Phylogenetic and Expression Analysis of Fos Transcription Factors in Zebrafish

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Abstract: Members of the FOS protein family regulate gene expression responses to a multitude of extracellular signals and are dysregulated in several pathological states. Whilst mouse genetic models have provided key insights into the tissue-specific functions of these proteins in vivo, little is known about their roles during early vertebrate embryonic development. This study examined the potential of using zebrafish as a model for such studies and, more broadly, for investigating the mechanisms regulating the functions of Fos proteins in vivo. Through phylogenetic and sequence analysis, we identified six zebrafish FOS orthologues, fosaa, fosab, fosb, fosl1a, fosl1b, and fosl2, which show high conservation in key regulatory domains and post-translational modification sites compared to their equivalent human proteins. During embryogenesis, zebrafish fos genes exhibit both overlapping and distinct spatiotemporal patterns of expression in specific cell types and tissues. Most fos genes are also expressed in a variety of adult zebrafish tissues. As in humans, we also found that expression of zebrafish FOS orthologs is induced by oncogenic BRAF-ERK signalling in zebrafish melanomas. These findings suggest that zebrafish represent an alternate model to mice for investigating the regulation and functions of Fos proteins in vertebrate embryonic and adult tissues, and cancer.

Keywords: Fos; activator protein-1; transcription factor; embryogenesis; zebrafish; development; ortholog

1. Introduction

The mammalian FOS proteins, c-FOS, FOSB, FOSL1, and FOSL2, belong to the activator protein-1 (AP-1) family of transcription factors that contain evolutionarily conserved basic leucine zipper (bZIP) domains [1]. FOS proteins regulate transcription by forming heterodimers with other AP-1 proteins, particularly members of the JUN, ATF, and MAF families [2–5]. AP-1 complexes regulate the expression of genes important for cell proliferation, differentiation, and apoptosis in response to a plethora of extracellular signals, including growth factors, cytokines, and hormones [3,5–11]. In addition to possessing a conserved basic leucine zipper (bZIP) domain mediating DNA binding and dimerisation, FOS isoforms have different N-terminal and C-terminal regions, providing a basis for their differential regulation and functional activities [1]. c-FOS and FOSB have N- and C-terminal transactivation motifs termed the N-TA, C-TM, and TBD that are absent in FOSL1 and FOSL2. Additional C-terminal motifs HOB1 and HOB2, which stabilise and facilitate assembly of the pre-initiation complex, are present solely in c-FOS [1,12,13]. In contrast, FOSB has a unique proline-rich functional module, PRM also within the C-terminal region [14]. Another critical regulatory region present in all FOS proteins is the C-terminal destabilising element (C-DEST). Phosphorylation of this domain is induced by ERK MAPK signalling and leads to protein stabilisation [15–17].
FOS family members are implicated with the pathogenesis of various diseases [18]. Aberrant expression and activation of FOS proteins is driven by dysregulation of key cancer-associated signalling pathways, most notably the ERK MAPK pathway [1]. The pro-tumorigenic actions of FOS family members have been examined in a variety of in vitro and in vivo models, which have identified roles for FOS proteins in oncogenic transformation [19] and cancer progression [20–23]. Early studies showed that rodent fibroblasts undergo transformation upon expression of c-FOS but not FOSL1 and FOSL2, which is attributed to differences in the N- and C-terminal regions of these proteins. Notably, c-FOS and FOSB harbour transactivation domains that are absent in FOSL1 and FOSL2 [1,12]. Consequently, AP-1 dimers containing c-FOS and FOSB show stronger transcription activation potential than those containing FOSL1 and FOSL2 [24,25].

Genetic knockout and transgenic mouse models have shown that individual Fos proteins have specific in vivo functions [26,27]. c-Fos deficiency perturbs normal development of bone, cartilage, and the haematopoietic system [28], whereas its transgenic expression in the bone induces the formation of osteosarcoma [29]. Fosb deficiency impairs brain development, leading to nurturing defects in the adult female mouse, while transgenic overexpression of Fosb2 in the thymus disrupts T-cell differentiation [30]. Deficiency of either Fosl1 and Fosl2 in mice caused embryonic lethality, while their overexpression resulted in abnormalities in bone and ocular development [3,27]. Fosl1 deletion also led to placental defects associated with the aberrant differentiation of trophoblasts [31,32].

Despite providing important insights into the in vivo functions of specific FOS proteins in normal and disease states, the use of mouse models to investigate the mechanisms regulating the activities of individual FOS isoforms in vivo is challenging. In addition, mouse models have provided limited insight into the functions of FOS isoforms during early vertebrate development. The zebrafish has emerged as a robust in vivo model for early developmental studies, as its embryos are transparent and develop externally, facilitating easy observation of key processes [33]. The zebrafish genome has been sequenced and about 70% of human genes have at least one ortholog in zebrafish. In addition, their rapid development, high fecundity, low maintenance costs, and amenability to genetic manipulation, including tissue-specific targeted genome editing and transgenesis, makes them well suited for investigating molecular mechanisms in vivo. Through phylogenetic and sequence analysis, we identified six zebrafish FOS homologues, fosaa, fosab, fosb, fosl1a, fosl1b, and fosl2, which encoded proteins showing high sequence conservation in key regulatory domains and post-translational modification sites compared to the equivalent human proteins. Spatiotemporal expression analysis revealed both overlapping and distinct patterns of fos gene in specific cell types and tissues during early embryonic development. Our data thus suggest that the zebrafish represents a valuable vertebrate model for investigating Fos protein regulation in vivo and defining their functions during embryogenesis.

2. Results
2.1. Identification of Zebrafish Fos Proteins

The human FOS gene family consists of FOS, FOSB, FOSL1, and FOSL2 [1]. Analysis of genomic databases identified six zebrafish FOS genes, which synteny analysis indicated were single fosb and fosl2 orthologues, but duplicated fosaa/fosab and fosl1a/fosl1b paralogues (Figure 1). Phylogenetic analysis of the encoded proteins showed they formed a distinct clade from another bZIP transcription factor (JDP2) outgroup (Figure 2) [34,35]. Within the FOS clades, there was a clear sub-clade for c-FOS, FOSB, and FOSL2 proteins, with the c-FOS parologue Fosaa being more divergent than Fosab. In contrast, while Fosl1a grouped with its mammalian counterparts, Fosl1b sat as an outlier to all subclades. In addition, clades of c-FOS and FOSB as well as FOSL1 and FOSL2 proteins were grouped under two separate clades, indicating that these pairs of proteins share a common ancestral origin.
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Figure 1. Synteny analysis of FOS genes. Human and zebrafish FOS (A), FOSB (B), FOSL1 (C), and FOSL2 (D) gene loci, indicating adjacent genes in their respective orientations. Zebrafish genes that share conserved synteny between human neighbouring genes are shown in black and non-syntenic genes in grey. The red line indicates the reference gene. Ch, chromosome.

2.2. Major Functional Domains and Post-Translationally Modified Residues in Human FOS Proteins Are Conserved in Their Zebrafish Counterparts

Sequence alignment of full-length human, mouse, and zebrafish FOS proteins revealed that, in addition to the bZIP domain, other major N- and C-terminal functional domains of human FOS proteins [1] were present in their corresponding zebrafish proteins (Figures 3 and S1). Protein BLAST analysis showed that human c-FOS was 48% identical to Fosaa and 55% to Fosab. For c-FOS, regions of significant homology were present throughout the protein, particularly in the bZIP motif, and to a lesser extent in the C- and N-terminal transactivation domains, N-TA, HOB1, HOB2, C-TM, and TBD (Figures 3A and S1A). Notably, previously characterised regulatory phosphorylation sites in mammalian FOS proteins are highly conserved in zebrafish Fosab but not in Fosaa, where only the C-terminal phosphorylation sites were conserved. The region of highest conservation in the c-FOS paralogs was the bZIP domain, while the C-DEST region of Fosab was more conserved to c-FOS than that of Fosaa (Figure S1A).

The overall sequence identity of zebrafish FosB and human FOSB was 65%, with the bZIP domain being 93% identical. Amongst FOS proteins, FosB showed the highest overall sequence and bZIP domain conservation. Zebrafish FosB showed high conservation of the bZIP motif with mammalian FOSB and moderate conservation of the C- and N-terminal transactivation domains (N-TA, PRM, C-TM, and TBM) (Figures 3B and S1B). Key regulatory phosphorylation sites in human FOSB were also conserved in its zebrafish ortholog. However, the C-DEST region in zebrafish FosB is least conserved amongst FOS proteins.

In contrast to c-FOS and FOSB, human FOSL1 and FOSL2 lack potent transactivation domains [1,12]. The overall sequence identities of Fosl1a and Fosl1b compared to human FOSL1 are 49% and 40%, respectively, with their bZIP domain identities being 78% and 59%, respectively (Figure 3C). Finally, zebrafish Fosl2 showed 65% overall identity and 85% bZIP domain identity compared to human FOSL2 (Figure 3D). Of the two zebrafish FOSL1 paralogs, Fosl1b showed weaker sequence conservation compared to Fosl1a, with Fosl1b lacking the C-DEST domain and C-terminal phosphorylation sites (Figures 3C and S1C). Amongst FOS proteins, the C-DEST domain of zebrafish Fosl2 showed highest identity to the human protein (Figure S1D). Collectively, these findings suggest that zebrafish and human FOS proteins share key functional and regulatory features.
Figure 2. Phylogeny of mammalian and zebrafish FOS proteins. Phylogenetic analysis of human, mouse, and zebrafish FOS proteins were performed using the neighbour-joining algorithm and visualised with TreeView, with bootstrap values represented as a percentage of 1000 replicates and the relative evolutionary distance represented at the bottom left corner. JDP2 proteins were used as a closely related outgroup. Clades of related proteins are indicated within pink coloured boxes.
2.2. Major Functional Domains and Post-Translationally Regulated Sites

Figure 3. Schematic representation of human and zebrafish FOS proteins. Depicted are human and zebrafish sequences related to c-FOS (A), FOSB (B), FOSL1 (C), and FOSL2 (D). Functional regions indicated are the basic leucine zipper (bZIP) including the basic region for DNA interaction (orange box) and leucine zipper for dimerization (red box), N-terminal transactivation domain (N-TA), homology box one and two (HOB1, HOB2), proline-rich motif (PRM), C-terminal transactivation motif (C-TM), transactivation domain (TBD), and destabiliser region (C-DEST). Previously characterised phosphorylation (P) sites curated in the Phosphosite database (phosphosite.org (accessed on 24 July 2022)) are indicated. The percentage identity within the bZIP domain and overall protein are indicated by blue dotted or solid lines and text, with the protein length indicated on the left in black text.

2.3. Zebrafish Fos Genes Show Distinct Expression Patterns during Embryonic Development

Wholemount in situ hybridisation (WISH) analysis was used to establish the pattern of expression of each zebrafish Fos isoform during early embryonic development. Maternal fossa transcripts were detected in 1-cell embryos (0 hpf), and by 12 hpf (6-somite stage), zygotic expression was observed in the brain (forebrain, midbrain, and hindbrain), pre-somitic mesoderm, somites, and tailbud (Figure 4A), and at 24 hpf, in the brain (forebrain, midbrain, and hindbrain), eye, migratory neural crest cells, melanoblasts, and notochord (Figure 5A,A'). From 48 hpf to 4 dpf, no specific fossa expression was observed compared to the sense controls (Figures 5B–C' and S2). These results suggest a role for fossa during early organ development (brain, eye) and formation of diverse cell lineages including melanocytes, smooth muscle, craniofacial bone and cartilage, cranial neurons, and glia. The pattern of fossb expression was similar to fossa in early embryos (Figure 4B) but persisted until 4 dpf in multiple regions of the embryo (Figure 5D–F'). These included the brain, eyes, migrating neural crest cells, melanoblasts, epidermis, liver, and pancreas at 24 hpf (Figures 5D,D' and S4A–C), and the brain, heart, gut, migratory neural crest cells, lateral line neuromasts, nephron (proximal straight tubule of nephron and distal early of nephron), cloaca, somites, and tailbud at 48 hpf (Figures 5E,E' and S4D–F), while at 4 dpf, it was expressed in the mouth, otic vesicle, peridermis, epidermis, jaws, pharyngeal arch, and caudal fin (Figures 5F,F' and S4G–K). These observations suggest a role for fossb during the development of multiple organs (eye, brain, heart, liver, and pancreas) and cell lineages (e.g., melanocytes and epidermal cells).

Zebrafish fosb was expressed from 8 hpf (75% epiboly stage) (Figure 4C), with specific expression observed in the hatching gland and eye at 24 hpf (Figure 5G,G'). At 48 hpf, fosb expression was present in the brain (midbrain, pallium), heart, hatching gland, and tailbud (Figures 5H,H' and S4L–N), while at 4 dpf, expression was also observed in the otic vesicle, pharyngeal arches, epidermis, and caudal fin, including the fin rays (Figures 5I,I' and S4O,P).
Fosl1b lacking the C-DEST domain and C-terminal phosphorylation sites (Figures 3C and S1D). Amongst FOS proteins, the C-DEST domain of zebrafish Fosl2 showed highest identity to the human protein (Figure S1D). Collectively, these findings suggest that zebrafish Fosl1b plays specific roles in the development of the brain, epidermis, and hatching gland.

Zebrafish fosl2 showed the highest early embryonic expression being detected at 12 hpf in the eyes, somites, presomitic mesoderm, mid and hindbrain, and tailbud (Figure 4F). At 24 hpf, fosl2 was expressed in the brain (forebrain, midbrain, hindbrain, and midbrain–hindbrain barrier), eye, otic vesicle, somites, and tailbud (Figures 5J,J’ and S4U,V). Additional staining was observed in the heart, hatching gland, and posterior notochord at 48 hpf (Figures 5Q,Q’ and S4W–Y), and in the epidermis, jaws, pharyngeal arch, cloaca, and fin rays at 4 dpf (Figures 5R,R’ and S4Z,Z’).

Figure 4. Spatiotemporal expression of fos genes during early zebrafish embryonic development. Lateral, dorsal, and frontal views of 0 hpf, 8 hpf, and 12 hpf zebrafish embryos analysed by WISH showing expression for fosaa (A), fosab (B), fosb (C), fosl1a (D), fosl1b (E), and fosl2 (F). 1-c, one cell; e, eye; epb, epiblast; f, forebrain; hyb, hypoblast; mh, mid and hindbrain; psm, presomitic mesoderm; s, somite; tb, tailbud. Black arrows within each panel point to the specific expression indicated by the abbreviation.

2.4. Expression of FOS Genes in Adult Zebrafish Tissues

To determine the levels of fosaa, fosab, fosb, fosl1a, fosl1b, and fosl2 transcripts in adult male and female zebrafish tissues, we performed qRT-PCR analysis (Figure 6) using optimised primers (Figure S3). In male zebrafish, all fos genes showed weak expression in the heart and liver (Figure 6A–F). All fos genes showed high expression in the brain, particularly fosb, fosl1b, and fosl2. All fos genes were expressed in the spleen, where fosb and
foslb levels were highest. In the intestine, fosab, fosb, and foslb levels were high, whereas fosaa, fosla, and fos2 showed modest expression (Figure 6). Low to moderate level of fos gene transcripts were observed for other tissues examined (eyes, kidney, skin, gills, and testis) (Figure 6).

Overall, the expression of fosaa, fosab, fosb, fosla, foslb, and fos2 was similar in adult zebrafish female tissues, but with modest variations compared to males (Figure 6A–F). All fos genes were weakly expressed in the liver of females, whereas fosla and foslb showed highest expression in the brain (Figure 6D,E). fosb and foslb showed moderate expression in the spleen, skin, and gills (Figure 6C,E). Amongst fos genes, foslb shows highest expression in the zebrafish adult female intestine (Figure 5E), followed by fosla and fosla, which shows moderate expression (Figure 6D,F). Moderate to low fos expression levels were observed in all other female tissues, including eyes, kidney, gills, spleen, and oocytes (Figure 6A–F).

Figure 5. Spatiotemporal expression of fos genes in later stages of zebrafish embryonic development. Lateral and dorsal views of zebrafish embryos analysed by WISH showing expression for fosaa at 24 hpf (A'), 48 hpf (B'), and 4 dpf (C'); fosab at 24 hpf (D'), 48 hpf (E'), and 4 dpf (F,F'); fosb at 24 hpf (G,G'), 48 hpf (H,H'), and 4 dpf (I,I'); fosla at 24 hpf (J,J'), 48 hpf (K,K'), and 4 dpf (L,L'); foslb at 24 hpf (M,M'), 48 hpf (N,N'), and 4 dpf (O,O') and fosla at 24 hpf (P,P'), 48 hpf (Q,Q'), and 4 dpf (R,R'). cf, caudal fin; cl, cloaca; de, distal early of nephron; e, eye; ep, epidermis; f, forebrain; fr, fin ray; g, gut; h, hindbrain; hg, hatching gland; ht, heart; j, jaw; li, liver; lln, lateral line neuromasts; m, midbrain; mb, melanoblast; mff, median fin fold; mhb, midbrain hind brain barrier; mnc, migratory neural crest cell; mo, medulla oblongata; mt, mouth; nt, notochord; ob, olfactory bulb; ot, optic tectum; ov, otic vesicle; p, pancreas; pa, pharyngeal arches; pal, pallium; pe, peridermis; pf, pectoral fin; pnc, posterior notochord; pst, proximal straight tubule of nephron; rhb, rostral hindbrain; s, somite; tb, tailbud. Black arrows within each panel point to the specific expression indicated by abbreviations.

2.5. Fos Genes Are Induced in BRAF-Driven Melanoma in Zebrafish

The ERK MAPK pathway is a key signalling pathway regulating the transcription of human Fos genes [1]. To determine if this pathway also regulates expression of zebrafish Fos genes, we expressed the human BRAFV600E to drive ERK MAPK activation in zebrafish melanocytes using the MiniCoopR transgenesis system [36], which allows rapid testing of candidate modifiers of melanoma development in F0 zebrafish generation (Figure 7A,B). Gene expression analysis on zebrafish melanoma samples revealed higher transcript levels of all fos genes except foslb, with fosb displaying the highest relative expression (Figure 7C). These findings indicate functional conservation of fos gene regulation by the ERK MAPK pathway in zebrafish.
2.4. Expression of FOS genes in Adult Zebrafish Tissues

At 24 hpf, a broad expression of all four zebrafish fos genes was observed in all male tissues, including eyes, kidney, gills, spleen, and oocytes which shows moderate expression (Figure 6A–F).

2.5. Fos Genes Are Induced in BRAF-Driven Melanoma in Zebrafish

Gene expression analysis on zebrafish melanoma samples revealed higher transcript levels in the zebrafish adult female intestine (Figure 5E), followed by the expression relative to the normal skin (fold change) (C).

Overall, the expression of fos genes in human BRAF V600E oncogene driven melanoma in zebrafish. mRNA expression levels of fos genes were determined in normal wildtype (WT) skin (A) and melanoma tumour from BRAF V600E; tp53−/− zebrafish line (B), with the graph showing the expression relative to the normal skin (fold change) (C). The relative mRNA levels were normalised to actb and ddct values determined by comparison with expression in the normal wildtype skin. Error bars indicate the standard error of mean for four biological replicates and the dotted line indicates the median in a tissue showing moderate expression.

Figure 6. Relative expression of fos genes in male and female zebrafish adult tissues. mRNA expression levels of fosaa (A), fosab (B), fosb (C), fosl1a (D), fosl1b (E), and fosl2 (F) in zebrafish adult male tissues eyes, brain, heart, kidney, spleen, intestine, skin, gills, testis, and liver were determined by qRT-PCR. The relative mRNA levels were normalised to actb and ddct values determined by comparison with expression in the male eyes, the tissue with moderate expression. The black error bars indicate the standard error of mean for four biological replicates and the dotted line indicates the median in a tissue showing moderate expression.

Figure 7. Relative expression of fos genes in human BRAF V600E oncogene driven melanoma in zebrafish. mRNA expression levels of zebrafish fos genes were determined in normal wildtype (WT) skin (A) and melanoma tumour from BRAF V600E; tp53−/− zebrafish line (B), with the graph showing the expression relative to the normal skin (fold change) (C). The relative mRNA levels were normalised to actb and ddct values determined by comparison with expression in the normal wildtype skin. Error bars indicate the standard error of mean for five biological replicates with statistical significance indicated (***p < 0.001, ns not significant). The white arrow indicates the tumour.
3. Discussion

Despite their well-documented roles as key regulators of cell fate in a multitude of normal and pathological contexts, several important aspects of FOS protein biology remain poorly understood, particularly in vivo, such as how specific FOS heterodimers regulate transcription and how their activities are regulated. Though possible, such studies are challenging to undertake in mice, the main in vivo model that has been used to study FOS protein biology. Our findings highlight the potential of zebrafish as a vertebrate model for such studies.

The six zebrafish FOS proteins share 40–65% sequence identity and 56–74% similarity to their respective human counterparts. As expected, sequence conservation was highest in the respective bZIP domains of zebrafish and human FOS proteins. However, we also found that zebrafish Fos proteins retained significant sequence conservation in other functional domains previously identified in human FOS isoforms [1], including the N- and C-terminal transcription activation domains of c-FOS and FOSB, and the C-terminal DEST that controls protein stability. The exception was Fosl1b, which lacked a C-terminal region, including the DEST domain. As FOS proteins are stabilised upon deletion or ERK pathway-dependent phosphorylation of this domain [15,17], it is likely that Fosl1b is inherently more stable than the other zebrafish FOS orthologs and that its expression is uncoupled from ERK pathway regulation. Such divergence has often been observed in paralogs arising from gene duplication events [37] and provides an evolutionary mechanism for creating unique protein functionalities.

The major mechanism of FOS protein regulation is through post-translational modification, primarily phosphorylation. We found that phosphorylation sites previously identified in human FOS proteins [1] are also conserved in zebrafish. Thus, the functionality of each FOS isoform is likely to be regulated via similar mechanisms in both species. Additionally, the conservation of regulatory elements, including phosphorylation sites, suggests that their functional impacts could be characterised in vivo using targeted genome editing approaches in zebrafish.

One of the least understood aspects of FOS biology is their roles during early embryonic development. Compared to mice, such investigations are much easier to undertake in zebrafish, as they have transparent embryos, develop externally, and are readily amenable to genetic manipulation. Our data suggest that zebrafish fos genes play distinct and temporally restricted roles in specific cell lineages and tissues during early development. For example, whereas sustained fosab and fosl2 expression was evident over 4 days of development in multiple lineages, fosaa, fosl1a, and fosl1b showed more transient expression. The pattern of fosab expression suggests it may play a role during early development of embryonic skin, liver, and pancreas. In addition to fosab, fosl1a also may play roles in pancreatic development. Interestingly, all fos genes except fosl1b showed transient expression in the embryonic eyes and brain, indicating potential roles for fos genes in development of these tissues. Expression of fosab, fosl2, and fosl2 in the jaws and pharyngeal arches suggests these genes may participate in bone and cartilage development, as has been previously reported in mice [27,38]. In addition to their distinct expression patterns in the early embryo, we found that fos genes were expressed in a variety of adult zebrafish tissues. Interestingly, fos gene expression appears to differ between some male and female tissues, most notably the spleen. This finding suggests a potential role for fos genes in regulating sexual dimorphism in the immune system, a possibility that warrants further investigation. Of note expression of fos has previously been shown to be sexually dimorphic in the zebrafish brain [39].

The data from our WISH analysis highlight the potential of zebrafish as an alternative in vivo model to mice for investigating the roles of Fos proteins in vertebrates. An important goal of future studies will be to use the powerful toolkit available for genetic manipulation in zebrafish to dissect the role of specific fos genes in the embryo. To date, few such analyses have been undertaken with the exception of the heart, where fosl2 was reported to potentiate the rate of myocardial differentiation from the zebrafish second heart field [40] and where cardiomyocyte-specific expression of a dominant negative AP-1 protein leads to defects in
cardiomyocyte proliferation following injury [41]. Consistent with these observations, we also noted expression of fosl2, as well as fosab, fosb, and fosl1a, in the embryonic heart.

In mammalian cells, Fos genes are classified as inducible immediate early genes transiently induced by extracellular signals such as growth factors, hormones, and cytokines [42]. Though limited, available evidence indicates that they are also likely to be inducible genes in zebrafish. For example, expression of zebrafish c-Fos orthologs has been shown to be induced in the dorsal telencephalon by cannabinoids [43]. Fosl1a was induced during skeletal muscle regeneration [44] while fosab, fosl1a, and fosl2 were induced during heart regeneration [45]. The major pathway regulating transcription of Fos genes in mammalian cells is the ERK MAPK pathway, whose activation in response to IGF and FGF signalling has been shown to control key developmental events in zebrafish embryos, including somite boundary formation [46], dorsoventral patterning [47], axial patterning [48], and the development of the subpallial telencephalon [49]. Interestingly, we found that fosaa, fosab, fosl1b, and fosl2 were expressed in the presomitic mesoderm, where ERK signalling has been shown to be critical for somitogenesis in both zebrafish and chick embryos [50]. In addition to developmental contexts, ERK signalling has also been reported to induce FOS gene expression downstream of oncogenic lesions, such as activating mutations in the BRAF gene, which frequently occur in human melanomas [51–54]. Consistent with these observations, we show that fos gene expression was also induced in zebrafish BRAF mutant melanomas, indicating functional conservation of key pathways regulating expression of these genes in humans and zebrafish. Thus, zebrafish may provide a useful model to dissect the role of specific Fos genes during development and progression of melanoma and other oncogene-driven cancers.

4. Materials and Methods

4.1. Identification of Zebrafish FOS Family Orthologues

Zebrafish orthologues of human FOS genes were identified by bioinformatics analysis using the National Centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov/, accessed on 9 May 2022) and the Zebrafish Information Network (https://zfin.org/, accessed on 9 May 2022). The FASTA sequences of human FOS mRNAs were obtained from the NCBI database and used for Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 9 May 2022) analysis to identify the zebrafish orthologues which were further crosschecked with the ZFIN database and confirmed by sanger sequencing.

4.2. Multiple Sequence Alignment (MSA) and Phylogenetic Comparison of FOS Family Orthologues in Human and Zebrafish

Sequence comparisons of human and zebrafish FOS protein orthologues were determined using pBLAST. Multiple sequence alignments (MSA) were performed using Clustal X version 2.1 software and bootstrapped phylogenetic trees of 1000 replicates generated using the neighbor-joining algorithm [55], then visualised in TreeView [56]. The genomic arrangement of human and zebrafish FOS gene loci, including syntenic genes, was determined using NCBI Map Viewer. Information on regulatory phosphorylation sites in human FOS proteins was obtained from the Phosphosite database (www.phosphosite.org, accessed on 9 May 2022). Synteny analysis for zebrafish and human FOSL1 and FOSL2 genes was performed using the Genomicus database [57].

4.3. Zebrafish Maintenance and Embryo Collection

Zebrafish were maintained under standard husbandry practices [39], following the national guidelines for animal use and care, with approval from the Deakin University Animal Welfare Committee. Embryos were collected manually and nurtured in a petri dish containing E3 media and incubated at 28.5 °C, with transparency maintained by adding 0.003% (w/v) 1-phenyl-2-thio-urea (PTU), a pigment inhibitor into the E3 media from 9 h post fertilization (hpf).
4.4. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Adult zebrafish were euthanised with benzocaine and their tissues were isolated by dissection. Total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer’s guidelines. qRT-PCR was performed using the iTaq™ Universal SYBRGreen Supermix (Biorad, South Granville, Australia) according to the manufacturer’s guidelines with the following primers:

- fosaa (5′-AACATCAAAGCGAGGCGT, 5′-CGGAGACTCGCCCTGTCG),
- fosab (5′-CGGAGACTCGCCCTGTCG),
- fosb (5′-CGGAGACTCGCCCTGTCG),
- fosl1a (5′-CACATCAAAGCGAGGCGT, 5′-CGGAGACTCGCCCTGTCG),
- fosl1b (5′-CACATCAAAGCGAGGCGT, 5′-CGGAGACTCGCCCTGTCG),
- fosl2 (5′-CACATCAAAGCGAGGCGT, 5′-CGGAGACTCGCCCTGTCG),
- actb (5′-GACACTGCTGTCTGGGAAT, 5′-GACACTGCTGTCTGGGAAT).

Standard and unknown samples were assayed in triplicate using the following thermocycle profile conditions: initial incubation at 95 °C for 5 s, 58 °C for 10 s, and then 72 °C for 20 s. Data was normalized to actb and the relative fold changes in adult male and female zebrafish tissue gene expression were determined by comparison with the expression in the male eyes (tissue with moderate overall fos gene expression) by using the ddct method [58].

4.5. Whole-Mount In Situ Hybridization (WISH)

Total RNA was isolated from zebrafish embryos using TRIzol (Bioline) according to the manufacturer’s instructions and cDNA synthesis was performed using an iScript cDNA synthesis kit (Promega). RT-PCR was performed to amplify cDNA by using the Go Taq Green Master Mix (Promega, Madison, USA) using the following primers: fosaa (5′-GGAAGAGCAAGAGCGAGCA, 5′-CTTGTAGAGCGTCTCCCAGTC), fosab (5′-GGAAGAGCAAGAGCGAGCA, 5′-CTTGTAGAGCGTCTCCCAGTC), fosl1a (5′-GGAAGAGCAAGAGCGAGCA, 5′-CTTGTAGAGCGTCTCCCAGTC),

PCR reactions were performed under the following conditions for 35 cycles: 2 min at 95 °C, 30 s at 95 °C, 30 s at 55 °C, 1.5 min s at 72 °C, and 10 min at 72 °C. RT-PCR amplified cDNA products of fosaa, fosab, fosl1a, fosl1b, and fosl2 were isolated, purified and cloned into pGEM-T Easy vectors. Purified plasmids with the gene insert were linearised, followed by transcription using either T7 or SP6 polymerase and DIG RNA labelling mix (Roche) to generate DIG-labelled anti-sense and sense probes, which was followed by their purification using Probe-Quant G-50 micro columns (Cytiva). At different time points during development, embryos were collected, dechorionated, and anesthetized with 0.4 mg/mL benzocaine before fixing with 4% w/v paraformaldehyde (PFA) at 4 °C. WISH was performed using digoxigenin (DIG)-labelled RNA probes as described in [38] and the images were obtained using an Olympus MVX10 monozoom microscope with a 1 × MVXPlan Apochromat lens (NA = 0.25) with an Olympus DP74 camera. Specific expression of each probe was confirmed by comparison to their corresponding sense controls.

4.6. Melanoma Model

Zebrafish melanoma formation was studied as described previously [36]. Briefly, 25 pg of a MiniCooP vector expressing BRAFV600E (MiniCooP mitfa:BRAFV600E) was microinjected along with 25 pg of Tol2 transposase mRNA into one-cell stage tp53 knockout embryos. The animals were monitored weekly for the presence of visible tumours. The tumours were dissected and RNA extracted for subsequent qRT-PCR analysis to determine the expression of zebrafish fos genes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms231710098/s1.
Author Contributions: Conceptualization, F.B. and A.S.D.; methodology, K.K., F.B., G.K.G. and S.H.; validation, K.K., F.B. and A.S.D.; formal analysis, K.K., F.B., A.S.D. and A.C.W.; investigation, K.K., F.B., A.S.D. and A.C.W.; resources, A.S.D. and F.B.; data curation, F.B. and K.K.; writing, original draft preparation, F.B., A.S.D. and A.C.W.; writing, review and editing, F.B., A.S.D., A.C.W. and C.L.; supervision, F.B. and A.S.D.; project administration, F.B. and A.S.D.; funding acquisition, F.B. and A.S.D. All authors have read and agreed to the published version of the manuscript. All authors contributed to data interpretation and drafting of the paper, and all approved the final manuscript.

Funding: This project was supported by National Health and Medical Research Council grant 1141906 and IMPACT Seed Funding from Deakin University, Australia.

Institutional Review Board Statement: This study was approved by the Deakin University Animal Ethics Committee under projects G13-2019 (29 August 2019) and G21-2019 (15 June 2020).

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analyzed during this study are included in this published article (and its Supplementary Materials).

Acknowledgments: This work was supported by grants from the National Health and Medical Research Council of Australia and from Deakin University (IMPACT). The authors also acknowledge the exceptional support provided by the Deakin University Animal Facility staff. We also thank members of the Zon Lab, Leonard Zon, Haley Noonan, Alicia McConell, and Georgia Stirtz for reagents and advice on the zebrafish melanoma model.

Conflicts of Interest: The authors declare that they have no competing interests.

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