New players in zebrafish caudal fin regeneration : differential requirement for protein-tyrosine phosphatases

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Chapter 6

An Essential Role for Shp2-MAPK Signalling during Zebrafish Embryo Caudal Fin-Fold Regeneration

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ABSTRACT

Zebrafish completely regrow their caudal fins after amputation. Following injury, wound healing occurs, followed by the formation of a blastema, which produces cells to replace the lost tissue in the final phase of regenerative outgrowth. Here, we show that *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos, which lack functional Shp2, did not regenerate their caudal fin-folds following amputation. Rescue experiments revealed that catalytic activity and the SH2 domains, but not the two tyrosine phosphorylation sites in the C-terminal domain of Shp2a were required for caudal fin-fold regeneration. Normal expression of the blastema marker *junbb* indicated normal blastema formation in *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos. Cell proliferation was arrested in zebrafish embryos lacking functional Shp2, and MAPK phosphorylation was reduced. Consistent with these Shp2 results, we found that inhibition of MEK1 in wild-type zebrafish embryos inhibited caudal fin-fold regeneration and arrested cell proliferation, indicating that MAPK activation was required for regenerative outgrowth. Collectively, our results suggest an essential role for Shp2a-MAPK signalling in promoting cell proliferation during the regenerative outgrowth phase of regenerating zebrafish embryo caudal fin-folds.

INTRODUCTION

In a process termed epimorphic regeneration, zebrafish (*Danio rerio*) fully regenerate their heart, retina, spinal cord, and caudal fin after injury (1, 2). Regeneration of the zebrafish caudal fin proceeds sequentially through three distinct phases: wound healing, blastema formation, and regenerative outgrowth. The mechanism of zebrafish embryonic caudal fin-fold regeneration is the same as adult zebrafish caudal fin regeneration, though reaches completion within 72 hours, instead of within two weeks (3). Following injury, nearby cells migrate to cover the wound and form an apical epidermal cap that is essential to initiate blastema formation and regenerative outgrowth (4). Many genes have been implicated in the regenerative process (5) and multiple signalling pathways have been validated to be essential for regeneration, including fibroblast growth factor (FGF), sonic hedgehog, bone morphogenetic protein, Wnt, and Notch (6).

Src homology 2 domain containing phosphatase (SHP2) is a protein tyrosine phosphatase (PTP), encoded by the *PTPN11* gene, that dephosphorylates phosphorylated tyrosine residues of target substrates. The zebrafish genome contains two *ptpn11* genes, *ptpn11a* and *ptpn11b*, encoding Shp2a and Shp2b, respectively. Both Shp2a and Shp2b are highly homologous to human SHP2 and harbour catalytic activity. Shp2b is dispensable, but Shp2a is not, which is due to differential expression of *ptpn11a* and *ptpn11b* during early development. *Ptpn11a*$^{+/+}$*ptpn11b*$^{-/-}$ and *ptpn11a*$^{+/-}$*ptpn11b*$^{-/-}$ zebrafish are viable and fertile, yet homozygous *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ double knock out zebrafish are embryonically lethal from 5-7 days post fertilization (dpf) onwards (7).

SHP2 is involved in signalling initiated by various growth factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), and insulin-like growth factor 1 (IGF1) (8, 9). SHP2 has a role in promoting mitogen activated protein kinase (MAPK) signalling in response to growth factor stimulation. Yet, SHP2-mediated regulation of phosphoinositide 3-kinase (PI3K) signalling depends on the growth factor stimulus. SHP2 inhibits PI3K signalling in response to EGF, but activates PI3K signalling in response to PDGF and IGF in the same cell type (10). Hence, the effects of SHP2 on PI3K signalling are stimulus and cell-type dependent. We refer to (11) for a recent overview of SHP2 signalling.

The SHP2 protein consists of two SH2 domains, followed by a catalytically active PTP domain, and a C-terminal domain (12). SHP2, like all classical PTPs, mediates dephosphorylation of its substrates through a mechanism involving a catalytic cysteine (C460 in zebrafish Shp2a) and an assisting arginine (R466 in zebrafish) in the PTP

domain (13). SHP2 requires catalytic activity to promote MAPK and PI3K signalling, as mutation of the catalytic cysteine results in reduced downstream signalling (14, 15). Importantly, the SH2 domains and C-terminal domain of SHP2 are required for the function of SHP2 in response to growth factor stimulation. Both SH2 domains contain conserved arginine residues (R32 and R138) that are required for binding to phospho-tyrosine-containing target proteins, such as insulin receptor substrate 1 (IRS-1), PDGF receptor, and GAB1, and are essential for the function of SHP2 (16). The SH2 domains also have an ERK-independent, anti-apoptotic function, as expression of a zebrafish Shp2 mutant lacking the PTP domain and C-terminal domain rescued apoptosis induced by zebrafish Shp2 knockdown. The C-terminal SH2 domain was particularly important for this function, as full-length zebrafish Shp2a R138M failed to block apoptosis (17). The C-terminal domain of SHP2 contains serine and tyrosine phosphorylation sites, as well as a proline-rich domain, that mediate interactions with other proteins. Two tyrosines (Y542 and Y580) are particularly important, because when phosphorylated, they constitute binding sites for SH2 domain containing proteins, such as growth factor receptor-bound protein 2 (GRB2) (18, 19). Binding of GRB2 modulates the interaction of the C-terminal domain with the SH2 domains of SHP2 (20), and is required for Shp2 mediated MAPK signalling in response to EGF (21).

Collectively, the studies on the function of the domains of SHP2 show that both the SH2 and C-terminal domain potentiate, but are not definitively required for the stimulation of MAPK and PI3K signalling by the PTP domain of SHP2. Activated MAPK promotes cell proliferation, migration, and differentiation (22) and activated PI3K/AKT promotes cell cycle progression to drive cell proliferation, and enhances cell survival by inhibiting pro-apoptotic transcription factors (23). It is not surprising that SHP2 has been implicated to play a role in a plethora of cellular processes, including proliferation (24, 25), cell migration (26–28), and stem cell self-renewal and differentiation (29–32). SHP2, MAPK, and PI3K signalling are indispensable for proper embryonic development (7, 16, 33–37).

Regeneration requires cell survival, migration, proliferation and differentiation for effective wound healing and replacement of the lost tissue (4, 38, 39). MAPK activation following injury is associated with regenerative competence across species (40, 41). The need for MAPK signalling in zebrafish caudal fin regeneration has been implicated by demonstrating that inhibition of FGF receptor (FGFR) signalling (42–44) impairs regeneration. However, activation of the FGFR results in the promotion of not only MAPK, but also PI3K, Phospholipase C γ (PLCγ), and signal transducer and activator of

transcription (STAT) (45), convoluting the conclusion that MAPK signalling is required. Similarly, the requirement for activation of the EGF receptor (EGFR) family member ErbB2 for zebrafish caudal fin regeneration (46) eludes to a requirement for MAPK and PI3K signalling (47). Whereas PI3K signalling is essential for zebrafish caudal fin regeneration (38, 46), the evidence supporting a role for MAPK signalling is inconclusive. Rojas-Muñoz et al. showed that treatment of zebrafish embryos with the ErbB2/3 inhibitor PD168393 or the PI3K inhibitor LY294002 effectively blocked caudal fin regeneration, but treatment with the MEK1 inhibitor PD098059 had no effect (46). However, it has recently been shown that MAPK signalling is essential for zebrafish cardiac regeneration, as inhibition with the MEK1 inhibitor AZD6244 or expression of dominant-negative MEK1 impaired zebrafish cardiac regeneration (48). Hence, the role of SHP2 and MAPK signalling in zebrafish caudal fin regeneration remains to be determined definitively.

We investigated the role of Shp2 in zebrafish embryo caudal fin-fold regeneration using homozygous *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos, that lack functional Shp2 (7), and found that Shp2 is required for normal caudal fin-fold regeneration. Re-expression of wild-type Shp2a rescued regeneration, indicating that the lack of regeneration in mutant zebrafish embryos was indeed attributable to lack of functional Shp2. Rescue experiments with mutant Shp2a indicated that Shp2a catalytic activity and functional SH2 domains were required, whereas the two tyrosine residues in the C-terminus of Shp2a that are essential for certain aspects of Shp2 signalling, were dispensable for its capacity to rescue regeneration. Characterization of the regeneration defect in *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos by *in situ* hybridization using a *junbb*-specific probe indicated normal blastema formation. PCNA staining indicated that cell proliferation during regenerative outgrowth was reduced in *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos. MAPK phosphorylation was reduced in amputated caudal fin-folds of *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos. Pharmacological inhibition of MEK1, upstream of MAPK, in wild-type zebrafish embryos inhibited regeneration and reduced proliferation during regenerative outgrowth. The similarity with the lack of regeneration in *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos is consistent with Shp2a acting through MAPK in regeneration. Collectively, our results demonstrate that catalytically active Shp2a is required for zebrafish embryo caudal fin-fold regeneration and may exert its effects through activation of MAPK.

MATERIALS AND METHODS

Zebrafish husbandry

All procedures involving experimental animals were performed under licence number GZB/VVB 2041019 of the Hubrecht Institute/ Royal Academy of Arts and Sciences (Koninklijke Nederlandse Akademie van Wetenschappen, KNAW), approved by the local animal experiments committee according to local guidelines and policies in compliance with national and European law.

The *ptpn11a*$^{+/-}$*ptpn11b*$^{-/-}$ zebrafish lines in the Tuebingen Long fin (TL) background were previously created by target-selected gene inactivation (TSGI), and both *ptpn11a*^{hu3459} and *ptpn11b*^{hu5920} alleles result from non-sense mutations that lead to a premature stop codon upstream of the catalytic cysteine, C460 (7). Adult *ptpn11a*$^{+/-}$*ptpn11b*$^{-/-}$ zebrafish were in-crossed to generate *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos for all experiments. Zebrafish were raised and maintained as described by Westerfield (49) under a 14 hours light/ 10 hours dark cycle at 28.5°C.

mRNA synthesis and micro-injections

All constructs contain a C-terminal eGFP connected by a peptide-2a cleavage sequence (50). The constructs pCS2+-eGFP-2a-Shp2a (WT), pCS2+-eGFP-2a-Shp2a-R466M (RM), pCS2+-eGFP-2a-Shp2a-R32M-R138M (SH), and pCS2+-eGFP-2a-Shp2a-Y542F-Y580F (YF) were obtained as described (51). Sense messenger RNA (mRNA) synthesis and micro-injection into one-cell stage zebrafish embryos was performed as described (52).

Caudal fin-fold amputation

Zebrafish embryos were amputated as previously described (53), amputations were performed at 2 days post fertilisation (dpf) for all experiments. Regeneration was allowed to proceed until analysis at 3 days post amputation (dpa) or fixation at 3 hours post amputation (hpa), 1dpa or 2dpa. PD184352 (Sigma) or Dimethylsulfoxide (Sigma) was administered directly following recovery of amputated zebrafish embryos in E3 medium. Whole zebrafish embryos were lysed for genotyping or fixed in 4% paraformaldehyde (PFA), in PBS, either 3hpa for *in situ* hybridization, or at 1dpa and 2dpa for immunohistochemistry.

In situ hybridization

In situ hybridizations were performed as previously described (54), using *junbb* digoxigenin-UTP-labelled anti-sense riboprobes. Parts of *junbb* were amplified from zebrafish cDNA using specific primers (**Table S1**) and the resulting PCR products were cloned into vectors and used as DNA templates for synthesis of riboprobes. Digoxigenin-UTP-labelled anti-sense riboprobes were synthesized from *junbb* PCR product, produced using *fwd: TACACGACGCTGAACGCATA* and *rev: TAATACGACTCACTA-TAGGTGTCCGTTCTCTTCCGTCC*. Caudal fin-folds of zebrafish embryos were severed and mounted in 70% glycerol in PBS for imaging on a Zeiss Axioskop 2 Mot Plus microscope with either a Plan-NEOFLUAR 10x/0.30 or 20x/0.50 objective. The rest of the zebrafish embryo was lysed for genotyping.

Immunohistochemistry

Zebrafish embryos fixed in 4% PFA were washed in PBS 0.1% Tween-20 and antigen retrieval performed depending on the antibody used: ice-cold acetone for 20mins for PCNA; and 10mM Tris, 1mM EDTA, pH 9.0 for phospho-p44/42 MAPK (Thr202/Tyr204). Whole zebrafish embryos were incubated overnight at 4°C in mouse anti-PCNA (1:200, #M0879; DAKO Agilent Pathology Solutions), or rabbit anti-phospho-p44/42MAPK (Thr202/Tyr204) (1:100, #4370 Cell Signaling Technology). Secondary antibodies conjugated to Cy5 goat anti-mouse or goat anti-rabbit IgG were used 1:500 and 1:200 respectively (#115-175-146 and #111-175-144; Jackson ImmunoResearch). Nuclei were shown by DAPI (4',6-diamidino-2-phenylindole) staining. Caudal fin-folds of zebrafish embryos were mounted for imaging in 70% glycerol in PBS, and the rest of the zebrafish embryo was lysed for genotyping. Z-stacks (6µm step size) of the caudal fin-fold were acquired for every zebrafish embryo with a Leica Sp8 confocal microscope. ImageJ software (http://rsb.info.nih.gov./ij/) was used to generate maximum intensity (z) projections and merge channels. Quantification was performed on the original z-projections (1024 x 1024px, 300dpi) following rolling ball background subtraction with an average from 50px. Mean intensity of phospho-p44/42 MAPK (Thr202/Tyr204) (hereafter p-MAPK) was measured from the wound margin inwards using an ROI with dimensions equivalent to: h: 2.08µm (625px) and varying widths for each sample: for zebrafish embryos at 24hpa: w: 0.32µm (95px) for amputated zebrafish embryos; w: 0.75µm (225px) for uncut zebrafish embryos; for embryos at 48hpa: w: 0.82µm (246px) for amputated zebrafish embryos; w: 1.00µm (300px) for uncut zebrafish embryos.

Genotyping

All zebrafish embryos that were used in these assays were genotyped to establish *ptpn11a* status. To this end, genomic zebrafish DNA was extracted through lysis of zebrafish embryos in 100µg/ml proteinase K (Sigma) diluted in SZL buffer (50mM KCl, 2.5mM MgCl, 10mM Tris pH 8.3, 0.005% NP40, 0.005% Tween-20, and 10% 0.1% Gelatine). Lysis was performed by incubating at 60°C for 1 hour, followed by 95°C for 15 minutes in a thermal cycler (BioRad T100). The *ptpn11a*^{hu1864} allele in non-fixed tissue was analysed by Kompetitive Allele Specific PCR (KASP): primers of *ptpn11a* containing the non-sense mutations of the *ptpn11a*^{hu1864} allele (**Table S1**), were mixed with genomic zebrafish DNA and KASP master mix (LGC Group). Amplification was carried out according to the manufacturer's instructions and the resulting PCR products were analysed in a PHERAstar microplate reader (BMG LABTECH). Klustercaller software (LGC Group) was used to identify the mutations. For fixed tissue genotyping for the *ptpn11a*^{hu1864} allele was performed by nested PCR with primer sets 1-4 (**Table S1**) followed by Sanger sequencing (Macrogen Inc., Europe) to detect the mutations.

Immunoblotting

Whole cell protein extracts from human embryonic 293 T cells transfected with and overexpressing zebrafish *ptpn11a* were prepared by lysis in ice-cold buffer (25mM HEPES pH 7.4, 150mM NaCl, 0.25% deoxycholate, 1% triton X-100, 10mM MgCl2, 1mM EDTA and 10% glycerol) containing protease and phosphatase inhibitors. Samples were centrifuged at 12,000g for 20mins and the supernatant collected. Proteins were resolved in 10% SDS-PAGE gels under reducing conditions and transferred to PVDF membranes (IPVH00010, Merck Millipore). Immunoblotting was performed using anti-SHP2 (SH-PTP2 C-18, #SC-280, Santa Cruz Technology) or anti-Tubulin (#CP06, Merck Millipore) antibodies followed by horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibodies, respectively. Detection was done by enhanced chemiluminescence (#34095, Thermo Fischer Scientific).

Statistics

For analysis of caudal fin-fold lengths, histograms of whole data sets were examined to determine non-normal distribution of the data. Statistical analysis of unequal variances was obtained through a Kruskall-Wallis test. Differences between different experimental conditions were assessed for significance using a Mann-Whitney U test. Differences were considered significant when $p<0.001$, and if they satisfied

a confidence interval of 95% in a Monte Carlo exact test. All tests for regenerating caudal fin-folds were performed in SPSS (IBM). For analysis of immunohistochemistry measurements, differences between different experimental condition were assessed for significance using a Mann-Whitney U test with a confidence level set to 95%. All tests for immunohistochemistry measurements were performed in GraphPad Prism (GraphPad Software). Differences were considered significant when $p<0.05$.

RESULTS

Functional Shp2a is required for zebrafish embryo caudal fin-fold regeneration

To investigate the role of Shp2 in regeneration, homozygous *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos, lacking functional Shp2, were analysed for the potential to regenerate their caudal fin-fold following amputation. The caudal fin-fold of offspring from a *ptpn11a*$^{+/-}$*ptpn11b*$^{-/-}$ in-cross were amputated at 2 dpf and allowed to regenerate for 3 days. Regeneration of individual zebrafish embryos was assessed at 5 dpf (i.e. 3 days post amputation, dpa), and, subsequently, the zebrafish embryos were genotyped. In contrast to sibling *ptpn11a*$^{+/+}$*ptpn11b*$^{-/-}$ and *ptpn11a*$^{+/-}$*ptpn11b*$^{-/-}$ zebrafish embryos, which do regenerate their caudal fin-folds, regeneration of the caudal fin-fold of *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos is severely impaired (**Fig. 1**). Quantification of caudal fin-fold lengths, obtained by measuring the distance from the tip of the notochord to the edge of the fin-fold, were determined and are presented as percentage caudal fin-fold growth, normalized to uncut controls of *ptpn11a*$^{+/+}$*ptpn11b*$^{-/-}$ zebrafish embryos (**Fig. 1B**). Regeneration of the caudal fin-fold of *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos is significantly impaired, compared with sibling embryos ($p<0.001$). Normal growth rate of the caudal fin-fold of *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos is unaffected by the lack of functional Shp2. These results demonstrate that functional Shp2 is required for zebrafish embryo caudal fin-fold regeneration.

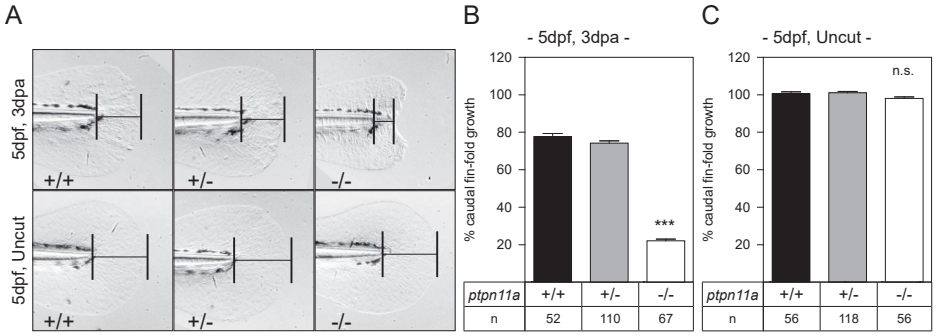

Fig. 1. Functional Shp2 is required for regeneration. Zebrafish embryos from a *ptpn11a*$^{+/-}$*ptpn11b*$^{-/-}$ in-cross were amputated at 2 dpf and caudal fin-fold regeneration was assessed at 3dpa (i.e. 5dpf, 3dpa). Equivalent uncut embryos were included as controls (i.e. 5dpf, uncut). All embryos were genotyped. (**A**) Representative images of amputated embryo caudal fin-folds at 3dpa are shown together with uncut controls for comparison. (**B**, **C**) Regeneration was quantified by measuring the distance from the tip of the notochord to the edge of the caudal fin-fold as indicated. The means of caudal fin-fold growth are depicted relative to caudal fin-fold growth of uncut *ptpn11a*$^{+/+}$*ptpn11b*$^{-/-}$ controls. Means of *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ embryos were compared *ptpn11a*$^{+/+}$*ptpn11b*$^{-/-}$ embryos using a Mann-Whitney U-test. Significance: *** p<0.001; n.s. not significant; error bars indicate standard error of the mean. Data pooled from multiple experiments.

Shp2a catalytic activity and the SH2 domains of Shp2a are required for zebrafish embryo caudal fin-fold regeneration

To validate that impaired regeneration in *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos is indeed due to the lack of functional Shp2, rescue experiments were performed. Zebrafish embryos from a *ptpn11a*$^{+/-}$*ptpn11b*$^{-/-}$ in-cross were micro-injected at the one-cell stage with synthetic mRNA encoding Shp2a, and at 2 dpf the caudal fin-fold was amputated and allowed to regenerate for 3 days. Expression of wild-type Shp2a resulted in a significant rescue (p<0.001) of regeneration in *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos (**Fig. 2A-B**).

The catalytic activity of SHP2 is important for the function of SHP2, although PTP-independent functions have been identified as well. The SH2 domains and the C-terminal domain of SHP2 are required for downstream signalling (17, 18, 21, 55–58). To determine which domains of Shp2a are required for caudal fin-fold regeneration, the following mutants of Shp2a were used in rescue experiments: catalytically inactive Shp2a-R466M (RM); the SH2 domain mutant Shp2a-R32M-R138M (SH), in which the essential arginine residues in both SH2 domains were mutated; and the C-terminal domain mutant Shp2a-Y542F-Y580F (YF), which lacks the two tyrosine phosphorylation sites that are important for signalling. We used the R466M mutant of Shp2a in our experiments rather than the catalytic cysteine mutant (C460S in zebrafish Shp2a), be-

cause Shp2a-C460S may trap substrates (59, 60), and thus have inadvertent dominant effects. Indeed, expression of Shp2a-C460S in wild-type zebrafish embryos induced developmental defects, and expression in *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos induced more severe developmental defects than non-injected Shp2 deficient zebrafish embryos (data not shown). In contrast, the Shp2a-R466M mutant is catalytically inactive and has no dominant-negative effects (17).

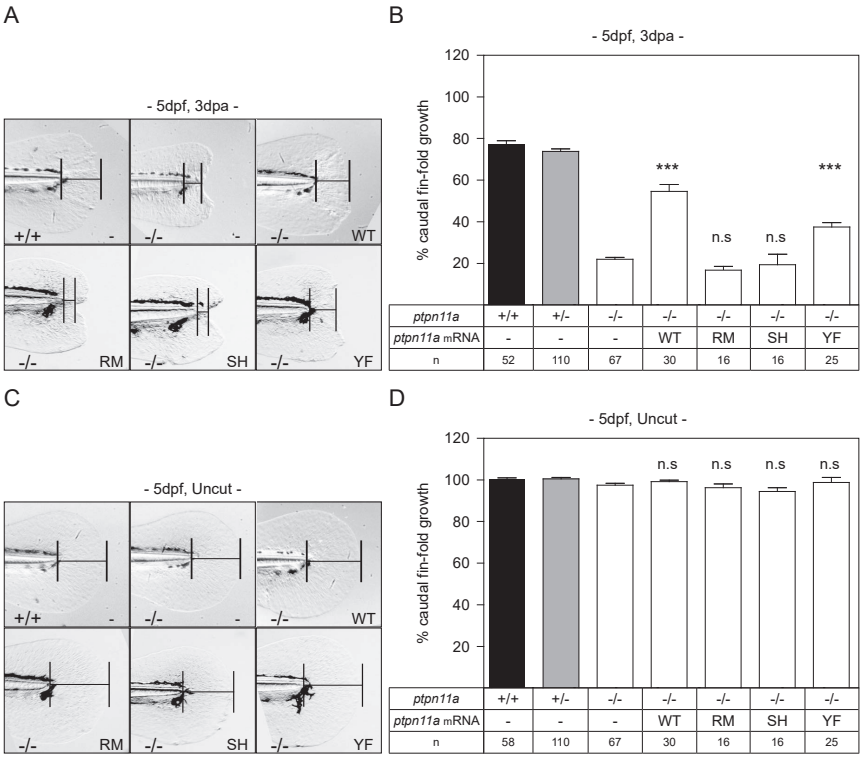

Fig. 2. The catalytic activity and SH2 domains of Shp2 are required to rescue caudal fin-fold regeneration in Shp2 deficient embryos. Embryos from a *ptpn11a*$^{+/-}$*ptpn11b*$^{-/-}$ in-cross were micro-injected at the one-cell stage with synthetic mRNA encoding wild-type Shp2a (WT), catalytically inactive Shp2a-R466M (RM), SH2 domain mutant Shp2a-R32M-R138M (SH), C-terminal tyrosines mutant Shp2a-Y542F-Y580F (YF), or were not injected (-). At 2dpf the caudal fin-fold was amputated and regeneration was assessed at 3dpa (i.e. 5dpf, 3dpa), equivalent uncut controls were included (i.e. 5dpf, uncut). All embryos were genotyped. (**A**) Representative images of amputated *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ embryos caudal fin-folds at 3dpa are shown together with a non-injected sibling (*ptpn11a*$^{+/+}$*ptpn11b*$^{-/-}$) for comparison. (**C**) Representative images of uncut *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ embryos caudal fin-folds at 3dpa. (**B**, **D**) Regeneration was quantified by measuring the distance from the tip of the notochord to the edge of the caudal fin-fold as indicated. The means of caudal fin-fold growth are depicted relative to caudal fin-fold growth of uncut *ptpn11a*$^{+/+}$*ptpn11b*$^{-/-}$ controls. Means of micro-injected amputated *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ embryos were compared to non-injected amputated *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ embryos using a Mann-Whitney U-test. Significance: *** $p<0.001$; n.s. not significant; error bars indicate standard error of the mean. Data pooled from multiple experiments.

The mRNAs encoding (mutant) Shp2a proteins also encode eGFP linked by a peptide-2a cleavage sequence, resulting in a single polypeptide that is cleaved prior to protein folding (50). At 2dpf, eGFP-positive zebrafish embryos were selected and their caudal fin-folds were amputated. Zebrafish embryos were allowed to regenerate for 3 days. Representative pictures of regenerated caudal fin-folds of *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos expressing mutant Shp2a protein at 3dpa are depicted (**Fig. 2A**). The equivalent uncut fin-folds (i.e. 5dpf, uncut) are all normal (**Fig. 2C**). Caudal fin-fold lengths were determined and are presented as percentage caudal fin-fold growth, normalized to uncut controls of *ptpn11a*$^{+/+}$*ptpn11b*$^{-/-}$ zebrafish embryos (**Fig. 2B, D**). In contrast to their siblings, *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos displayed severely impaired regeneration, which was rescued by expression of wild-type Shp2a (WT), but not by catalytically inactive Shp2a-R466M (RM). Furthermore, expression of the SH2 domain mutant of Shp2a (SH) was unable to rescue regeneration. In contrast, the mutant lacking two tyrosine phosphorylation sites in its C-terminal domain (YF) rescued regeneration of *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos (**Fig. 2B**). A trivial explanation for the inability of the catalytically inactive mutant of Shp2a (RM), or the SH2 domain mutant of Shp2a (SH) to rescue caudal fin-fold regeneration might be that these proteins have reduced stability. Due to low expression levels of all mutant Shp2a proteins in zebrafish embryos, it was not possible to monitor protein expression in these zebrafish embryos. However, transfection of constructs encoding the mutant Shp2a proteins in human embryonic kidney 293 T cells revealed similar expression levels of all mutant Shp2a proteins (**Fig. S1**). This suggests that the differences in ability to rescue caudal fin-fold regeneration are not due to large differences in protein expression or stability, but rather to differences in Shp2a function. Together, these results demonstrate that although the two tyrosine phosphorylation sites in the C-terminal domain are dispensable, the catalytic activity as well as the SH2 domains of Shp2 are required for zebrafish embryo caudal fin-fold regeneration.

Normal blastema formation in zebrafish embryos deficient for Shp2

Following amputation, three sequential steps can be discerned in the regeneration process: wound healing, blastema formation and regenerative outgrowth. Following wound healing, an apical epidermal cap is produced that signals for the formation of the blastema, and, thus, successful blastema formation is indicative of successful wound healing (1, 2). In the *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos, we assessed blastema formation at 3hpa by *in situ* hybridization using a *junbb*-specific probe, which

has previously been shown to be upregulated in the blastema (61, 62). All zebrafish embryos were subsequently genotyped. *Junbb* was clearly expressed in amputated caudal fin-folds, but not in uncut controls, and *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ mutant zebrafish embryos expressed *junbb* to a similar extent as their siblings following caudal fin-fold amputation (**Fig. 3**). These results indicate that blastema formation was normal in Shp2 deficient zebrafish embryos.

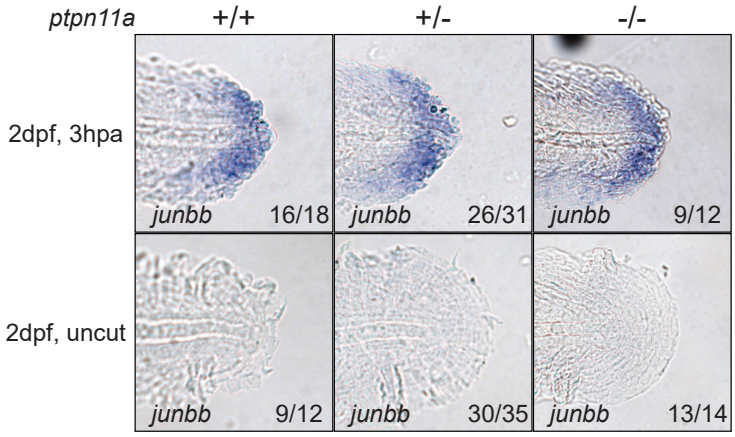

Fig. 3. Normal blastema formation in zebrafish embryos lacking functional Shp2. At 2dpf the caudal fin-folds of embryos from a *ptpn11a*$^{+/-}$*ptpn11b*$^{-/-}$ in-cross were amputated and allowed to regenerate. Embryos were fixed at 3hpa, or equivalent for uncut controls, and subjected to hybridization for *junbb*. Representative images of caudal fin-folds of genotyped embryos are shown, and the number of embryos showing similar patterns/ total number of embryos analysed is indicated in the bottom right corner of each panel.

Arrested proliferation in zebrafish embryos deficient for Shp2 during regenerative outgrowth

Proliferation is upregulated during regenerative outgrowth to generate the cells required to form and replace the lost tissue (2, 3). We analysed cell proliferation during the regenerative outgrowth stage by immunohistochemistry using an antibody specific for proliferating cell nuclear antigen (PCNA). Zebrafish embryos from a *ptpn11a*$^{+/-}$ *ptpn11b*$^{-/-}$ in-cross were fixed at 1dpa or 2dpa and subjected to whole-mount immunohistochemistry for detection of PCNA expression. All zebrafish embryos were subsequently genotyped. By 2dpa, PCNA immunofluorescence was dispersed at the edge of the amputated caudal fin-fold of *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos, whereas in siblings that regenerated normally, PCNA immunofluorescence was concentrated between the amputation plane and the wound margin (**Fig. 4**). Because PCNA positive

cells were predominantly concentrated in the regenerating tissue in siblings, which was absent in *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos, quantification of PCNA staining was not meaningful. Nevertheless, from the representative images in **Fig. 4** it is evident that proliferation was severely arrested in *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos at 2dpa. PCNA immunofluorescence remained low in uncut controls (**Fig. S2**). At 1dpa PCNA immunofluorescence was found between the amputation plane and the wound margin at the edge of the caudal fin-fold of siblings, whereas PCNA immunofluorescence was less pronounced in the same region of Shp2 deficient zebrafish embryos (**Fig. S2**). These results indicate that proliferation is reduced in *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos compared to siblings in the regenerative outgrowth phase following caudal fin-fold amputation.

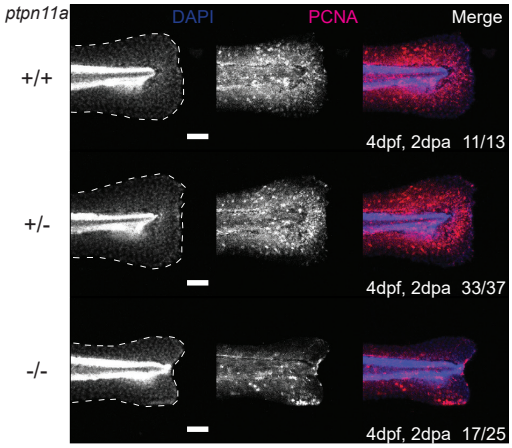

Fig. 4. Proliferation arrest at the amputated caudal fin-fold margin of Shp2 deficient embryos. At 2dpf the caudal fin-folds of embryos from a *ptpn11a*$^{+/-}$*ptpn11b*$^{-/-}$ in-cross were amputated and allowed to regenerate. Embryos were fixed at 2dpa (i.e. 4dpf, 2dpa) and subjected to whole-mount immunohistochemistry using an antibody specific for the cell proliferation marker PCNA (red). The embryos were counterstained with DAPI (blue). Maximum intensity projection images were taken of the caudal fin-folds and all embryos were genotyped. Representative images of amputated embryo caudal fin-folds are shown, and in the left panels the edge of the fin-fold is indicated with a dashed line. The number of embryos showing similar patterns/ total number of embryos analysed is indicated in the bottom right corner of the right panels. The scale bar represents 100µm. Representative pictures of equivalent uncut controls are depicted in **Fig. S2**.

Reduced MAPK signalling in zebrafish embryos deficient for Shp2

Loss of SHP2 in tissue culture cells or in knock-out mice results in reduced MAPK signalling, leading to reduced proliferation, differentiation, and developmental defects (22, 29, 30, 63–65). Furthermore, activated MAPK signalling following injury is asso-

ciated with regenerative competence across species (40, 41). To determine if loss of Shp2 affected MAPK signalling in *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos, we performed whole-mount immunohistochemistry using a phospho-MAPK-specific antibody (p-MAPK, phospho-p44/42 MAPK (Thr202/Tyr204)). In comparison to their siblings, *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos displayed significantly reduced p-MAPK levels following caudal fin-fold amputation at 4 dpf (2 dpa) as well as 3 dpf (1 dpa) (**Fig. 5, S3**). Note that p-MAPK levels were very low in the caudal fin-folds of uncut control zebrafish embryos (**Fig. S3**). These immunohistochemistry experiments indicate that MAPK signalling following caudal fin-fold amputation is reduced in zebrafish embryos lacking functional Shp2.

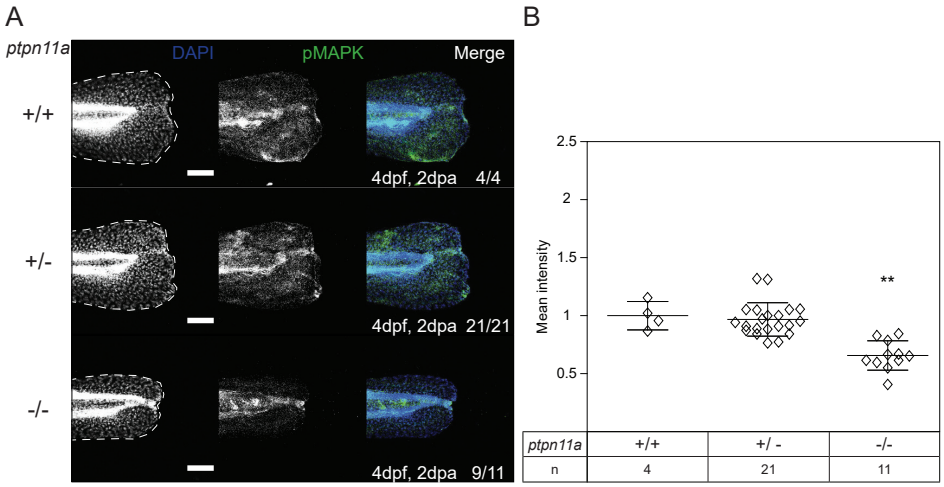

Fig. 5. Reduced p-MAPK in regenerating caudal fin-folds of Shp2 deficient embryos. At 2dpf the caudal fin-folds of embryos from a *ptpn11a*$^{+/-}$*ptpn11b*$^{-/-}$ in-cross were amputated and allowed to regenerate. Embryos were fixed at 2dpa (i.e. 4dpf, 2dpa) and subjected to whole-mount immunohistochemistry using a p-MAPK-specific antibody (Thr202/Tyr204) (green). The embryos were counterstained with DAPI (blue). Maximum intensity projection images were taken of the caudal fin-folds and all embryos were genotyped. (**A**) Representative images of amputated embryo caudal fin-folds are shown and in the left panels the edge of the fin-fold is indicated with a dashed line. The number of embryos showing similar patterns/ total number of embryos analysed is indicated in the bottom right corner of the right panels. The scale bar represents 100µm. (**B**) p-MAPK was quantified by mean intensity of the region between the notochord and the edge of the caudal fin-fold. Equivalent uncut controls were also quantified (representative pictures depicted in **Fig. S3**). Means within amputated groups are compared to *ptpn11a*$^{+/+}$*ptpn11b*$^{-/-}$ embryos using a Mann-Whitney U-test. Significance: ** $p<0.01$; error bars represent standard deviation.

Impaired caudal fin-fold regeneration and reduced cell proliferation by inhibition of MAPK signalling

To test if reduced MAPK signalling by itself is sufficient to inhibit zebrafish embryo caudal fin-fold regeneration, the caudal fin-folds of wild-type zebrafish embryos (*ptpn11a*$^{+/+}$*ptpn11b*$^{+/+}$) were amputated at 2dpf and allowed to regenerate for 3 days in the presence of 50nM MEK1 inhibitor PD184352 (also known as CI-1040) or solvent (1% DMSO) as a control. Treatment of zebrafish embryos with PD184352 significantly impaired caudal fin-fold regeneration (**Fig. 6A**). Uncut controls showed that PD184352 treatment by itself did not affect normal caudal fin-fold growth.

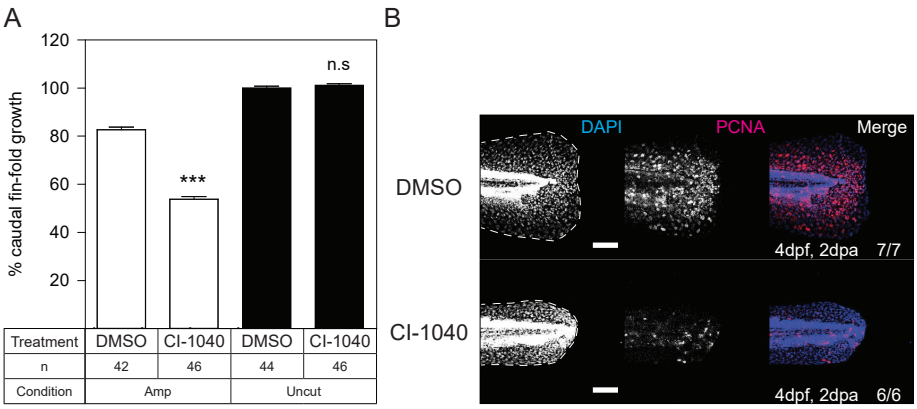

Fig. 6. Impaired regeneration and arrested cell proliferation by MAPK inhibition during caudal fin-fold regeneration. At 2dpf the caudal fin-folds of wild-type embryos were amputated and allowed to regenerate in the presence of 50nM PD184352 or 1% DMSO (solvent control). (**A**) Regeneration was quantified by measuring the distance from the tip of the notochord to the edge of the caudal fin-fold. The means of caudal fin-fold growth are depicted relative to caudal fin-fold growth of DMSO treated uncut controls. Mean of PD184352 treated amputated embryos is compared to DMSO treated amputated embryos using a Mann-Whitney U-test. The number of embryos is indicated (n). Significance: *** p<0.001; n.s. not significant; error bars indicate standard error of the mean. (**B**) Embryos were fixed at 2dpa (i.e. 4dpf, 2dpa) and subjected to whole-mount immunohistochemistry using an antibody specific for the cell proliferation marker PCNA (red). The embryos were counterstained with DAPI (blue). Maximum intensity projection images were taken of the caudal fin-folds. Representative images of amputated embryo caudal fin-folds are shown, and in the left panels the edge of the fin-fold is indicated with a dashed line. The number of embryos showing similar patterns/ total number of embryos analysed is indicated in the bottom right corner of the right panels. The scale bar represents 100µm. Representative pictures of equivalent uncut controls are depicted in **Fig. S4**.

We next investigated if the impaired regeneration of wild-type zebrafish embryos treated with PD184352 was associated with arrested cell proliferation by whole-mount immunohistochemistry for PCNA expression. At both 3dpf and 4dpf PCNA immunof-

luorescence was dispersed in zebrafish embryos treated with PD184352 compared to control zebrafish embryos treated with DMSO (**Fig. 6B, S4**). Baseline PCNA staining in the caudal fin-folds of uncut control zebrafish embryos at 3dpf and 4dpf was low, but also appeared to be reduced following PD184352 treatment (**Fig. S4**). These results demonstrate that MAPK signalling is required for normal caudal fin-fold regeneration and promotes proliferation during regenerative outgrowth.

DISCUSSION

Our results demonstrate a critical role for Shp2 in zebrafish embryo caudal fin-fold regeneration. Zebrafish embryos lacking functional Shp2 (*ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$) show severely impaired regeneration of their caudal fin-folds following amputation (**Fig. 1**). Expression of wild-type Shp2a (WT) rescues regeneration, which relies on its catalytic activity and SH2 domains (**Fig. 2**). Whilst blastema formation was normal in Shp2 deficient zebrafish embryos (**Fig. 3**), proliferation was arrested during the regenerative outgrowth phase (**Fig. 4**). We propose that the reduced p-MAPK levels in *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos (Fig. 5) cause impaired caudal fin-fold regeneration, which is consistent with our observation that regeneration is inhibited by MEK1 inhibition (**Fig. 6**).

As the catalytic activity of SHP2 is often paramount to regulating MAPK or PI3K signalling (14, 15), we first asked whether catalytic activity is required for zebrafish embryo caudal fin-fold regeneration. Caudal fin-fold regeneration was rescued by expressing wild-type Shp2a (WT), but not the catalytically inactive Shp2a R466M mutant (**Fig. 2A-B**), indicating that Shp2a catalytic activity is required for zebrafish embryo caudal fin-fold regeneration. As the Shp2a R466M mutant still retains functional SH2 domains, we exclude the possibility that the SH2 domains alone are sufficient for caudal fin-fold regeneration. In addition, caudal fin-fold regeneration was rescued by expressing the C-terminal domain mutant (YF), but not the SH2 domain mutant (SH), indicating that the SH2 domains are required for the function of Shp2a in zebrafish embryo caudal fin-fold regeneration, but that the two tyrosine phosphorylation sites, Y542 and Y580, in the C-terminus are not (**Fig. 2A-B**). Mutating R32 or R138 impairs the association of SHP2 with phosphorylated growth factor receptors and substrates (56), and has been shown to inhibit EGF stimulation of MAPK activation in cells (55). Thus, the inability of Shp2a-R32M-R138M to rescue caudal fin-fold regeneration suggests

that Shp2a requires recruitment or binding to a substrate for its function in caudal fin-fold regeneration. In comparison, mutating Y542 or Y580 prevents binding of SHP2 to GRB2, and reduces, but importantly does not abolish, the activation of MAPK in response to stimulation by some growth factors in tissue culture cells, suggesting that the SHP2-GRB2 interaction is dispensable in some contexts (14, 18, 58). We conclude that expression of Shp2a-Y542F-Y580F (YF) in *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos apparently mediated sufficient MAPK activation to rescue caudal fin-fold regeneration.

Following amputation, regeneration proceeds through wound healing, blastema formation, and then regenerative outgrowth to replace the lost tissue (1, 2). *Junbb* expression is maintained well into the initial stage of regenerative outgrowth (62), indicating that *junbb* is a definitive blastema marker. We show that the amputated caudal fin-folds of *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos express *junbb*, like their siblings (**Fig. 3**), indicating that both wound healing and blastema formation occur normally in the absence of Shp2. This appears to be in contrast with previous results showing that Fgfr1 signalling is required for blastema formation (43, 66). Whereas there are overlaps in FGFR1 and SHP2 signalling (45), apparently, Fgfr1 and Shp2a signalling in zebrafish differ to such an extent that blastema formation is dependent on Fgfr1, but not on Shp2. In addition to promoting MAPK signalling, SHP2 has been shown to promote or inhibit phosphoinositide 3-kinase (PI3K) signalling (10, 67). Interestingly, the symptoms that present *in vivo* as a result of loss of *ptpn11* or activating mutations of SHP2 (11) appear to be primarily due to the effect on MAPK signalling. For example, mice expressing the activating SHP2 mutant Q79R display MAPK hyperphosphorylation and congenital heart defects, whilst both these phenotypes are ameliorated in Q79R x *Erk1*$^{-/-}$ mice (68). In comparison, genetic ablation of PTPN11 in retinal cells results in reduced MAPK phosphorylation but does not affect AKT phosphorylation (64). The defects that result are also not rescued by mutating the antagonist of PI3K signalling, phosphatase and tensin homolog (PTEN), which normally increases PI3K signalling (23). However, hyper-active KRas, which has been shown to alleviate the requirement for SHP2 in the maintenance of hematopoietic stem cells (29), does rescue the retinal defects. As PI3K signalling has previously been shown to be required for blastema formation (38), and blastema formation was normal in zebrafish embryos lacking functional Shp2, we conclude that it is unlikely that Shp2 acts through PI3K signalling.

Regenerative outgrowth is characterized by proliferation and differentiation of cells to replace the lost tissue. Our results from whole-mount immunohistochemistry using

an antibody specific for PCNA, suggest that cell proliferation is arrested in the regenerating caudal fin-folds of *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos, compared to siblings (**Fig. 4, S2**). In addition, p-MAPK staining in *ptpn11a*$^{-/-}$*ptpn11b*$^{-/-}$ zebrafish embryos is reduced compared to siblings (**Fig. 5, S3**), suggesting the arrested proliferation is due to reduced MAPK signalling. Moreover, inhibiting MAPK signalling using an inhibitor of MEK1, upstream of MAPK, was sufficient to inhibit regeneration and arrest cell proliferation during caudal fin-fold regeneration (**Fig. 6, S4**). Our results are consistent with previous work demonstrating a requirement for Fgfr1 signalling in proliferation during zebrafish caudal fin regeneration (42, 44, 66), and reduced proliferation and regeneration in response to MEK1 inhibition during zebrafish heart regeneration (48). Recent work shows that loss of SHP2 in mice, resulting in reduced MAPK signalling and reduced proliferation, leads to impaired muscle regeneration, and this was attributed to satellite cell quiescence (69). Possibly, the loss of Shp2 in zebrafish embryo caudal fin-folds induces quiescence in dedifferentiated cells of the blastema. This would certainly be in concordance with our results showing that regenerative outgrowth was impaired, despite normal blastema formation. Collectively, our results suggest an essential role for Shp2a-MAPK signalling in promoting cell proliferation during the regenerative outgrowth phase of regenerating zebrafish embryo caudal fin-folds.

In conclusion, we demonstrate for the first time that Shp2 is required for embryonic zebrafish embryo caudal fin-fold regeneration. Loss of Shp2 inhibits zebrafish embryo caudal fin-fold regeneration associated with reduced proliferation and reduced MAPK phosphorylation. Rescue of regeneration by re-expression of Shp2a is dependent on the catalytic activity and the SH2 domains of Shp2a. Consistent with Shp2a promoting MAPK signalling, zebrafish embryo caudal fin-fold regeneration is inhibited by MEK1 inhibition, and is associated with reduced proliferation. Hence, our results suggest Shp2 is indispensable during zebrafish embryo caudal fin-fold regeneration, and is required to coordinate proper proliferation mediated by MAPK signalling during the regenerative outgrowth of the zebrafish embryo caudal fin-fold.

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SUPPORTING INFORMATION

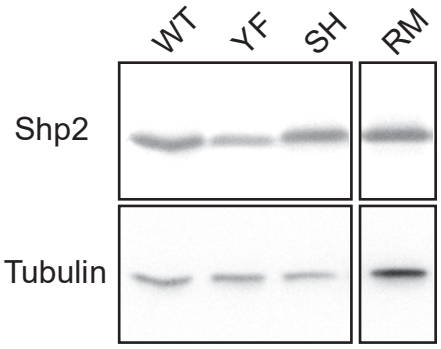

Fig. S1. Similar expression levels of *ptpn11a* mutants. Human embryonic kidney 293 T cells were transfected with CMV-promoter driven expression vector for zebrafish Shp2 mutants: Shp2a wild-type (WT); Shp2a Y542F, Y580F (YF); Shp2a R32M, R138M (SH); or Shp2a R466M (RM). Cells were lysed and the lysates run on an SDS-PAGE gel. Blots were probed using a SHP2-specific antibody and developed using enhanced chemiluminescence. Blots were stripped and re-probed for Tubulin as a loading control. All samples were loaded on the same blot, samples between SH and RM were removed as they were not relevant.

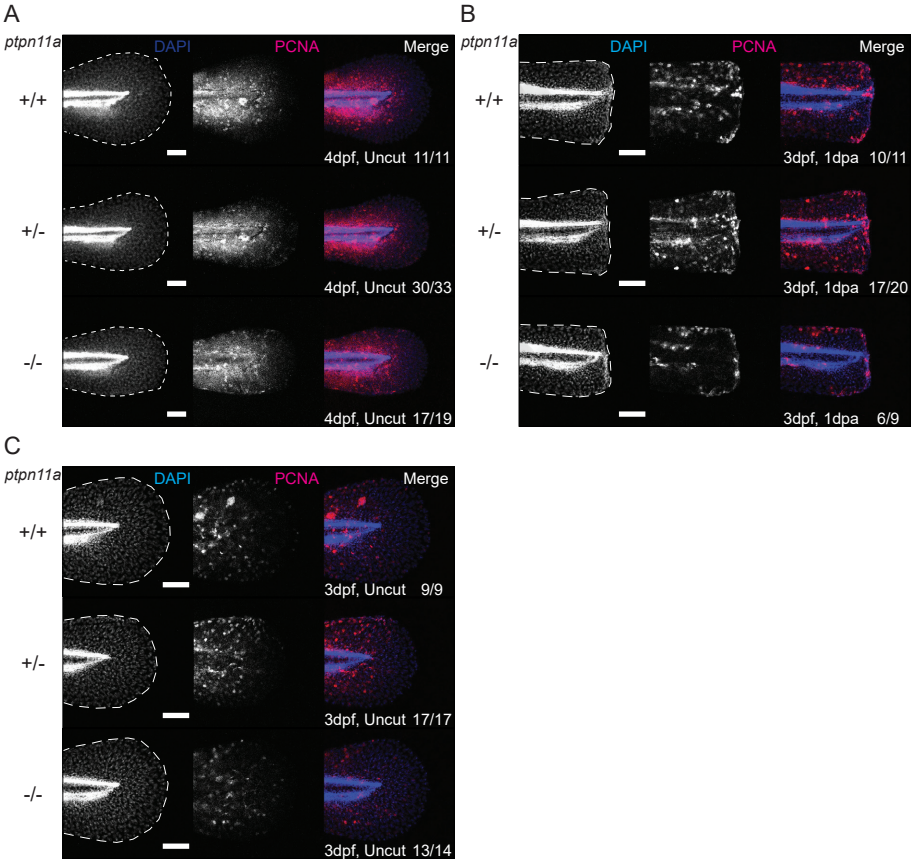

Fig. S2. Normal proliferation in uncut caudal fin-folds of Shp2 deficient embryos. Uncut embryos from a *ptpn11a*$^{+/-}$*ptpn11b*$^{-/-}$ in-cross were fixed at 4dpf (4dpf, uncut) in parallel to the embryos depicted in **Fig. 4** (**A**). At 2dpf the caudal fin-fold of embryos from a *ptpn11a*$^{+/-}$*ptpn11b*$^{-/-}$ in-cross was amputated and allowed to regenerate. Embryos were fixed at 1dpa (i.e. 3dpf, 1dpa) (**B**), or equivalent for uncut controls (3dpf, uncut) (**C**). Embryos were subjected to whole-mount immunohistochemistry using an antibody specific for the cell proliferation marker PCNA (red). The embryos were counterstained with DAPI (blue). Maximum intensity projection images were taken of the caudal fin-folds and all embryos were genotyped. Representative images of embryo caudal fin-folds are shown, and in the left panels the edge of the fin-fold is indicated with a dashed line. The number of embryos showing similar patterns/ total number of embryos analysed is indicated in the bottom right corner of the right panels. The scale bar represents 100µm.

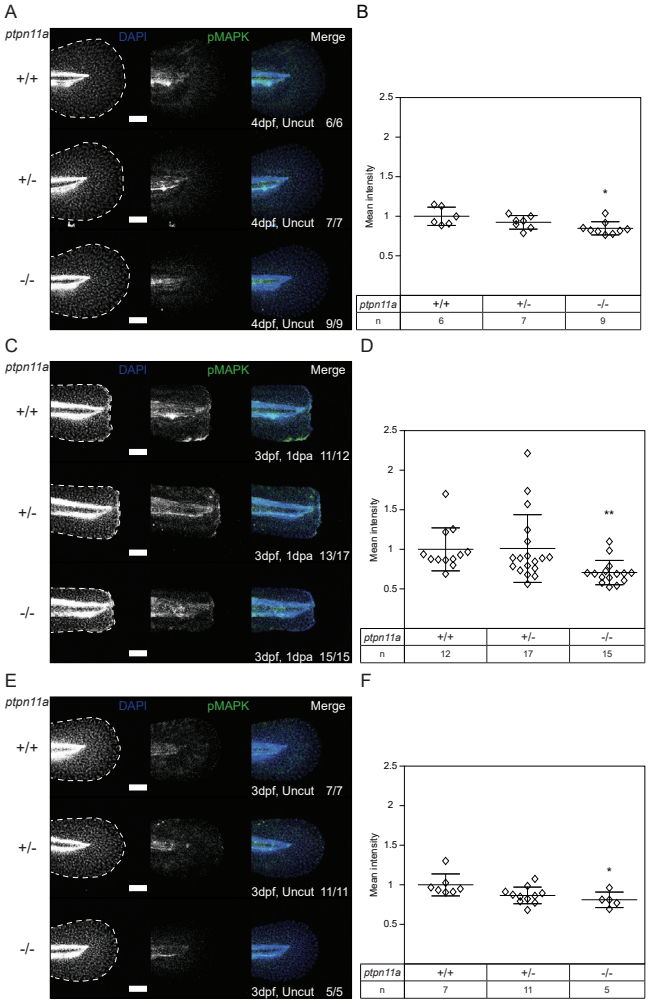

Fig. S3. Reduced p-MAPK in the caudal fin-folds of Shp2 deficient embryos. Uncut embryos from a *ptpn11a*$^{+/-}$*ptpn11b*$^{-/-}$ in-cross were fixed at 4dpf (4dpf, uncut) in parallel to the embryos depicted in **Fig. 5A** (**A**, **B**). At 2dpf the caudal fin-fold of embryos from a *ptpn11a*$^{+/-}$*ptpn11b*$^{-/-}$ in-cross was amputated and allowed to regenerate. Embryos were fixed at 1dpa (i.e. 3dpf, 1dpa) (**C**, **D**), or equivalent for uncut controls (3dpf, uncut) (**E**, **F**). Embryos were subjected to whole-mount immunohistochemistry using a p-MAPK-specific antibody (Thr202/Tyr204) (green). The embryos were counterstained with DAPI (blue). Maximum intensity projection images were taken of the caudal fin-folds and all embryos were genotyped. Representative images of embryo caudal fin-folds are shown, and in the left panels the edge of the fin-fold is indicated with a dashed line. The number of embryos showing similar patterns/ total number of embryos analysed is indicated in the bottom right corner of the right panels. The scale bar represents 100µm. p-MAPK immunofluorescence was quantified by mean intensity of the region between the notochord and the edge of the caudal fin-fold. Means within amputated or uncut groups are compared to *ptpn11a*$^{+/+}$*ptpn11b*$^{-/-}$ embryos using a Mann-Whitney U-test. The number of embryos analysed is indicated (n). Significance: ** $p<0.01$; * $p<0.05$; error bars represent standard deviation (**D**). Quantification of p-MAPK immunofluorescence at 4dpf, 2dpa is depicted in **Fig. 5B**.

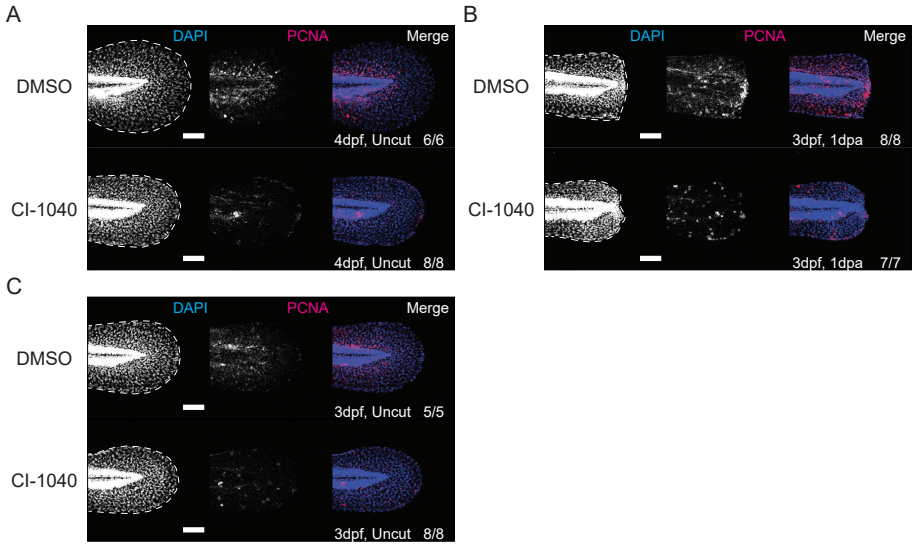

Fig. S4. Arrested proliferation in the caudal fin-folds of wild-type embryos following MAPK inhibition. Uncut wild-type embryos treated with 50nM PD184352 (CI-1040) were fixed at 4dpf (4dpf, uncut) in parallel to the embryos depicted in **Fig. 6A** (**A**). At 2dpf the caudal fin-fold of wild-type embryos was amputated and allowed to regenerate whilst incubated with 50nM PD184352 (CI-1040). Embryos were fixed at 1dpa (i.e. 3dpf, 1dpa) (**B**), or equivalent for uncut controls (3dpf, uncut) (**C**). Embryos were subjected to whole-mount immunohistochemistry using an antibody specific for the cell proliferation marker PCNA (red). The embryos were counterstained with DAPI (blue). Maximum intensity projection images were taken of the caudal fin-folds. Representative images of embryo caudal fin-folds are shown, and in the left panels the edge of the fin-fold is indicated with a dashed line. The number of embryos showing similar patterns/ total number of embryos analysed is indicated in the bottom right corner of the right panels. The scale bar represents 100µm. PCNA immunofluorescence at 4dpf, 2dpa is depicted in **Fig. 6B**.

Table S1. Primers used for amplifying *junbb* from zebrafish embryo cDNA, and *ptpn11a* from genomic zebrafish DNA.

Primer	Sequence
junbb FWD 1	TGGGTTACGGTCACAACGAC
junbb REV 1	CAGTGTCCGTTCTCTTCCGT
BamHI-*junbb* FWD (nested)	ATAGGATCCTACACGACGCTGAACGCATA
junbb-EcoRI REV (nested)	CTCGAATTCGTGTCCGTTCTCTTCCGTCC
junbb FWD 2	TACACGACGCTGAACGCATA
junbb-T7 REV 2	TAATACGACTCACTATAGGTGTCCGTTCTCTTCCGTCC
Ptpn11a WT FWD	GAAGGTGACCAAGTTCATGCTACATGGAGCATCATGGAC
Ptpn11a KO FWD	GAAGGTCGGAGTCAACGGATTACATGGAGCATCATGGAT
Ptpn11a REV	AGCTCAATGACGTCTCCGTTTTTCTCTTT
Ptpn11a FWD 1	GCGCTGTCACACACATTAAGA
Ptpn11a REV 2	TCACAGCCAATAAAGAGAAGC
Ptpn11a FWD (nested) 3	CGACCTGTATGGTGGAGAGAA
Ptpn11a REV (nested) 4	TCCCAAATTGTCATGTAAGG