hpf) and 4, 7, 11, 14, 16, 21, 24, 28, 29, 32, 36, 39 and 45 days post hatching (dph) for turbot and 2, 3, 5, 8, 10, 19, 24 and 29 dph for sole were extracted and cDNA was primed as before.

### Skin RNA isolation and absolute-quantitative real time PCR (qRT-PCR)

Dorsal and ventral skin samples from control and pseudoalbino turbot and sole were collected and total RNA was extracted as before. cDNA was synthesized from total RNA using superscript III (Invitrogen) according to manufacturer's recommendations.

Absolute quantification was used as a method to analyse the skin spatially specific expression of *asip1* genes. Sole and turbot *asip1* cDNAs cloned into pGEM-T easy were used as standards. 10-fold serial dilutions of *asip1* into pGEM-T, ranging from  $1 \times 10^5$  to  $1 \times 10^{10}$  copies/ $\mu$ L, were used to construct standard curves for both *asip1* genes. The concentration of the dsDNA standards was measured using a fluorometer and the corresponding copy number was calculated following the Whetlan method [49]. Real time PCR quantification (qRT-PCR) was performed in 96-well optical plates in triplicate on an Applied Biosystems 7500 analyzer with Maxima SYBR Green qPCR master mix (Fermentas, Life Science). The total reaction volume was 25  $\mu$ L with 12.5  $\mu$ L of SYBR green, 0.5  $\mu$ L of each primer, 9.5  $\mu$ L of nuclease free water and 1  $\mu$ L of cDNA template. After denaturation at 95°C for 10 min, 40 cycles of amplification were carried out with denaturation at 95°C for 15 s, annealing and elongation at 60°C for 1 min, followed by the melting curve analysis. The following primer sequences were used for qRT-PCR: for turbot *asip1* (5' primer/3' primer) 5' CTGCGAACTGCATTCCCTTGT 3' and 5' TCAGCAGCGAGGGTTGCC 3', for sole *asip1* (5' primer/3' primer) 5' GCACTCCCTTGTGGGGAAG 3' and 5' TCAGCAGTGTGGGTTGCC 3'. A standard curve was drawn by plotting the natural logarithms of the threshold cycle ( $C_T$ ) against the number of molecules, respectively.  $C_T$  was calculated under default settings for the real-time sequence detection software (Applied Biosystems). The equation drawn from the graph was used to calculate the precise number of specific *Asip1* cDNA molecules present per microgram of total primed cDNA, tested in the same reaction plate as the standard.

Turbot Tyrp1 gene expression was quantified by relative qRT-PCR. The level of  $\beta$ -actin mRNA was used as an internal reference for sample normalization. Two pairs of primers were used for amplification: Tyrp1 forward (5' CCAGTTCAG-CAATGTATCC 3') and Tyrp1 reverse (5' GCCATTCGGCTT-CATAAGAG 3'). Data were analyzed using the comparative cycle threshold method (CT method). Characteristics of the real time PCR (qRT-PCR) system was the same as used above.

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### Capped mRNA synthesis, injection and electroporation

To generate capped mRNA, DNA fragments containing the Kozak sequence followed by entire ORF of turbot and sole *asip1* were generated by PCR. These DNA fragments were subcloned into the pCS2+ vector to generate the *asip1* overexpression plasmid DNAs (pCS2+*asip1*-Turbot and pCS2+*asip1*-Sole). The purified plasmids were dissolved in DNase free water and stored at  $-20^\circ\text{C}$  until use. The pCS2+*asip1* plasmids were linearized by restriction with NotI and used for capped sense or antisense *asip1* mRNA synthesis using mMessage Machine kit (Ambion). Five and seven month-old turbot and sole, respectively were anesthetized and *asip1* capped mRNA was injected into the dorsal skin area using a 1 ml Omnifix®-F syringes. Approximately 10  $\mu$ g of capped-mRNA was injected per  $\text{cm}^2$  of dorsal skin. Immediately following injection, both dorsal and ventral halves were electroporated using a ECM 830 BTX electroporator (Harvard apparatus, Inc.). Electric pulses were applied by a pair of electrode disks (7 mm diameter) rigged on the tips of tweezers (pinsettes-Type electrode 524, BTX instrument). The following parameters were used: 5-msec pulses of 10 V with a 200 msec pause between pulses. Fish were then rapidly returned to their tanks for skin coloration analysis at 4 days post-electroporation (dpe). The mRNA for green fluorescent protein (eGFP), which was synthesized from pCS2+eGFP, was injected-electroporated into the skin as control.

At 4 dpe, fluorescein uptake was monitored. Five fish were tested in all experiments.

### Data analysis and statistics

Specimens were observed and photographed under a Leica M165FC fluorescence stereoscope (Leica Microsystems, Germany) equipped Leica DFC 500 digital camera. Adobe Photoshop<sup>TM</sup> software was used to adjust contrast levels in all images.

Flatfish sequences were compiled with GenRunner free software and compared with known *asip1* and agouti-related protein (*agr*) sequences from the National Center for Biotechnology Information (NCBI) and ENSEMBL databases. Sequence alignments were performed using public domain ClustalX 2.1 and edited with GeneDoc software. Phylogenetic tree was derived using ClustalX and SeaView that uses the Neighbor-Joining method on a matrix of distances and maximum likelihood, respectively. The cleavage site for removal of the hydrophobic signal peptide was predicted using SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>). Differences in gene expression were assayed by Student t-test and statistical significance was considered at  $p < 0.05$ . Results are given as mean  $\pm$  SEM.

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### Author Contributions

Conceived and designed the experiments: JMC-R JR. Performed the experiments: RG RMC RC JR JMC-R. Analyzed the data: RG RMC JR JMC-R. Contributed reagents/materials/analysis tools: RC. Wrote the paper: JMC-R JR.

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