Live-imaging of endothelial Erk activity reveals dynamic and sequential signalling events during regenerative angiogenesis

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Abstract

The formation of new blood vessel networks occurs via angiogenesis during development, tissue repair and disease. Angiogenesis is regulated by intracellular endothelial signalling pathways, induced downstream of Vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs). A major challenge in understanding angiogenesis is interpreting how signalling events occur dynamically within endothelial cell populations during sprouting, proliferation and migration. Erk is a central downstream effector of Vegf-signalling and reports the signalling that drives angiogenesis. We generated a vascular Erk biosensor transgenic line in zebrafish using a kinase translocation reporter that allows live-imaging of Erk-signalling dynamics. We demonstrate the utility of this line to live-image Erk activity during physiologically relevant angiogenic events. Further, we reveal dynamic and sequential endothelial cell Erk-signalling events following blood vessel wounding. Initial signalling is dependent upon Ca\(^{2+}\) in the earliest responding endothelial cells, but is independent of Vegfr-signalling and local inflammation. The sustained regenerative response however, involves a Vegfr-dependent mechanism that initiates concomitant with the wound inflammatory response. This work reveals a highly dynamic sequence of signalling events in regenerative angiogenesis and validates a new resource for the study of vascular Erk-signalling in real-time.
Introduction

The formation of new blood vessels from pre-existing vasculature (angiogenesis) is a fundamental process central in the formation of a viable embryo and in the pathogenesis of many diseases (Carmeliet and Jain, 2011; Chung and Ferrara, 2011; Potente et al., 2011). Angiogenesis is controlled by intricately regulated cell-cell, cell-matrix and intracellular signalling events. The activity of extracellular signal-regulated kinase (ERK) downstream of the vascular endothelial growth factor A (VEGFA)/VEGF receptor 2 (VEGFR2) signalling pathway is essential for both developmental and pathological angiogenesis (Koch and Claesson-Welsh, 2012; Simons et al., 2016). ERK-signalling is also required downstream of VEGFC/VEGFR3 signalling in lymphangiogenesis (Deng et al., 2013). ERK is required for angiogenic sprouting, proliferation and migration, with genetic or pharmacological inhibition of ERK-signalling resulting in impaired blood vessel development in zebrafish and mice (Srinivasan et al., 2009; Costa et al., 2016; Nagasawa-Masuda and Terai, 2016; Shin et al., 2016a). Cancer-associated vessels have high ERK activity and inhibition of ERK-signalling blocks cancer-associated angiogenesis in mice (Wilhelm et al., 2004; Murphy et al., 2006). Beyond the formation of new vessels, ERK-signalling is also essential to maintain vascular integrity in quiescent endothelial cells (ECs) (Ricard et al., 2019), altogether demonstrating a central role for ERK in vascular biology.

Despite its importance, vascular ERK-signalling has largely been examined with biochemical assays or in tissues in static snapshots. Numerous studies have suggested that ERK-signalling is likely to be highly dynamic during angiogenic events, for example studies that examine Erk activation using antibodies to detect phosphorylated Erk (pErk) have observed changes associated with increased EC signalling, EC motility and specific EC behaviours (Costa et al., 2016; Nagasawa-Masuda and Terai, 2016; Shin et al., 2016a). In zebrafish, live-imaging of blood ECs at single cell-resolution coupled with carefully staged immunofluorescence staining for pErk suggested that an underlying dynamic Erk-signalling event may control tip-cell maintenance in angiogenesis (Costa et al., 2016). Nevertheless, EC signalling dynamics at the level of key intracellular kinases, such as ERK, remain poorly understood. This gap in our understanding is largely due to a gap in our ability to live-image changes in signalling as they occur.
A number of new biosensors have now been applied in vitro and in vivo that allow live-imaging of proxy readouts for intracellular signalling events (reviewed in detail in (Shu, 2020)). One approach used, has involved application of biosensors that utilise fluorescence resonance energy transfer (FRET)-based readouts. The first ERK FRET-based biosensor (ERK activity reporter (EKAR)) was developed in 2008 (Harvey et al., 2008). Since then, modifications had been made to improve sensitivity and dynamic range and to generate other ERK FRET-based biosensors such as EKAR-EV, RAB-EKARev, and sREACH (Komatsu et al., 2011; Ding et al., 2015; Tang and Yasuda, 2017; Mehta et al., 2018). Importantly, these ERK FRET-based biosensors had been applied in vivo to visualise ERK-signalling dynamics in various cell types during development, cell migration, and wound healing (Kamioka et al., 2012; Mizuno et al., 2014; Goto et al., 2015; Hiratsuka et al., 2015; Kamioka et al., 2017; Takeda and Kiyokawa, 2017; Sano et al., 2018; Wong et al., 2018). While ERK FRET-based biosensors have been widely reported, they are limited in requiring extensive FRET controls and a low speed of acquisition for FRET based imaging.

More recently, Regot and colleagues generated the ERK-kinase translocation reporter (KTR)-Clover construct (hereafter referred to as EKC), that allows for dynamic analysis of ERK activity using a readout not involving FRET. A fluorescence-based kinase activity reporter translates ERK phosphorylation events into a nucleo-cytoplasmic shuttling event of a synthetic reporter (Regot et al., 2014). Thus, the KTR system allows rapid quantifiable measurements of ERK activity based upon subcellular localisation of a fluorescent fusion protein, and is more sensitive to phosphatase-mediated kinase activity downregulation when compared to other commonly used reporters. This has been applied to enable dynamic ERK-signalling pulses to be analysed at single-cell resolution both in vitro and also in vivo (Regot et al., 2014; de la Cova et al., 2017; Mayr et al., 2018; Goglia et al., 2020; Pokrass et al., 2020; De Simone et al., 2021), where it has demonstrated to be of high utility.

In this study, we generated a vascular EC-restricted EKC zebrafish transgenic strain and assessed its utility to study angiogenesis in vivo. We apply real-time quantification of Erk-signalling dynamics during developmental angiogenesis and vessel regeneration. We validate methods to quantify Erk activity during real time imaging that will be applicable in a variety of settings in vascular biology and beyond.
Demonstrating the promise of this approach, we here identify an immediate early Erk-signalling response to wounding of vasculature that is Ca\(^{2+}\) signalling dependent and distinct from a later Vegfr-driven regenerative response. Overall, this work reports a unique resource for imaging of vascular signalling and further illuminates mechanisms of vascular regeneration following wounding.

**Results**

**Generation of a zebrafish EC-EKC transgenic line**

KTRs utilise a kinase docking and target site that is juxtaposed to a phospho-inhibited nuclear localization signal (NLS) and attached to a fluorescent tag (Regot et al., 2014). Upon kinase activity the NLS is inactive and the fluorescent tag detected in the cytoplasm; when the kinase is not active, dephosphorylated NLS leads to increased nuclear localisation. The EKC module that we took advantage of here relies upon the well characterised ERK-dependent transcription factor ELK1, utilising the ERK docking site (**Figure 1A**) (Chang et al., 2002;Regot et al., 2014) This reporter has previously been shown to report Erk activity in vivo (de la Cova et al., 2017;Mayr et al., 2018;Pokrass et al., 2020;De Simone et al., 2021). To visualise real-time Erk-signalling in ECs, we expressed this reporter under the control of an EC-specific promoter (**fli1aep** (Villefranc et al., 2007)) (**Figure 1A-E**). Blood vessel development was unaffected in **Tg(fli1aep:EKC)** transgenic embryos and larvae (**Figure 1B-E**). Furthermore, transgenic adults displayed no adverse morphological features and were fertile (data not shown), indicating that EKC does not inhibit Erk-signalling in vivo, or cause developmental phenotypes and consistent with previous findings (Mayr et al., 2018;De Simone et al., 2021).

To test if the **Tg(fli1aep:EKC)** line reports vascular Erk-signalling, embryos were treated with either DMSO, mitogen-activated protein kinase kinase (MEK) inhibitor SL327, or pan-VEGFR inhibitor SU5416, and vascular EKC localisation examined at 27 hpf. Tip ECs in developing ISVs have been shown to have high Erk activity (Costa et al., 2016;Nagasawa-Masuda and Terai, 2016;Shin et al., 2016a) and we observed nuclear depleted EKC localisation in leading angiogenic ISV cells including at the level of the dorsal longitudinal anastomosing vessel (DLAV) in DMSO treated embryos. (**Figure 1F-F'',I**). In contrast, ISV ECs of embryos treated with either
SL327 or SU5416 had nuclear enriched EKC localisation indicating inactive Erk-signalling (Figure 1G-I). To best visualise these differences in signalling and differences shown below, we used a heat map of nuclear EC EKC intensity that is inverted so that blue-scale indicated low signalling (nuclear enriched) and red-scale indicates high signalling (nuclear depleted) (Figure 1F"'-H"'). Therefore, we confirmed that the Tg(fli1aep:EKC) (hereafter EC-EKC) transgenic line enables quantification of Erk activity in developing ECs.

The EC-EKC line enables visualisation and quantification of dynamic Erk activity during primary angiogenesis

We next sought to determine whether the EC-EKC line reports physiologically relevant Erk-signalling events. Using immunofluorescence staining, ISV tip cells that sprout from the dorsal aorta (DA) have been shown to have higher Erk-signalling than ECs that remain in the DA during the initiation of angiogenesis (Nagasawa-Masuda and Terai, 2016; Shin et al., 2016a). We examined 22 hpf embryos and indeed observed that sprouting ISV ECs display high Erk activity based upon EKC localisation (Figure 1-figure supplement 1A-B). However, many DA ECs also appeared to have nuclear depleted EKC localisation (Figure 1-figure supplement 1A, yellow arrows). To compare EKC and Erk-signalling levels between sprouting tip-cells and the DA, we utilised multiple methods. We found that measuring the nuclear/cytoplasm EKC intensity ratio in DA ECs was inaccurate because DA ECs are irregular in morphology, making cytoplasmic quantification unreliable (Figure 1-figure supplement 1A'). Previous studies have compared nuclear EKC with nuclear H2B-mCherry intensity in the same cell as a ratio to measure Erk activity (e.g. in vulval precursor cells in the worm (de la Cova et al., 2017)). We assessed the ratio of nuclear EKC/H2B-mCherry intensity in double transgenic EC-EKC; Tg(fli1a:H2B:mCherry) embryos and found that the ISV tip cells had higher Erk activity than adjacent DA “stalk” ECs (Figure 1-figure supplement 1A’ and C). We used a stable Tg(fli1a:H2B-mCherry) transgenic line with consistent H2B-mCherry intensity. Next, we investigated whether nuclear EKC intensity alone was sufficient to compare Erk-signalling between ECs. The ratio of nuclear EKC intensity of the sprouting ISV tip-cell and the adjacent DA “stalk” EC clearly showed higher signalling in tip-cells and was consistent with EKC/H2B-mCherry measurements (Figure 1-
figure supplement 1C). Thus, we establish that both methods can be reliably used, when measurement of nuclear/cytoplasm EKC intensity is not possible because of difficulty identifying a cell's cytoplasm. We compare nuclear EKC intensities for analyses hereafter.

Next, we correlated EC Erk-signalling state (based on EKC intensity) with a cell's migratory state (based on nuclear ellipticity) as previous studies have suggested a correlation (Costa et al., 2016). At 28 hpf, ISV tip cells were either located above the horizontal myoseptum with elliptical nuclei indicative of a migrating EC, or at the level of the future DLAV, with less-elliptical (oblate) nuclei indicative of a non-migrating EC (Figure 1-supplement 1D-F). We found that migrating ECs had higher Erk activity than non-migrating ECs, irrespective of their tip or stalk cell location in an ISV (Figure 1-figure supplement 1D-G). This is consistent with previous studies of Vegfa/Kdr/Kdrl/Erk signalling in zebrafish ISVs (Yokota et al., 2015; Costa et al., 2016; Nagasawa-Masuda and Terai, 2016; Shin et al., 2016a) and confirms a strong correlation between ISV EC motility and EC Erk-signalling.

Using carefully staged immunofluorescence analyses, it was previously suggested that when tip cells divide in ISV angiogenesis, daughter cells show asymmetric Kdrl/Erk signalling that re-establishes the tip/stalk EC hierarchy (Costa et al., 2016). However, an analysis of fixed material can only ever imply underlying dynamics. To investigate the dynamics of Erk-signalling upon tip-cell division, we performed high-speed time-lapse imaging of ISV tip ECs as they undergo mitosis in 24 hpf embryos. Immediately preceding cell-division, ECs display cytoplasmic localisation of H2B-mCherry due to the disruption of the nuclear membrane (Figure 2A, yellow arrow). High-speed live-imaging of ISV tip ECs revealed nuclear enriched EKC localisation during cell division (Figure 2A-C), which was maintained until cytokinesis (Figure 2B, Video 1) but may reflect nuclear membrane breakdown rather than altered cellular signalling. Subsequently, daughter ECs in the tip position progressively increased their Erk activity, while ECs in the trailing stalk daughter position remained nuclear enriched, showing asymmetric Erk-signalling activity rapidly following cell division (Figure 2B-I, Video 1). To accurately assess this across multiple independent tip-cell divisions, we measured the ratio of tip/stalk daughter cell nuclear EKC intensity over time. This revealed that tip cells consistently increased their Erk
activity relative to stalk cells in a progressive manner with the most dramatic
asymmetry observed ~21 minutes post-cytokinesis (Figure 2J,K, Video 1).
Collectively, the EC-EKC line enabled quantitative assessment of physiologically
relevant Erk activity by real-time live imaging and confirmed previously suggested
asymmetric signalling post tip-cell division.

Vessel wounding induces rapid Erk activation

As a major downstream target for VEGFA/VEGFR2 signalling, ERK is essential for
stimulating ectopic sprouting of otherwise quiescent mature vessels (Wilhelm et al.,
2004;Murphy et al., 2006). However, Erk-signalling dynamics during pathological
angiogenesis have not been analysed in detail. To determine whether the EC-EKC
line can be used to dynamically visualise Erk activation in ECs in pathological
settings, we analysed EC Erk activity following vessel wounding using a laser
ablation method. We chose this model because vessel wounding in 4 dpf larvae
results in highly reproducible Vegfa/Kdr/Kdrl signalling-dependent vessel
regeneration (Gurevich et al., 2018). Importantly, cell wounding induces ERK-
signalling in vitro and in vivo in other settings (Matsubayashi et al., 2004;Li et al.,
2013;Hiratsuka et al., 2015;Aoki et al., 2017;Mayr et al., 2018).

To visualise Erk-signalling dynamics following cellular ablation and vessel wounding,
we time-lapse imaged both ablated ISV ECs and the adjacent non-ablated ISV ECs
in 4 dpf EC-EKC larvae for 20 minutes before and for 22 minutes after vessel
wounding (Figure 3A-C). As a control, ISV ECs of unablated 4 dpf larvae were time-
lapse imaged for the same period. EKC localisation in the majority of ISV ECs
indicated low basal Erk-signalling in ECs of mature vessels (Figure 3D,D’,F,F’,H,I,
Videos 2-5). Upon vessel wounding, Erk activity in ablated ISV ECs immediately
increased (Figure 3E,E’,H,I, Videos 3 and 4). Surprisingly, Erk activity in ECs of
ISVs located adjacent to the ablated ISV (termed adjacent ISV) also rapidly
increased (Figure 3G,G’,H,I, Videos 3 and 5). Although the activation of Erk-
signalling in adjacent ISV ECs was slower than in ablated ISV ECs, the level of Erk
activation in ablated and adjacent vessels was comparable by 15 minutes post-
ablation (mpa, green dotted line) and consistent up to 22 mpa (Figure 3I). Both
venous and arterial ECs equally showed Erk activation 15 mpa in ablated ISVs post-
vessel wounding, suggesting that both venous and arterial ECs are able to rapidly activate Erk-signalling (Figure 3J). Finally, to understand the relationship between Erk activation in vessels and distance from the wound, we measured the response of ECs in immediately adjacent, 2nd adjacent and 3rd adjacent ISVs from the ablated ISV (in an anterior direction). We found that the activation of Erk signalling was largely limited to the wounded and immediately adjacent ISVs (Figure 3-figure supplement 1).

The initial rapid Erk-signalling response is not induced by macrophages or Vegfr activity

Macrophages recruited to a wound site have been shown to provide a local source of Vegfa that stimulates vessel regeneration (Gurevich et al., 2018). Therefore, we investigated whether macrophages are required for rapid Erk activation in ISV ECs. As previously reported (Gurevich et al., 2018), macrophage recruitment to the wound was minimal at 15 mpa, while robust macrophage recruitment was observed 3 hours post-ablation (hpa), suggesting that macrophages may not contribute to rapid Erk activation (Figure 3-figure supplement 2A-D). We depleted macrophages by knocking down Spi-1 proto-oncogene b (Spi1b) and Colony stimulating factor 3 receptor (Csf3r) using established morpholino oligomers (Rhodes et al., 2005;Ellett et al., 2011;Pase et al., 2012) (Figure 3-figure supplement 2E-G). We found that depletion of macrophages led to a quantifiable but mild reduction in normal vessel regeneration measured at 24 hpa in this model (Figure 3-figure supplement 2H-J). The rapid EC Erk activation post-wounding was unaffected upon macrophage depletion (Figure 3K, Figure 3-figure supplement 2K-V'). We next tested whether Vegfr-signalling was required for this rapid Erk activation. Erk activation in both ablated and adjacent ISV ECs 15 mpa was blocked in larvae treated with SL327, indicating that it requires upstream Mek activation (Figure 3L, Figure 3-figure supplement 3D-M'). However, treatment with two independent and validated VEGFR-inhibitors, SU5416 (Figure 1H-I) and AV951 (Figure 3-figure supplement 3A-C), did not impair the rapid Erk activation at 15 mpa (Figure 3L, Figure 3-figure supplement 3O-Z'). Therefore at 15 mpa, Erk activation in both ablated and adjacent ISV ECs is induced independently of either macrophages or Vegfr-signalling, suggesting an initial response to vessel wounding that has not been previously examined.
Following the initial rapid Erk activation, signalling is progressively restricted to regenerating vessels

Previous studies have shown that local wounding induces a rapid burst in ERK-signalling in surrounding cells (Matsubayashi et al., 2004; Li et al., 2013; Hiratsuka et al., 2015; Aoki et al., 2017; Mayr et al., 2018). To determine whether the initial Erk activation in ISV ECs post-vessel wounding was maintained, Erk activity was followed over a longer time-course until 3 hpa, when robust macrophage recruitment was observed (Figure 3-figure supplement 2C,D). Erk activity was again increased upon vessel wounding in both ablated and adjacent ISV ECs at 15 mpa (Figure 4A-D, Figure 4-figure supplement 1A-I’). Erk activity was maintained until 30 mpa in adjacent ISV ECs, but then gradually decreased and returned to non-ablated control levels by 1 hpa (Figure 4B-D). By contrast, high Erk activity was maintained for the duration in ablated ISV ECs (Figure 4A,A’,C,D). To test if this difference in Erk activity was influenced by long-term time-lapse imaging, Erk-signalling was analysed in ISV ECs of 3 hpa larvae. Similar to the time-course analysis, Erk activity in ablated ISV ECs was high at 3 hpa, while ECs in adjacent ISVs were at non-ablated control level (Figure 4-figure supplement 1J-N).

Given that the initial rapid burst of Erk activation progressively returns to basal levels in unwounded vessels, we assessed if this was a general wound response. We examined the initial Erk-signalling burst in muscle and skin cells following a large puncture wound using a ubiquitous EKC strain (Mayr et al., 2018). This confirmed that an initial activation of Erk signalling in cells surrounding the puncture wound was only transient (Video 6) and in this case was progressively lost even in cells at the immediate site of the wound, unlike in regenerating vessels. To further investigate whether only regenerating ISVs maintain high Erk activity after wounding, tissue in between the ISVs was ablated without injuring the ISVs in 4 dpf EC-EKC larvae (termed control ablation hereafter). Erk activity in surrounding ISV ECs was analysed at 15 mpa and 3 hpa. Similar to vessel ablation, this adjacent tissue ablation resulted in rapid activation of Erk-signalling in ISV ECs (Figure 4-figure supplement 2A-C). Erk activity in these ECs decreased to non-ablated control levels by 3 hpa (Figure 4-figure supplement 2A-C). Therefore, Erk-signalling is immediately activated in
muscle, skin epithelial and ECs upon injury, but only regenerating vessels retain this high activity at 3 hpa upon vessel wounding.

**Vegfr-signalling drives ongoing Erk activity to control vessel regeneration**

We next examined if ongoing Erk activity in ablated ISV ECs was maintained by Vegfr-signalling consistent with earlier reports (Gurevich et al., 2018). To test this, we analysed Erk activity of ablated ISV ECs in 3 hpa larvae treated with inhibitors of the Kdr/Kdrl/Mek/Erk signalling pathway. Treatment with SL327 inhibited Erk activation at 3 hpa, as did treatment with the Vegfr-inhibitor SU5416 ([Figure 5A, Figure 5-figure supplement 1A-F',I-J']). Furthermore, we used an F0 CRISPR approach (Wu et al., 2018) to generate kdrl knockout embryos (termed kdrl crispant hereafter). These embryos phenocopied earlier reported mutant and morphant phenotypes ([Figure 5-figure supplement 1K,L]) (Habeck et al., 2002; Covassin et al., 2006). 3 hpa F0 crispant larvae displayed reduced Erk activity in EC-EKC measurements compared with ISV ablation control larvae ([Figure 5B, Figure 5-figure supplement 1M-T']). Unlike drug treated larvae, kdrl crispants displayed a mild reduction in Erk activity, likely due to compensation from other Vegfrs, such as Kdr, and/or Flt4 (zebrafish orthologue of VEGFR3) (Covassin et al., 2006; Shin et al., 2016b). Overall, these genetic and pharmacological approaches indicate that Vegfr/Mek signalling is required for sustained high Erk activity in ablated ISV ECs at 3 hpa. To determine the functional relevance of this in ongoing regeneration, we treated embryos following ablation-based wounding with SU5416 or two independent Mek inhibitors: SL327 and Trametinib. We observed that inhibition of Vegfr- or Erk-signalling completely blocked all ongoing vessel regeneration ([Figure 5C, Figure 5-figure supplement 1U-X]). Finally, we found no difference in EC-EKC activation at 3 hpa in the absence of macrophages, suggesting that macrophages play a modulatory role in vessel regeneration and are not the sole source of Vegfs in this laser ablation model ([Figure 5D, Figure 5-figure supplement 2]).

Interestingly, we noted that while treatment with SU5416 at 10 μM blocked ongoing Erk activation ([Figure 5A, Figure 5-figure supplement 1I-J']), treatment with the same inhibitor at a lower dose of 4 μM did not completely block Erk activity ([Figure 5A, Figure 5-figure supplement 1G-H']). To further investigate this with more spatial
resolution, we examined Erk activity in ISV ECs relative to their distance from the cellular ablation site. Erk-signalling in the first, second, and third ISV ECs from the wound was activated 3 hpa in control larvae, while treatment with 10 μM SU5416 inhibited signalling in ECs located in all of these positions (**Figure 5E,G,H, Figure 5-figure supplement 1C-D',I-J'**). However, with the intermediate dose of 4 μM SU5416, while the closest cell to the wound site still displayed Erk activity, as did the second cell from the wound site, the third and furthest from the wounded sites were now inhibited (**Figure 5F,H, Figure 5-figure supplement 1G-H'**). These results suggest that there is a gradient of Vegfr/Erk signalling activity in the ablated ISV ECs resulting in higher Vegfr/Erk activity in ECs closer to the wounded site, which can only be inhibited with SU5416 at higher concentrations. To test this, we examined the EC EKC levels relative to cell position and directly confirmed this graded activation at 3 hpa (**Figure 5I, Figure 4-figure supplement 1J-M'**). Together, these analyses confirm that during the ongoing response to vessel wounding, Vegfr-signalling is crucial and drives a positionally graded signalling response to regulate regenerating vessels.

**Ca**²⁺ signalling is required for initial rapid Erk activation upon vessel wounding

Although Vegfr-signalling is required for sustaining high Erk activity in ablated ISV ECs, it is not required for inducing the initial rapid Erk-signalling response. Activated by ATP released by damaged cells, Ca²⁺ signalling is one of the first intra-cellular mechanisms to be activated post-wounding in many cell types (reviewed in detail in (Ghilardi et al., 2020)). Accordingly, mechanical injury of blood vessels has been shown *in situ* to rapidly activate Ca²⁺ signalling in neighbouring endothelial cells in excised rat aorta (Berra-Romani et al., 2008;Berra-Romani et al., 2012). Although Ca²⁺ signalling activates Erk-signalling in endothelial cells downstream of the Vegfa/Vegfr2 signalling pathway (Koch and Claesson-Welsh, 2012;Moccia et al., 2012), Ca²⁺ signalling alone can also activate Erk-signalling (Xiao et al., 2011;Handly et al., 2015).

To determine whether Ca²⁺ signalling is rapidly activated in ablated ISV ECs in our model, we measured the dynamic expression of a ubiquitously expressed GCamp, a GFP-based Ca²⁺ probe, using the *Tg(actb2:GCaMP6f);Tg(kdrl:mCherry-CAAX)*
transgenic line (Herzog et al., 2019). We used a validated transgenic line which has previously demonstrated a general Ca$^{2+}$ wound response and Ca$^{2+}$ signalling in brain tumours and associated microglia (Chia et al., 2019; Herzog et al., 2019). We observed a general response in tissue surrounding the ablated site (data not shown), as well as active Ca$^{2+}$ signalling in immune cells (Figure 6A, Videos 7,8, as previously described in (Yoo et al., 2012; Razzell et al., 2013; de Oliveira et al., 2014; Beerman et al., 2015; Herzog et al., 2019; Poplimont et al., 2020)) in the same movies analysed below, validating the utility of this line. ISVs in non-ablated 4 dpf larvae did not show Ca$^{2+}$ signalling, indicating low Ca$^{2+}$ activity in stable ISVs (Figure 6B, Video 7). In contrast, ablated ISV ECs showed a rapid pulse of active Ca$^{2+}$ signalling at 5mpa, which progressively decreased and returned to the level of the surrounding tissue (Figure 6A,B, Video 8). Active Ca$^{2+}$ signalling was not observed in adjacent ISVs (Figure 6A,B, Video 8). To determine whether Ca$^{2+}$ signalling is required for rapid Erk activation in ablated ISV ECs, 4 dpf EC-EKC larvae were treated with either DMSO or a potent Ca$^{2+}$ signalling inhibitor Nifedipine for 30 minutes. Nifedipine treatment did not inhibit Erk-signalling activation in adjacent ISV ECs resulting in similar Erk activity as DMSO treated larvae 15 mpa (Figure 6C, Figure 6-figure supplement 1A-B',G-J'). However, Erk activation in ablated ISV ECs (where we observed the GCaMP signal above) was significantly reduced when compared to DMSO treated larvae (Figure 6C, Figure 6-figure supplement 1C-F'). This was reproduced in an independent experiment using Amlodipine, an alternative Ca$^{2+}$ signalling inhibitor (Figure 6D, Figure 6-figure supplement 1K-T'). This indicates that Ca$^{2+}$ signalling plays a crucial role upstream of Erk in the wound response, but also that the response is differentially regulated in ablated compared with adjacent vessels, indicative of additional underlying signalling complexity.

We next tested whether Ca$^{2+}$ signalling is required for maintaining Erk activity in ablated ISV ECs 3 hpa. To assess ongoing signalling, 4 dpf EC-EKC larvae were treated with either DMSO or Nifedipine 30 minutes prior to the 3 hpa timepoint. Activation of Erk-signalling in ablated ISV ECs 3 hpa was not inhibited by Nifedipine (Figure 6E, Figure 6-figure supplement 2A-G'). Inhibition of Ca$^{2+}$ signalling immediately following wounding between 0 and 30 mpa also had no impact on later Erk signalling at 3 hpa (Figure 6F, Figure 6-figure supplement 2H-N'). Thus, Ca$^{2+}$
signalling is required for rapid Erk activation, but not for maintaining Erk activity in ablated ISV ECs. In the analysis of Ca\(^{2+}\) signalling following vessel wounding, we noted that this transient pulse of Ca\(^{2+}\) signalling was highest in the ECs closest to the wounded site (Video 8). Thus, we further sought to determine if Erk-signalling in ECs closest to the wound activates first during the initial dynamic induction. Quantitative analysis based on multiple movies (including Video 3), showed that Erk-signalling in ECs proximal to the wounded site (first and second positioned ECs) activated first, followed by ECs further away from the wounded site (third, fourth and fifth ECs) (Figure 6G). Quantitatively the ECs proximal to the ablation site (first and second positioned ECs) showed the highest magnitude of difference from control, and this difference reduced as ECs were positioned further from the ablation site (Figure 6H). This shows that like the initial burst in Ca\(^{2+}\) signalling post-vessel wounding, Erk-signalling is activated progressively in ECs closest to the wounded site first, followed by those further away.

**Discussion**

ERK-signalling is a downstream target for a number of pathways essential for development (including VEGFA/VEGFR2, EGF/EGFR, FGF/FGFR pathways) and plays a central role in organ development by promoting proliferation, growth, migration and differentiation (Hogan and Schulte-Merker, 2017; Lavoie et al., 2020). As such, Erk-signalling must be tightly regulated in both its spatial and temporal activation. To understand how dynamically Erk activity is regulated in developing vasculature, we generated the EC-EKC transgenic line and validated its use as a proxy readout of active Erk-signalling in vasculature. We found that it both provided a valid readout for physiological Erk-signalling and uncovered previously unappreciated Erk-signalling dynamics during vessel regeneration (Figure 7). In the context of tip cell proliferation in angiogenesis, we revealed very rapid post-cell division signalling asymmetry, confirming previous work based on static imaging (Costa et al., 2016). In regenerative angiogenesis, we reveal a two-step mechanism for Erk-signalling activation post-vessel wounding, that involves an immediate and an ongoing signalling response that progressively limits Erk-signalling to vessels that are regenerating. Importantly, this study shows the utility of this new transgenic line to elucidate dynamic Erk-signalling events in vertebrate ECs and we suggest it will be a useful tool for diverse future studies of development and disease.
At a technical level, we used various quantification methods for measuring Erk activity in ECs and all generated valid results. The ratio of nuclear/cytoplasm EKC localisation gives the most accurate readout (Regot et al., 2014), but can only be used when a cell’s cytoplasmic fluorescence can be accurately measured. This is especially challenging for ECs which overlap and have unpredictable morphology in vascular tubes. De la Cova and colleagues, used a second generation ERK KTR which includes a nuclear localised H2B-mCherry expressed from the same promoter, allowing them to quantify Erk activity based on the Clover/mCherry ratio in *C. elegans* (de la Cova et al., 2017). We used a similar approach here with two independent transgenes driving EKC and H2B-mCherry and produced highly consistent results. It is worth noting that inter-embryo/larvae variations in H2B-mCherry intensity need to be considered, hence transgenic lines that express both ERK KTR and H2B-mCherry under a single promoter would be ideal. Finally, we also used the measurement of nuclear EKC normalised to the average EKC intensity of the DA to normalise for embryo to embryo variation. This approach also provided data consistent with the other two methods. Thus, overall this EC-EKC model is highly robust with multiple methods to quantify and normalise sensor localisation. As KTR reporters are used more frequently *in vivo* in the future, the quantification methods used here may be applied to many scenarios analysing cellular Erk activity in cells with complex 3D morphology.

Studies in zebrafish and *Xenopus* have demonstrated rapid Erk activation in epithelial cells upon local wounding, which subsides relatively quickly (within 1hpa) as tissue repair progresses (Li et al., 2013; Mayr et al., 2018). Interestingly, our work shows a similar, very rapid, Erk activation in all vasculature in proximity of a wound. This suggests a common, initial, rapid Erk-signalling response immediately post-wounding in many different cell types and tissues – as if cells adjacent to a wound are rapidly primed to respond. However, in the vasculature this signalling returned to pre-ablation levels by 1 hpa, while Erk activity was maintained for a longer timeframe only in the wounded vessels. This ongoing, later signalling was maintained through Vegfr activity, likely stimulated in part by Vegfa secreted from macrophages (Gurevich et al., 2018) and our data suggests other local sources of Vegfs (see Figure 7). Thus, Erk-signalling dynamics between wounded (ablated) and
unwounded (adjacent) vessels differed significantly. We suggest this difference represents an initial priming of the wounded tissue (the rapid Erk response) that is replaced overtime with sustained vascular Erk-signalling that is essential in the regenerative response.

Rapid Ca²⁺ signalling post-wounding is observed in multiple systems *in vitro and in vivo* (reviewed in detail in (Ghilardi et al., 2020)). Using both quantitative live imaging and pharmacological inhibition, we found that Ca²⁺ signalling is required for Erk activation in ablated ISV ECs. Taking advantage of the high spatial and temporal resolution in our model, we found that Ca²⁺-dependent Erk-signalling is activated progressively from cells closest to the wound to cells further away. This may be consistent with a wave of Ca²⁺ signalling through the wounded vessel. Activation of Erk-signalling at 2 mpa in wounded epithelial cells in *Xenopus* promotes actomyosin contraction and wound closure (Li et al., 2013). Therefore, rapid Ca²⁺ signalling-mediated Erk activation in the wounded vessel may ensure efficient wound closure in ablated ISVs. At a molecular mechanistic level, it seems likely that EC Ca²⁺ signalling is influenced by either the activity of transient receptor potential (TRP) channels (Smani et al., 2018) or P2X receptors (P2X4 or P2X7) (Surprenant and North, 2009) which are active in ECs and can influence angiogenesis, cytoskeletal remodelling and vascular permeability. We found no evidence that Ca²⁺ signalling influenced the broader, rapid Erk-signalling response in unwounded but adjacent vasculature. One interesting candidate to contribute to this broader mechanism is altered tissue tension associated with the tissue ablation, which had been shown in some contexts to modulate ERK-signalling (Rosenfeldt and Grinnell, 2000; Hirata et al., 2015). Perhaps consistent with this idea, we did not identify a mechanism required for rapid Erk activation in adjacent ISV ECs and vessel wounding was not required - tissue wounding in between ISVs alone activated Erk-signalling in surrounding ECs. Further work is needed to fully appreciate the role of mechanical contributions in this response. Nevertheless, rapid Erk activation in ECs upon wounding seems likely to potentiate these ECs to more rapidly respond to external growth factors such as Vegfa upon the later activation of the inflammatory response and initiation of sustained regenerative angiogenesis.
Taking advantage of spatial information in the imaging data, we showed that ECs in wounded ISVs that are actively regenerating at 3 hpa display a graded signalling response along the vessel at the level of Vegfr/Erk activity. This is likely due to a local source (or sources) of Vegfa and may explain why unwounded ISV ECs, which are further away from the Vegfa source, do not sustain high Erk activity at 3 hpa. In bigger wounds, excessive angiogenesis has been previously reported to occur from adjacent ISVs and macrophage-dependent vascular regression is then required to ensure vessel patterns return to their original state (Gurevich et al., 2018). Therefore, we hypothesise that maintaining Erk activity only in ECs of vessels that need to regenerate in this laser ablation model, ensures EC proliferation and migration only occurs in regenerating vessels, and prevents excessive angiogenesis. Further studies could investigate Erk-signalling dynamics of ECs in bigger wounds, which more closely resemble traumatic injury in humans and could further assess Erk-signalling dynamics in excessive angiogenesis and regression.

Blood vessels constantly remodel to accommodate for the needs of the human body during development and disease (Carmeliet and Jain, 2011; Chung and Ferrara, 2011; Potente et al., 2011). It is therefore not surprising that Erk-signalling, which is a key modulator of angiogenesis, is highly dynamic in ECs. As a novel tool that allows real-time analysis of Erk activity, EC-EKC biosensors will be useful for elucidating Erk-signalling events in vasculature in an array of settings and different vertebrate models. Importantly, in zebrafish the EC-EKC transgenic line can be coupled with both established and novel mutants with vascular phenotypes to investigate how real-time EC Erk-signalling dynamics is affected in the absence of key vascular genes. Further, dynamic Erk-signalling events in ECs in zebrafish disease models associated with increased angiogenesis such as in cancer (Nicoli et al., 2007) and tuberculosis (Oehlers et al., 2015) can be analysed using this EC-EKC model. This could highlight novel pathological Erk-signalling events in ECs, that could be normalised using drugs shown to modulate Erk-signalling (Goglia et al., 2020). Of note, KTR constructs for other kinases such as AKT, JNK and p38 are also now available (Regot et al., 2014; Maryu et al., 2016). Other types of fluorescence-based kinase activity reporters such as separation of phases-based activity reporter of kinases (SPARK), could also be applied (Zhang et al., 2018). Future studies will inevitably combine multiple signalling biosensors to elucidate real-time interactions...
between signalling pathways as they decipher incoming signals and drive development and disease.
### Materials and methods

#### Key resources table

| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information/reagent source |
|-----------------------------------|-------------|---------------------|-------------|---------------------------------------|
| Genetic reagent (D.rerio)         | Tg(fli1a:H2B-mCherry)$^{uq37bh}$ | (Baek et al., 2019) | RRID:ZFIN_ZDB-ALT-191011-5 | Ben M Hogan (Organogenesis and Cancer Program, Peter MacCallum Cancer Centre, Australia) |
| Genetic reagent (D.rerio)         | Tg(fli1a:EGFP)$^{y1}$ | (Lawson and Weinstein, 2002) | RRID:ZFIN_ZDB-ALT-011017-8 | Brant M Weinstein (National Institute of Child Health and Human Development, Bethesda, USA) |
| Genetic reagent (D.rerio)         | Tg(fli1aep:ERK-KTR-Clover)$^{uq39bh}$ | This study | | Ben M Hogan (Organogenesis and Cancer Program, Peter MacCallum Cancer Centre, Australia) |
| Genetic reagent (D.rerio)         | Tg(ubb:Mmu.Elk1-KTR-mClover)$^{vi1}$ | (Mayr et al., 2018) | ZFIN ID: ZDB-ALT-190211-6 | Martin Distel (Children’s Cancer Research Institute, Austria) |
| Genetic reagent (D.rerio)         | Tg(actb2:GCaMP6f)$^{zd3076}$ | (Herzog et al., 2019) | ZFIN ID: ZDB-ALT-200610-2 | Leah Herrgen (Centre for Discovery Brain Sciences, University of Edinburgh, Germany) |
| Genetic reagent | Tg(kdrl:mCherry-CAAX)\textsuperscript{y171} | (Fujita et al., 2011) | RRID:ZFIN_ZDB-ALT-110429-3 | Brent M Weinstein (National Institute of Child Health and Human Development, Bethesda, USA) |
|-----------------|-----------------------------------------|----------------------|-----------------------------|---------------------------------------------------------------------------------|
| Genetic reagent | Tg(mpeg1:mCherry)\textsuperscript{gl23} | (Ellett et al., 2011) | RRID:ZFIN_ZDB-ALT-120117-2 | Graham Lieschke (Australian Regenerative Medicine Institute, Monash University, Australia) |
| Genetic reagent | Tg(kdrl:EGFP)\textsuperscript{g843} | (Beis et al., 2005) | RRID:ZFIN_ZDB-ALT-050916-14 | Didier Stainier (Max Planck Institute for Heart and Lung Research, Germany) |
| Sequence-based reagent | MO1-spi1b | (Rhodes et al., 2005) | ZFIN ID: ZDB-MRPHLNO-050224-1 | Genetools, LLC, OR, USA |
| Sequence-based reagent | MO3-csf3r | (Ellett et al., 2011) | ZFIN ID: ZDB-MRPHLNO-111213-1 | Genetools, LLC, OR, USA |
| Software, algorithm | FIJI | ImageJ (http://imagej.nih.gov/ij/) | RRID:SCR_002285 | Image processing and analysis, Version Fiji version 1 |
| Software, algorithm | Imaris x64 | Bitplane, Belfast, UK | RRID:SCR_007370 | Image processing and analysis, Version 9.5.1 |
| Software, algorithm | GraphPad Prism | GraphPad Prism (http://graphpad.com) | RRID:SCR_002798 | Statistics, Prism8: Version 8.3.0 |
| Chemical compound, drug | SL327 (MEK signalling inhibitor) | Merck, Darmstadt, Germany | S4069 | Diluted in DMSO |
|-------------------------|----------------------------------|---------------------------|-------|-----------------|
| Chemical compound, drug | Trametinib (MEK signalling inhibitor) | Selleck chemicals, TX, USA | S2673 | Diluted in DMSO |
| Chemical compound, drug | SU5416 | Merck, Darmstadt, Germany | S8442 | Diluted in DMSO |
| Chemical compound, drug | AV951 | Adooq Bioscience, CA, USA | 475108-18-0 | Diluted in DMSO |
| Chemical compound, drug | Nifedipine | Bio-Techne, MN, USA | 1075 | Diluted in DMSO |
| Chemical compound, drug | Amlodipine | Merck, Darmstadt, Germany | A5605 | Diluted in DMSO |
Zebrafish

All zebrafish work was conducted in accordance with the guidelines of the animal ethics committees at the University of Queensland (AE54297), University of Melbourne, Peter MacCallum Cancer Centre (E634 and E643), University of Bristol (3003318), and the Children’s Cancer Research Institute (GZ:565304/2014/6 and GZ:534619/2014/4). The transgenic zebrafish lines used were published previously as following: Tg(fli1a:H2B-mCherry)$^{uq37bh}$ (Baek et al., 2019), Tg(fli1a:EGFP)$^{y1}$ (Lawson and Weinstein, 2002), Tg(ubb:Mmu.Elk1-KTR-mClover)$^{v1}$ (Mayr et al., 2018), Tg(actb2:GCaMP6f)$^{zf3076}$ (Herzog et al., 2019), Tg(kdrl:mCherry-CAAX)$^{y171}$ (Fujita et al., 2011), Tg(mpeg1:mCherry)$^{gl23}$ (Ellett et al., 2011), and Tg(kdrl:EGFP)$^{s843}$ (Beis et al., 2005). The Tg(fli1aep:ERK-KTR-Clover)$^{uq39bh}$ transgenic line (referred to as Tg(fli1aep:EKC)/EC-EKC in this study) was generated for this study using Gateway cloning and transgenesis. The pENTR-ERKKTRClover plasmid (#59138) was purchased from Addgene.

Live imaging and laser-inflicted vessel/tissue wounding

Embryos/Larvae at indicated stages were immobilised with Tricaine (0.08 mg/ml) and mounted laterally in either 1.2% ultra-low gelling agarose (specifically for Video 6), 0.25% low melting agarose (specifically for Videos 7 and 8, and Figure 6A), or 0.5% low melting agarose (Merck, Darmstadt, Germany, A9414-100G) as previously described (Okuda et al., 2018). Images were taken at indicated timepoints/timeframe using either a Zeiss LSM 710 confocal microscope using either a Zeiss Plan Apochromat 10X objective (dry, N.A. 0.45, specifically for Figure 1B-E) or a Zeiss Plan Apochromat 20X objective (dry, N.A. 0.8, specifically for Figure 3A,B), Zeiss Elyra 780 confocal microscope using either a Zeiss Apochromat 10X objective (dry, N.A. 0.45, specifically for Figure 5-figure supplement 1K,L) or a Zeiss Plan Apochromat 40X objective (water, N.A. 1.1, specifically for Figure 3-figure supplement 1A-B’, Figure 3-figure supplement 2H,I, Figure 5-figure supplement 1M-T’, Figure 5-figure supplement 2A-H’, and for Figure 6-figure supplement 2I-N’), Leica SP8 X WLL confocal microscope using a Leica HC PL APO CS2 40X objective (water, N.A. 1.1, specifically for Video 6), Leica TCS SP8 multiphoton microscope using a Leica HC Fluotar 25X objective (water, N.A. 0.95, specifically for Videos 7 and 8, and Figure 6A), Olympus Yokogawa CSU-W1 Spinning Disc Confocal microscope using a UPLSAPO 40X objective (silicon, N.A. 1.25,
specifically for Figure 6-figure supplement 1K-T), or an Andor Dragonfly Spinning Disc Confocal microscope using a Nikon Apo λ LWD 40X objective (water, N.A. 1.15).

Muscle wounding in 30 hpf Tg(ubb:Mmu.Elk1-KTR-mClover) embryos were conducted as previously described (specifically for Video 6) (Mayr et al., 2018). Briefly, a laser-inflicted wound was introduced on mounted embryos using the Leica SP8 X FRAP module with the UV laser line of 405 nm at 85% laser power. Vessel wounding in 4 dpf Tg(actb2:GCaMP6f);Tg(kdrl:mCherry-CAAX) larvae were conducted as previously described (specifically for Video 7 and 8, and Figure 6A) (Gurevich et al., 2018). Briefly, a laser-inflicted wound was introduced on mounted larvae using a Micropoint laser (Spectra-Physics, CA, USA) connected to a Zeiss Axioplan II microscope with a laser pulse at a wavelength of 435 nm. All other tissue/vessel wounding in either 3 dpf (specifically for Figure 3-figure supplement 2B,C,H,I,P,R,T,V and Figure 5-figure supplement 2F,H) or 4 dpf Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry) or Tg(kdrl:EGFP);Tg(mpeg1:mCherry) larvae were conducted using either a Zeiss LSM 710 confocal microscope or a Olympus FVMPE-RS multiphoton microscope. Briefly, a laser-inflicted wound was introduced on mounted larvae using a two-photon laser at 790 nm (Zeiss LSM 710 confocal microscope) or 900 nm (Olympus FVMPE-RS multiphoton microscope) at 80% laser power (Mai Tai, Spectra-Physics, CA, USA). The area of laser ablation for vessel wounding experiments was made consistent for all experiments (height: 40 μm, width: 15 μm). All vessel wounding was conducted on the ISV dorsal to the cloaca.

For Video 1, time-lapse images of ISVs in 24-25 Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry) embryos were acquired every 14-17 seconds for 40 minutes using an Andor Dragonfly Spinning Disc Confocal microscope. Difference in time intervals were due to difference in z section number in different embryos. Pre-division ISV tip ECs with cytoplasmic H2B-mCherry localisation were selected for imaging. For Videos 3-5, time-lapse images of ISVs in 4 dpf Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry) larvae were taken every minute for 20 minutes using an Andor Dragonfly Spinning Disc Confocal microscope, wounded as described above using a Zeiss
LSM 710 confocal microscope, transferred to an Andor Dragonfly Spinning Disc Confocal microscope (allowing for 2 minutes to transfer the larvae and initiate imaging) and re-imaged every minute for another 20 minutes. As a control (Video 2), time-lapse images of ISVs in 4 dpf Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry) larvae were taken every minute for 41 minutes. For Video 6, time-lapse images of the trunk in a 30 hpf Tg(ubb:Mmu.Elk1-KTR-mCherry) embryo were acquired every 21 minutes from 5 mpa until 3 hpa using a Leica SP8 X WLL confocal microscope. For Video 8, time-lapse images of ISVs in 4 dpf Tg(actb2:GCaMP6f);Tg(kdrl:mCherry-CAAX) larvae were acquired every minute from 5 mpa until 20 mpa using a Leica SP8 confocal microscope. As a control (Video 7), time-lapse images of ISVs in 4 dpf Tg(actb2:GCaMP6f);Tg(kdrl:mCherry-CAAX) larvae were acquired every minute for 15 minutes using a Leica SP8 confocal microscope.

Morpholino injections
The spi1b and csf3r morpholinos used in this study have been validated and described previously (Rhodes et al., 2005;Ellett et al., 2011;Pase et al., 2012). A cocktail of spi1b (5ng) and csf3r (2.5ng) morpholinos were injected into 1-4 cell stage EC-Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry) or Tg(mpeg1:mCherry) embryos as previously described (Pase et al., 2012). ISVs of 3 dpf morphants/uninjected controls were imaged before vessel wounding, wounded as described above, and reimaged either at 15 mpa or at 3 hpa. To measure vessel regeneration, ISVs of 3 dpf morphants/uninjected controls were wounded as described above and imaged at 24 hpa. Non-ablated 3 dpf Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry) morphants/uninjected controls were imaged, and re-imaged either 15 minutes or 3 hours later. Macrophage numbers (mpeg1:mCherry-positive) in 3 dpf embryos (Figure 3-figure supplement 2E,F) or 4 dpf larvae (Figure 3-figure supplement 2A-C) were manually quantified using the cell counter tool in FIJI.

Drug treatments
For investigating Erk activity in ISV tip ECs in 28 hpf embryos following drug treatment, 27 hpf Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry) embryos were treated for an hour with either 0.5% DMSO (vehicle control), 15 μM SL327, 4 μM SU5416, or 500 nM AV951 diluted in E3 medium with 0.003% 1-phenyl-2-thiourea (PTU) and
imaged as described above at 28 hpf. Up to 5 ISV tip ECs were quantified per embryo.

For investigating the role of prolonged EC Erk activity in vessel regeneration, ISVs of 4 dpf *Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry)* larvae were wounded as described above and were treated with either 0.5% DMSO (vehicle control), 4 μM SU5416, 15 μM SL327, or 1 μM Trametinib for 24 hours and imaged as described above at 5 dpf (24 hpa). For measuring Erk activity in ECs pre- and post-ablation in 4 dpf larvae following drug treatment, 4 dpf *Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry)* larvae were first treated for an hour with either 0.5% DMSO, 15 μM SL327, 4 μM or 10 μM SU5416, or 500 nM AV951. ISVs of these larvae were imaged then wounded as described above in the presence of respective drugs at indicated concentrations in the mounting media. The same larvae were reimaged at 15 mpa. Alternatively, larvae were removed from mounting media following vessel wounding and incubated in respective drugs at indicated concentrations in E3 media, before being remounted and imaged at 3 hpa.

For Nifedipine and Amlodipine treatment, 4 dpf *Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry)* larvae were first treated for 30 minutes with either 1% DMSO, 50 μM Nifedipine, or 100 μM Amlodipine. This was because treatment for 1 hour with either 50 μM Nifedipine or 100 μM Amlodipine resulted in mortalities due to reduced cardiac function. The ISVs of these larvae were imaged and wounded as described above and reimaged 15 mpa. Alternatively, 4 dpf *Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry)* larvae were imaged before vessel wounding, and removed from mounting media following vessel wounding and incubated in 1% DMSO. 30 minutes before 3 hpa, larvae were treated with 50 μM Nifedipine or continued its treatment with 1% DMSO, before being remounted in the presence of respective drugs at indicated concentrations and reimaged 3 hpa. To treat the larvae for 30 minutes with 50 μM Nifedipine following vessel wounding, 4 dpf *Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry)* larvae were mounted with either 1% DMSO or 50 μM Nifedipine, imaged before vessel wounding, and removed from mounting 30 minutes following vessel wounding. These larvae were incubated in 1% DMSO and reimaged 3 hpa. Non-ablated 4 dpf *Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry)* larvae controls were imaged, then reimaged either 15 minutes or 3 hours later.
Guide RNA synthesis and injection

kdrl guide RNA (gRNA) sequences were designed previously (Wu et al. 2018). Kdrl gRNA
oligonucleotide 1:
TAATACGACTCAGGTTTTCTGTTCGATGGCAGTTTTAGAGCTAGAAATA

Kdrl gRNA oligonucleotide 2:
TAATACGACTCAGGCTGAGACCCCTCTCCGTTTTAGAGCTAGAAAT

Kdrl gRNA oligonucleotide 3:
TAATACGACTCAGGCACTAGCGAGTTTTAGAGCTAGAAAT

Kdrl gRNA oligonucleotide 4:
TAATACGACTCAGGCTCAGGTTTTAGAGCTAGAAAT

Guide RNAs were synthesised as described previously (Gagnon et al., 2014) with modifications. Briefly, kdrl gRNA oligonucleotides were annealed to a constant oligonucleotide, ssDNA overhangs were filled in with T4 DNA polymerase (New England Biolabs, Victoria, Australia), and gRNA templates were purified using the DNA Clean and Concentrator Kit (Zymo Research, D4014, CA, USA). Kdrl four-guide RNA cocktail were transcribed with Ambion Megascript T7 promoter kit and cleaned using the RNA clean and concentrator™ Kit (Zymo Research, R1014, CA, USA). One-cell stage Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry) embryos were injected with a cocktail of Cas9 protein (Integrated DNA Technologies, 1081059, IA, USA) and the guide RNAs. Only kdrl crispsants with clear vascular phenotypes (Figure 5-figure supplement 1L) were used for all experiments. ISVs of 4 dpf crispsants/uninjected controls were imaged before vessel wounding, wounded as described above, and reimaged at 3 hpa. Non-ablated 4 dpf Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry) crispsants/uninjected controls were imaged, and re-imaged 3 hours later. As vessel wounding often resulted in no ECs in ISVs, ECs from ablated connecting horizontal vessels were used for quantification (Figure 5-figure supplement 1T).

Image processing and analysis

Images were processed with image processing software FIJI version 1 (Schindelin et al., 2012) and Imaris x64 (Bitplane, Version 9.5.1). Erk activity in ECs was measured by either comparing nuclear/cytoplasm EKC intensity, nuclear EKC/H2B-mCherry intensity, or nuclear EKC intensity. In Figures, EC-EKC intensity in nuclei is represented after masking nuclear expression using H2B-mCherry and presenting
EC-EKC intensity in 16 colour LUT (Fiji). The nuclear/cytoplasm EKC intensity was quantified as described before (Kudo et al., 2018) with modifications, using a semi-autonomous custom written script in the ImageJ macro language. Briefly, z stack images were first processed into a maximum intensity z-projection. H2B-mCherry-positive EC nuclei underwent thresholding and were selected as individual regions of interest (ROI). The EKC channel was converted to a 32-bit image with background (non-cell associated) pixels converted to NaN. The average pixel intensity of EKC in the nuclei ROIs were measured (nuclear EKC intensity). Nuclei ROIs were then expanded and converted to a banded selection of the adjacent cytoplasmic area and the average pixel intensity of EKC within the expanded ROIs were measured (cytoplasm EKC intensity). The custom written ImageJ macro is available here: [https://github.com/NickCondon/Nuclei-Cyto_MeasuringScript].

The average pixel intensity of either nuclear EKC or H2B-mCherry of ECs in 3D was quantified using Imaris software. The entire EC nucleus was masked using the H2B-mCherry signal. **Figure 2J and K** represent averages of data within each minute. For Embryos/larvae exposed to long-term time-lapse (for example Videos 2-5), or ablated with high-powered multiphoton laser for ablation studies, difference in photostability between fluorophores could significantly alter the ratio of nuclear EKC/H2B-mCherry intensity (Lam et al., 2012). Therefore, we either compared the ratio of nuclear EKC intensities between ECs within the same fish (for example Video 1), or we normalised EC nuclear EKC intensity with the average EKC intensity of another EKC-expressing structure (for example Videos 2-5). For larvae that underwent laser-inflicted wounding, nuclear EKC intensity pre- and post-ablation was normalised with the average pixel intensity of EKC of the entire DA within 2 somite length. The ROI that covers the same DA region in pre- and post-wounded larvae was manually selected on a maximum intensity z-projection of the EKC channel, and average pixel intensity was calculated using FIJI. Datasets were presented as either the ratio of post/pre-ablation normalised nuclear EKC intensity, or as normalised nuclear EKC intensity further normalised to normalised nuclear EKC intensity in 2 mpa ECs (specifically for Figure 6H). 3 closest ECs from the wounded site in both ablated and adjacent ISVs were quantified, except for Figures 5I and 6H, where 5 closest ECs from the wounded site in ablated ISVs were analysed. For Videos 2-5, reduction in EKC intensity due to photobleaching was
minimised using the bleach correction tool (correction method: Histogram Matching) in FIJI, however quantifications were all done using raw data.

GCaMP6f average pixel intensity on ISVs and unablated tissue in 4 dpf Tg(actb2:GCaMP6f);Tg(kdrl:mCherry-CAAX) larvae was measured using FIJI. Maximum intensity z-projection images of both GCaMP6f and mCherry-CAAX channels were first corrected for any drift in x/y dimensions. A ROI was drawn around the mCherry-CAAX-positive ISV segment nearest to the site of injury (an area consistently between 100-150 μm²) and the average pixel intensity of GCaMP6f within the ROI at each timepoints were measured using FIJI. Similar measurements were acquired for adjacent ISVs, ISVs in unablated control larvae, and uninjured tissue, maintaining consistent size of ROI within each biological replicate. ISV GCaMP6f average pixel intensity was normalised to the average pixel intensity in uninjured tissue GCaMP6f within the same larvae.

The percentage of ISV height was measured by dividing the total horizontal height of the ISV with the prospective total horizontal height of the ISV (the horizontal height from the base ISV/DA intersection to the tip of the ISV/DLAV intersection. Ellipticity (elliptic) of ISV tip ECs were quantified using Imaris software. Original raw data with relevant acquisition metadata can be provided upon request.

Statistics
Graphic representations of data and statistical analysis was performed using either Prism 8 Version 8.3.0 or R software. Mann-Whitney test was conducted when comparing two datasets and Kruskal-Wallis test was conducted when comparing multiple datasets using Prism 8 (except for Figure 5B, which conducted an ordinary one-way ANOVA test, following confirmation of normality of all datasets using Anderson-Darling, D’Agostino and Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov tests). Natural permutation test (Figure 3H and Figure 4C) or two-sample Kolmogorov-Smirnov test (Figure 6H) was used to test for differences between the population mean curve for datasets using R statistical software. For Figure 6H, we applied the non-parametric two sample Kolmogorov-Smirnov test to evaluate whether the distribution of Erk activity for each position differed from that of the
Null hypothesis was rejected where the D-statistic (maximum difference between two ECDF) exceeded the critical threshold (critical D) for each comparison and p-value < 0.001. D statistic indicates magnitude of change for each curve compared with control. Critical D varied for each position as follows: control vs 1st EC from wound, 0.166; control vs 2nd EC from wound, 0.166; control vs 3rd EC from wound, 0.166; control vs 4th EC from wound, 0.173; control vs 5th EC from wound, 0.209. P-value below 0.05 was considered statistically significant for all data. Error bars in all graphs represent standard deviation.

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References

Aoki, K., Kondo, Y., Naoki, H., Hiratsuka, T., Itoh, R.E., and Matsuda, M. (2017). Propagating Wave of ERK Activation Orients Collective Cell Migration. Dev Cell 43, 305-317 e305.

Baek, S., Oh, T.G., Secker, G., Sutton, D.L., Okuda, K.S., Paterson, S., Bower, N.I., Toubia, J., Koltowska, K., Capon, S.J., Bailie, G.J., Muscat, G.E.O., Lagendijk, A.K., Smith, K.A., Harvey, N.L., and Hogan, B.M. (2019). The Alternative Splicing Regulator Nova2 Constrains Vascular Erk Signaling to Limit Specification of the Lymphatic Lineage. Dev Cell 49, 279-292 e275.

Beerman, R.W., Matty, M.A., Au, G.G., Looger, L.L., Choudhury, K.R., Keller, P.J., and Tobin, D.M. (2015). Direct In Vivo Manipulation and Imaging of Calcium Transients in Neutrophils Identify a Critical Role for Leading-Edge Calcium Flux. Cell Rep 13, 2107-2117.

Beis, D., Bartman, T., Jin, S.W., Scott, I.C., D’amico, L.A., Ober, E.A., Verkade, H., Frantsve, J., Field, H.A., Wehman, A., Baier, H., Tallafuss, A., Bally-Cuijf, L., Chen, J.N., Stainier, D.Y., and Jungblut, B. (2005). Genetic and cellular analyses of zebrafish atrioventricular cushion and valve development. Development 132, 4193-4204.

Berra-Romani, R., Raqeeb, A., Torres-Jacome, J.E., Guzman-Silva, A., Guerra, G., Tanzi, F., and Moccia, F. (2012). The mechanism of injury-induced intracellular calcium concentration oscillations in the endothelium of excised rat aorta. J Vasc Res 49, 65-76.

Beis, D., Bartman, T., Jin, S.W., Scott, I.C., D’amico, L.A., Ober, E.A., Verkade, H., Frantsve, J., Field, H.A., Wehman, A., Baier, H., Tallafuss, A., Bally-Cuijf, L., Chen, J.N., Stainier, D.Y., and Jungblut, B. (2005). Genetic and cellular analyses of zebrafish atrioventricular cushion and valve development. Development 132, 4193-4204.

Carmeliet, P., and Jain, R.K. (2011). Molecular mechanisms and clinical applications of angiogenesis. Nature 473, 298-307.

Chang, C.I., Xu, B.E., Akella, R., Cobb, M.H., and Goldsmith, E.J. (2002). Crystal structures of MAP kinase p38 complexed to the docking sites on its nuclear substrate MEF2A and activator MKK3b. Mol Cell 9, 1241-1249.

Chia, K., Keatinge, M., Mazzolini, J., and Sieger, D. (2019). Brain tumours repurpose endogenous neuron to microglia signalling mechanisms to promote their own proliferation. Elife 8.

Chung, A.S., and Ferrara, N. (2011). Developmental and pathological angiogenesis. Annu Rev Cell Dev Biol 27, 563-584.

Costa, G., Harrington, K.I., Lovegrove, H.E., Page, D.J., Chakravartula, S., Bentley, K., and Herbert, S.P. (2016). Asymmetric division coordinates collective cell migration in angiogenesis. Nat Cell Biol 18, 1292-1301.

Covassin, L.D., Villefranc, J.A., Kacergis, M.C., Weinstein, B.M., and Lawson, N.D. (2006). Distinct genetic interactions between multiple Vegf receptors are required for development of different blood vessel types in zebrafish. Proc Natl Acad Sci U S A 103, 6554-6559.

De Oliveira, S., Lopez-Munoz, A., Candel, S., Pelegrin, P., Calado, A., and Mulero, V. (2014). ATP modulates acute inflammation in vivo through dual oxidase 1-derived H2O2 production and NF-kappaB activation. J Immunol 192, 5710-5719.
De Simone, A., Evanitsky, M.N., Hayden, L., Cox, B.D., Wang, J., Tornini, V.A., Ou, J., Chao, A., Poss, K.D., and Di Talia, S. (2021). Control of osteoblast regeneration by a train of Erk activity waves. Nature 590, 129-133.

Deng, Y., Atri, D., Eichmann, A., and Simons, M. (2013). Endothelial ERK signaling controls lymphatic fate specification. J Clin Invest 123, 1202-1215.

Ding, Y., Li, J., Enterina, J.R., Shen, Y., Zhang, I., Tewson, P.H., Mo, G.C., Zhang, J., Quinn, A.M., Hughes, T.E., Maysinger, D., Alford, S.C., Zhang, Y., and Campbell, R.E. (2015). Ratiometric biosensors based on dimerization-dependent fluorescent protein exchange. Nat Methods 12, 195-198.

Ellett, F., Pase, L., Hayman, J.W., Andrianopoulos, A., and Lieschke, G.J. (2011). mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish. Blood 117, e49-56.

Fujita, M., Cha, Y.R., Pham, V.N., Sakurai, A., Roman, B.L., Gutkind, J.S., and Weinstein, B.M. (2011). Assembly and patterning of the vascular network of the vertebrate hindbrain. Development 138, 1705-1715.

Gagnon, J.A., Valen, E., Thyme, S.B., Huang, P., Akhmetova, L., Pauli, A., Montague, T.G., Zimmerman, S., Richter, C., and Schier, A.F. (2014). Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. PLoS One 9, e98186.

Ghilardi, S.J., O'reilly, B.M., and Sgro, A.E. (2020). Intracellular signaling dynamics and their role in coordinating tissue repair. Wiley Interdiscip Rev Syst Biol Med 12, e1479.

Goglia, A.G., Wilson, M.Z., Jena, S.G., Silbert, J., Basta, L.P., Devenport, D., and Toettcher, J.E. (2020). A Live-Cell Screen for Altered Erk Dynamics Reveals Principles of Proliferative Control. Cell Syst 10, 240-253 e246.

Goto, A., Nakahara, I., Yamaguchi, T., Kamioka, Y., Sumiyama, K., Matsuda, M., Nakaniishi, S., and Funabiki, K. (2015). Circuit-dependent striatal PKA and ERK signaling underlies rapid behavioral shift in mating reaction of male mice. Proc Natl Acad Sci U S A 112, 6718-6723.

Gurevich, D.B., Severn, C.E., Twomey, C., Greenhough, A., Cash, J., Toye, A.M., Mellor, H., and Martin, P. (2018). Live imaging of wound angiogenesis reveals macrophage orchestrated vessel sprouting and regression. EMBO J 37.

Habeck, H., Odenthal, J., Walderich, B., Maischein, H., Schulte-Merker, S., and Tubingen Screen, C. (2002). Analysis of a zebrafish VEGF receptor mutant reveals specific disruption of angiogenesis. Curr Biol 12, 1405-1412.

Handly, L.N., Pilko, A., and Wollman, R. (2015). Paracrine communication maximizes cellular response fidelity in wound signaling. Elife 4, e09652.

Harvey, C.D., Ehrhardt, A.G., Cellurale, C., Zhong, H., Yasuda, R., Davis, R.J., and Svoboda, K. (2008). A genetically encoded fluorescent sensor of ERK activity. Proc Natl Acad Sci U S A 105, 19264-19269.

Herzog, C., Pons Garcia, L., Keatinge, M., Greenald, D., Moritz, C., Peri, F., and Herrgen, L. (2019). Rapid clearance of cellular debris by microglia limits secondary neuronal cell death after brain injury in vivo. Development 146.

Hirata, H., Gupta, M., Vedula, S.R., Lim, C.T., Ladoux, B., and Sokabe, M. (2015). Actomyosin bundles serve as a tension sensor and a platform for ERK activation. EMBO Rep 16, 250-257.
Hiratsuka, T., Fujita, Y., Naoki, H., Aoki, K., Kamioka, Y., and Matsuda, M. (2015). Intercellular propagation of extracellular signal-regulated kinase activation revealed by in vivo imaging of mouse skin. *Elife* 4, e05178.

Hogan, B.M., and Schulte-Merker, S. (2017). How to Plumb a Pisces: Understanding Vascular Development and Disease Using Zebrafish Embryos. *Dev Cell* 42, 567-583.

Kamioka, Y., Sumiyama, K., Mizuno, R., Sakai, Y., Hirata, E., Kiyokawa, E., and Matsuda, M. (2012). Live imaging of protein kinase activities in transgenic mice expressing FRET biosensors. *Cell Struct Funct* 37, 65-73.

Kamioka, Y., Takakura, K., Sumiyama, K., and Matsuda, M. (2017). Intravital Forster resonance energy transfer imaging reveals osteopontin-mediated polymorphonuclear leukocyte activation by tumor cell emboli. *Cancer Sci* 108, 226-235.

Koch, S., and Claesson-Welsh, L. (2012). Signal transduction by vascular endothelial growth factor receptors. *Cold Spring Harb Perspect Med* 2, a006502.

Komatsu, N., Aoki, K., Yamada, M., Yukinaga, H., Fujita, Y., Kamioka, Y., and Matsuda, M. (2011). Development of an optimized backbone of FRET biosensors for kinases and GTPases. *Mol Biol Cell* 22, 4647-4656.

Kudo, T., Jeknic, S., Macklin, D.N., Akhter, S., Hughey, J.J., Regot, S., and Covert, M.W. (2018). Live-cell measurements of kinase activity in single cells using translocation reporters. *Nat Protoc* 13, 155-169.

Lam, A.J., St-Pierre, F., Gong, Y., Marshall, J.D., Cranfill, P.J., Baird, M.A., Mckeown, M.R., Wiedenmann, J., Davidson, M.W., Schnitzer, M.J., Tsien, R.Y., and Lin, M.Z. (2012). Improving FRET dynamic range with bright green and red fluorescent proteins. *Nat Methods* 9, 1005-1012.

Lavoie, H., Gagnon, J., and Therrien, M. (2020). ERK signalling: a master regulator of cell behaviour, life and fate. *Nat Rev Mol Cell Biol*.

Lawson, N.D., and Weinstein, B.M. (2002). In vivo imaging of embryonic vascular development using transgenic zebrafish. *Dev Biol* 248, 307-318.

Li, J., Zhang, S., Soto, X., Woolner, S., and Amaya, E. (2013). ERK and phosphoinositide 3-kinase temporally coordinate different modes of actin-based motility during embryonic wound healing. *J Cell Sci* 126, 5005-5017.

Maryu, G., Matsuda, M., and Aoki, K. (2016). Multiplexed Fluorescence Imaging of ERK and Akt Activities and Cell-cycle Progression. *Cell Struct Funct* 41, 81-92.

Matsubayashi, Y., Ebisuuya, M., Honjo, S., and Nishida, E. (2004). ERK activation propagates in epithelial cell sheets and regulates their migration during wound healing. *Curr Biol* 14, 731-735.

Mayr, V., Sturtzel, C., Stardler, M., Grissenberger, S., and Distel, M. (2018). Fast Dynamic in vivo Monitoring of Erk Activity at Single Cell Resolution in DREKA Zebrafish. *Front Cell Dev Biol* 6, 111.

Mehta, S., Zhang, Y., Roth, R.H., Zhang, J.F., Mo, A., Tenner, B., Huganir, R.L., and Zhang, J. (2018). Single-fluorophore biosensors for sensitive and multiplexed detection of signalling activities. *Nat Cell Biol* 20, 1215-1225.

Mizuno, R., Kamioka, Y., Kabashima, K., Imajo, M., Sumiyama, K., Nakasho, E., Ito, T., Hamazaki, Y., Okuchi, Y., Sakai, Y., Kiyokawa, E., and Matsuda, M. (2014). In vivo imaging reveals PKA regulation of ERK activity during neutrophil recruitment to inflamed intestines. *J Exp Med* 211, 1123-1136.
Moccia, F., Berra-Romani, R., and Tanzi, F. (2012). Update on vascular endothelial Ca(2+) signalling: A tale of ion channels, pumps and transporters. *World J Biol Chem* 3, 127-158.

Murphy, D.A., Makonnen, S., Lassoued, W., Feldman, M.D., Carter, C., and Lee, W.M. (2006). Inhibition of tumor endothelial ERK activation, angiogenesis, and tumor growth by sorafenib (BAY43-9006). *Am J Pathol* 169, 1875-1885.

Nagasawa-Masuda, A., and Terai, K. (2016). ERK activation in endothelial cells is a novel marker during neovasculogenesis. *Genes Cells* 21, 1164-1175.

Nicoli, S., Ribatti, D., Cotelli, F., and Presta, M. (2007). Mammalian tumor xenografts induce neovascularization in zebrafish embryos. *Cancer Res* 67, 2927-2931.

Okuda, K.S., Baek, S., and Hogan, B.M. (2018). Visualization and Tools for Analysis of Zebrafish Lymphatic Development. *Methods Mol Biol* 1846, 55-70.

Pase, L., Layton, J.E., Wittmann, C., Ellett, F., Nowell, C.J., Reyes-Aldasoro, C.C., Varma, S., Rogers, K.L., Hall, C.J., Keightley, M.C., Crosier, P.S., Grabher, C., Heath, J.K., Renshaw, S.A., and Lieschke, G.J. (2012). Neutrophil-delivered myeloperoxidase dampens the hydrogen peroxide burst after tissue wounding in zebrafish. *Curr Biol* 22, 1818-1824.

Pokras, M.J., Ryan, K.A., Xin, T., Pielstick, B., Timp, W., Greco, V., and Regot, S. (2020). Cell-Cycle-Dependent ERK Signaling Dynamics Direct Fate Specification in the Mammalian Preimplantation Embryo. *Dev Cell* 55, 328-340 e325.

Poplimont, H., Georgantzoglou, A., Boulch, M., Walker, H.A., Coombs, C., Papaleonidopoulou, F., and Sarris, M. (2020). Neutrophil Swarming in Damaged Tissue Is Orchestrated by Connexins and Cooperative Calcium Alarm Signals. *Curr Biol* 30, 2761-2776 e2767.

Potente, M., Gerhardt, H., and Carmeliet, P. (2011). Basic and therapeutic aspects of angiogenesis. *Cell* 146, 873-887.

Razzell, W., Evans, I.R., Martin, P., and Wood, W. (2013). Calcium flashes orchestrate the wound inflammatory response through DUOX activation and hydrogen peroxide release. *Curr Biol* 23, 424-429.

Regot, S., Hughey, J.J., Bajar, B.T., Carrasco, S., and Covert, M.W. (2014). High-sensitivity measurements of multiple kinase activities in live single cells. *Cell* 157, 1724-1734.

Rhodes, J., Hagen, A., Hsu, K., Deng, M., Liu, T.X., Look, A.T., and Kanki, J.P. (2005). Interplay of pu.1 and gata1 determines myelo-erythroid progenitor cell fate in zebrafish. *Dev Cell* 8, 97-108.

Ricard, N., Scott, R.P., Booth, C.J., Velazquez, H., Clifone, N.A., Baylon, J.L., Gulcher, J.R., Quaggin, S.E., Chittenden, T.W., and Simons, M. (2019). Endothelial ERK1/2 signaling maintains integrity of the quiescent endothelium. *J Exp Med* 216, 1874-1890.

Rosenfeldt, H., and Grinnell, F. (2000). Fibroblast quiescence and the disruption of ERK signaling in mechanically unloaded collagen matrices. *J Biol Chem* 275, 3088-3092.

Sano, T., Kobayashi, T., Ogawa, O., and Matsuda, M. (2018). Gliding Basal Cell Migration of the Urothelium during Wound Healing. *Am J Pathol* 188, 2564-2573.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri,
K., Tomancak, P., and Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. Nat Methods 9, 676-682.

Shin, M., Beane, T.J., Quillien, A., Male, I., Zhu, L.J., and Lawson, N.D. (2016a). Vegfa signals through ERK to promote angiogenesis, but not artery differentiation. Development 143, 3796-3805.

Shin, M., Male, I., Beane, T.J., Villefranc, J.A., Kok, F.O., Zhu, L.J., and Lawson, N.D. (2016b). Vegfc acts through ERK to induce sprouting and differentiation of trunk lymphatic progenitors. Development 143, 3785-3795.

Shu, X. (2020). Imaging dynamic cell signaling in vivo with new classes of fluorescent reporters. Curr Opin Chem Biol 54, 1-9.

Simons, M., Gordon, E., and Claesson-Welsh, L. (2016). Mechanisms and regulation of endothelial VEGF receptor signalling. Nat Rev Mol Cell Biol 17, 611-625.

Smani, T., Gomez, L.J., Regodon, S., Woodard, G.E., Siegfried, G., Khatib, A.M., and Rosado, J.A. (2018). TRP Channels in Angiogenesis and Other Endothelial Functions. Front Physiol 9, 1731.

Srinivasan, R., Zabuawala, T., Huang, H., Zhang, J., Gulati, P., Fernandez, S., Karlo, J.C., Landreth, G.E., Leone, G., and Ostrowski, M.C. (2009). Erk1 and Erk2 regulate endothelial cell proliferation and migration during mouse embryonic angiogenesis. PLoS One 4, e8283.

Surprenant, A., and North, R.A. (2009). Signaling at purinergic P2X receptors. Annu Rev Physiol 71, 333-359.

Takeda, H., and Kiyokawa, E. (2017). Activation of Erk in ileal epithelial cells engaged in ischemic-injury repair. Sci Rep 7, 16469.

Tang, S., and Yasuda, R. (2017). Imaging ERK and PKA Activation in Single Dendritic Spines during Structural Plasticity. Neuron 93, 1315-1324 e1313.

Villefranc, J.A., Amigo, J., and Lawson, N.D. (2007). Gateway compatible vectors for analysis of gene function in the zebrafish. Dev Dyn 236, 3077-3087.

Wilhelm, S.M., Carter, C., Tang, L., Wilkie, D., Mcnabola, A., Rong, H., Chen, C., Zhang, X., Vincent, P., McHugh, M., Cao, Y., Shujath, J., Gawlak, S., Eveleigh, D., Rowley, B., Liu, L., Adnane, L., Lynch, M., Auclair, D., Taylor, I., Gedrich, R., Voznesensky, A., Riedl, B., Post, L.E., Bollag, G., and Trail, P.A. (2004). BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res 64, 7099-7109.

Wong, K.L., Akiyama, R., Bessho, Y., and Matsui, T. (2018). ERK Activity Dynamics during Zebrafish Embryonic Development. Int J Mol Sci 20.
Zhang, Q., Huang, H., Zhang, L., Wu, R., Chung, C.I., Zhang, S.Q., Torra, J., Schepis, A.,
Coughlin, S.R., Kornberg, T.B., and Shu, X. (2018). Visualizing Dynamics of Cell
Signaling In Vivo with a Phase Separation-Based Kinase Reporter. *Mol Cell* 69, 334-
346 e334.
**Figures and figure legends**

**Figure 1: The EC-EKC transgenic line enables quantification of vascular Erk activity during development.**

(A) Schematic representation of the fli1aep:ERK-KTR-Clover (EKC) construct, and ECs with nuclear enriched EKC (bottom left, inactive Erk-signalling) and nuclear depleted EKC localisation (bottom right, active Erk-signalling).

(B-E) Lateral confocal images of the EC-EKC (B,D) and Tg(fli1a:EGFP) (C,E) embryos/larvae at 24 hpf (B,C) and 5 dpf (D,E). Blood vessel development is not altered in EC-EKC embryos/larvae.

(F-H”) Lateral spinning disc confocal images of ISV ECs in 28 hpf EC-EKC embryos treated for 1 hour with either 0.5% DMSO (F-F”), with active EC Erk-signalling; or 15 μM SL327 (G-G”), or 4 μM SU5416 (H-H”); all of which with inactive EC Erk-signalling. Images F-H show the fli1aep:EKC expression, while images F’-H’ show both the fli1aep:EKC and the fli1a:H2B-mCherry expression. Images F”-H” show the nuclear fli1aep:EKC expression with intensity difference represented in 16 colour LUT (Fiji). The fli1a:H2B-mCherry signal was used to mask the nucleus.

(I) Quantification of nucleus/cytoplasm EKC intensity in ISV tip ECs of 28 hpf embryos treated with either 0.5% DMSO (0.881, 93 ISV tip ECs, n=20 embryos), 15 μM SL327 (1.419, 114 ISV tip ECs, n=27 embryos), or 4 μM SU5416 (1.591, 118 ISV tip ECs, n=27 embryos).

ISV: intersegmental vessel. Statistical test: Kruskal Wallis test was conducted for graph I. Error bars represent standard deviation. Scale bars: 200 μm for images B and D, 25 μm for image F.
Figure 2: Tip cells show asymmetric Erk activity immediately following cell division.

(A-I) Still images from Video 1 showing ISV ECs in a 24-25 hpf EC-EKC embryo at indicated time points. The tip daughter cell has higher Erk activity when compared to the stalk daughter cell immediately after cell division. Left panels show fli1aep:EKC expression, middle panels show the fli1a:H2B-mCherry expression, and right panels show the nuclear fli1aep:EKC intensity. The fli1a:H2B-mCherry signal was used to mask the nucleus. The yellow arrow indicates a tip ISV EC with cytoplasmic fli1a:H2B-mCherry expression. The light blue arrow indicates a tip ISV EC that has undergone cytokinesis.

(J,K) Quantification of tip/stalk nuclear EKC intensity of daughter ECs post-cytokinesis (14 EC division events, n=14 embryos). Graph J shows quantification of individual biological replicates and graph K shows the average of all biological replicates.

ISV: intersegmental vessel; DA: dorsal aorta. Error bars represent standard deviation. Scale bar: 25 μm.
Figure 3: Wounded vessels rapidly activate Erk independent of macrophages or Vegfr-signalling.

(A,B) Lateral confocal images of a 4 dpf Tg(kdrl:EGFP) larva following vessel wounding (post-ablation). Image A shows the kdrl:EGFP expression and image B shows the trans-light image of image A. Ablated ISV, adjacent ISVs, wounded site indicated with white arrows.

(C) Schematic representation of imaging schedule for larvae in images D-G and Videos 3-5.

(D-G') Still images from Video 4 (D-E') and Video 5 (F-G') showing ISV ECs before (pre-ablation) and after vessel wounding. Ablated and adjacent ISV ECs rapidly activate Erk-signalling. D-G = fli1aepp:EKC expression, D’-G’ = nuclear intensity.

(H,I) Quantification of post/pre-ablation nuclear EKC intensity of ECs in non-ablated control ISVs (black, 24 ECs, n=8 larvae), ablated ISVs (red, 27 ECs, n=9 larvae), and adjacent ISVs (light blue, 27 ECs, n=9 larvae). H shows quantification of individual ECs, I shows the average of all biological replicates. Green dotted line indicates 15 minutes post-ablation (mpa).

(J) Quantification of post/pre-ablation nuclear EKC intensity 15 mpa in ECs of non-ablated control ISVs (103 ECs, n=34 larvae), ablated venous ISVs (75 ECs, n=25 larvae), and ablated arterial ISVs (57 ECs, n=19 larvae). Both venous and arterial ISV ECs activate Erk-signalling.

(K) Quantification of post/pre-ablation nuclear EKC intensity 15 mpa in ECs of non-ablated uninjected control ISVs (45 ECs, n=15 larvae), non-ablated spil1/csf3r morphant ISVs (42 ECs, n=14 larvae), uninjected control ISVs (45 ablated/adjacent ISV ECs, n=15 larvae), and spil1/csf3r morphant ISVs (56 ablated ISV ECs and 57 adjacent ISV ECs, n=19 larvae). Macrophages are not required to rapidly activate Erk-signalling in ablated or adjacent ISV ECs.

(L) Quantification of post/pre-ablation nuclear EKC intensity 15 mpa in ECs of 0.5% DMSO-treated non-ablated control ISVs (33 ECs, n=11 larvae), and ISVs of larvae treated with either 0.5% DMSO (42 ablated/adjacent ISV ECs, n=14 larvae), 15 μM SL327 (39 ablated/adjacent ISV ECs, n=13 larvae), 4 μM SU5416 (36 ablated/adjacent ISV ECs, n=12 larvae), 10 μM SU5416 (42 ablated/adjacent ISV ECs, n=14 larvae), or 500 nM AV951 (42 ablated/adjacent ISV ECs, n=14 larvae).
Vegfr-signalling is not required to rapidly activate Erk-signalling in ablated or adjacent ISV ECs. ISV: intersegmental vessel. Statistics: Permutation test was conducted for graph H. Kruskal Wallis test was conducted for graphs J-L. Error bars represent standard deviation. White dotted lines/circle show the wounded sites of each larvae. Scale bar: 100 μm for image A, 20 μm for image D.
Figure 4: Wounded but not adjacent vessels maintain high Erk activity as the regenerative response proceeds.

(A-B') Lateral spinning disc confocal images of ablated (A) and adjacent ISVs (B) of a 4 dpf EC-EKC larva before and following vessel wounding at indicated timepoints. Erk activity is progressively lost in the adjacent but retained in the wounded ISV ECs.

Images A and B show fli1aep:EKC expression, images A' and B' show nuclear fli1aep:EKC intensity. White dotted lines show the wounded site.

(C,D) Quantification of post/pre-ablation nuclear EKC intensity of ECs in non-ablated control ISVs (black, 24 ECs, n=8 larvae), ablated ISVs (red, 30 ECs, n=10 larvae), and adjacent ISVs (light blue, 30 ECs, n=10 larvae) before and after vessel wounding at indicated timepoints. Graph C shows the quantification of individual ECs and graph D shows the average of all biological replicates. At 1 hpa: Control vs Ablated ISV ECs: p>0.001; Control vs Adjacent ISV ECs: p = 0.108 (Kruskal Wallis test).

ISV: intersegmental vessel. Statistical test: Permutation test was conducted for graph C. Error bars represent standard deviation. Scale bar: 20 μm.
Figure 5: Erk activity in ablated vessels is maintained through the Vegfr pathway.

(A) Ongoing Erk-signalling requires Vegfr and Mek activity. Quantification of post/pre-ablation nuclear EKC intensity 3 hpa in ECs of 0.5% DMSO-treated non-ablated control ISVs (33 ECs, n=11 larvae), and ablated ISVs of larvae treated with either 0.5% DMSO (51 ECs, n=17 larvae), 15 µM SL327 (42 ECs, n=14 larvae), 4 µM SU5416 (47 ECs, n=16 larvae), or 10 µM SU5416 (32 ECs, n=11 larvae).

(B) Kdrl is required for full induction of Erk activity in ablated ISV ECs. Quantification of post/pre-ablation nuclear EKC intensity 3 hpa in non-ablated control ISVs ECs of uninjected control (27 ECs, n=9 larvae) and kdrl crispants (26 ECs, n=9 larvae), and ablated ISV ECs of uninjected control (22 ECs, n=8 larvae) and kdrl crispants (27 ECs, n=9 larvae).

(C) Quantification of ISV horizontal length (as percentage of control) for ablated ISVs in 24 hpa, 5 dpf, EC-EKC larvae treated with either 0.5% DMSO (n=18 larvae), 4 µM SU5416 (n=12 larvae), 15 µM SL327 (n=15 larvae), or 1 µM Trametinib (n=13 larvae).

(D) Macrophages are not required for maintaining Erk activity in ablated ISV ECs. Quantification of post/pre-ablation nuclear EKC intensity 3 hpa in non-ablated control ISVs ECs of uninjected control (24 ECs, n=8 larvae) and sp1/csf3r morphants (21 ECs, n=7 larvae), and ablated ISV ECs of uninjected control (29 ECs, n=10 larvae) and sp1/csf3r morphants (31 ECs, n=11 larvae).

(E-G) Lateral spinning disc confocal images of ablated ISV ECs in 4 dpf 3 hpa EC-EKC larvae treated with either 0.5% DMSO (E), 4 µM SU5416 (F), or 10 µM SU5416 (G). EC Erk activity was consistently higher and more Vegfr-dependent closer to the wound. Arrows indicate first (white), second (yellow), and third (green) ECs from the wounded site. Full images: Figure 5-figure supplementary 1D',H',J'.

(H) Quantification of post/pre-ablation nuclear EKC intensity at 3 hpa in first (dark grey), second (red) and third (light blue) ECs from wound. Treatments were: 0.5% DMSO-treated non-ablated control ISVs (11 first, second and third ECs, n=11 larvae), and ablated ISVs of larvae treated with either 0.5% DMSO (17 first, second and third ECs, n=17 larvae), 4 µM SU5416 (16 first and second ECs, and 15 third ECs, n=16 larvae), or 10 µM SU5416 (11 first and second ECs, and 10 third ECs, n=11 larvae). The same embryos were used in A.
Quantification of post/pre-ablation nuclear EKC intensity at 3 hpa in first (14 ECs, n=14 larvae), second (14 ECs, n=14 larvae), third (14 ECs, n=14 larvae), forth (11 ECs, n=11 larvae), and fifth (8 ECs, n=8 larvae) ECs from the wounded site of ablated ISVs in 4 dpf EC-EKC larvae. Data for the first, second, and third ECs were taken from Figure 4-figure supplement 1N.

ISV: intersegmental vessel. DA: dorsal aorta. Statistical test: Kruskal Wallis test was conducted for graphs A,C,D,H,I. ordinary one-way ANOVA test was conducted for graph B. Error bars represent standard deviation. 15 μm for image E.
Figure 6: Ca^{2+} signalling is required for rapid Erk activation in ablated vessels.

(A) Still images from Video 8 demonstrating a pulse of Ca^{2+} signalling immediately adjacent to the wound (4 dpf). Left panels show actb2:GCaMP6f and kdr:mCherry-CAAX, right panels show actb2:GCaMP6f. Yellow arrows show ISV ECs with active Ca^{2+} signalling. Blue arrows show Ca^{2+} signalling in recruited immune cells.

(B) Quantification of actb2:GCaMP6f intensity in unablated control ISVs (black, n=4 larvae), ablated (red, n=10 larvae) and adjacent (light blue, n =10 larvae) ISVs following wounding. Intensity was normalised to actb2:GCaMP6f intensity in unablated tissue in the same larvae.

(C) Ca^{2+} signalling is required for rapid activation of Erk signalling in ablated ISV ECs. Quantification of post/pre-ablation nuclear EKC intensity at 15 mpa in ECs of 1% DMSO-treated non-ablated control ISVs (39 ECs, n=13 larvae), and ISVs of larvae treated with either 1% DMSO (39 ablated/adjacent ISV ECs, n=13 larvae) or 50 µM Nifedipine (36 ablated/adjacent ISV ECs, n=12 larvae).

(D) Quantification of post/pre-ablation nuclear EKC intensity at 15 mpa in ECs of 1% DMSO-treated non-ablated control ISVs (18 ECs, n=6 larvae), and ISVs of larvae treated with either 1% DMSO (27 ablated/adjacent ISV ECs, n=9 larvae) or 100 µM Amplopidine (31 ablated ISV ECs and 33 adjacent ISV ECs, n=11 larvae).

(E) Ca^{2+} signalling is not required for sustaining Erk activity in ablated ISV ECs. Quantification of post/pre-ablation nuclear EKC intensity at 3 hpa in ECs of 1% DMSO-treated non-ablated control ISVs (24 ECs, n=8 larvae), and ablated ISVs of larvae treated with either 1% DMSO (42 ECs, n=14 larvae) or 50 µM Nifedipine (39 ECs, n=13 larvae) for 30 minutes before 3 hpa (Figure 6-figure supplement 2A).

(F) Quantification of post/pre-ablation nuclear EKC intensity at 3 hpa in ECs of 1% DMSO-treated non-ablated control ISVs (21 ECs, n=7 larvae), and ablated ISVs of larvae treated with either 1% DMSO (27 ECs, n=9 larvae) or 50 µM Nifedipine (27 ECs, n=9 larvae) for 30 minutes after vessel wounding (Figure 6-figure supplement 2H).

(G,G') Still images from Video 3 showing ablated ISV ECs of a 4 dpf EC-EKC larva at after vessel wounding. Activation of Erk progresses from the wound to the vessel base. Image G show the fli1aep:EKC expression, G' shows nuclear fli1aep:EKC intensity. Arrows indicate first (white), second (yellow), third (green), forth (red), and fifth (orange) ECs from the wounded site.
(H) Quantification of nuclear EKC intensity (normalised to nuclear EKC intensity at 2 mpa) in ECs of ISVs in non-ablated control larvae (black, 24 ECs, n=8 larvae), and the first (red, 9 ECs, n=9 larvae), second (blue, 9 ECs, n=9 larvae), third (green, 9 ECs, n=9 larvae), fourth (orange, 8 ECs, n=8 larvae), and fifth (purple, 5 ECs, n=5 larvae) ablated ISV ECs from the wounded site following vessel wounding.

ISV: intersegmental vessel. Statistical test: Kruskal Wallis test was conducted for graphs C-F. two-sample Kolmogorov-Smirnov test was conducted for graph H. Error bars represent standard deviation. Scale bars: 50 μm for image A, 15 μm for image G.
**Figure 7: A two-step mechanism for activating and maintaining Erk activity in regenerating vessels.**

Schematic representation of the two-step mechanism employed by ECs to activate Erk-signalling following vessel wounding. Pre-ablation (left), the majority of ECs are Erk-signalling inactive. Following vessel wounding (middle), both ablated and adjacent ISV ECs rapidly activate Erk-signalling. Ca^{2+} signalling is also rapidly activated following vessel wounding but only in ablated ISV ECs, particularly in ECs close to the wounded site. Ca^{2+} signalling activity contributes to the activation of Erk-signalling in ablated ISV ECs in a sequential manner, starting from ECs close to the wounded site. Erk-signalling in adjacent ISV ECs has returned to pre-wound levels by 3 hpa (right). Erk activity in ablated vessel ISV ECs is sustained through Vegfr-signalling. ECs closer to the wounded site are less sensitive to Vegfr-signalling inhibition, with higher signalling compared to ECs further away. Recruited macrophages are essential for vessel regeneration but not the sole source of Vegfs at 3 hpa.
Supplementary figures and figure legends

Figure 1 - figure supplement 1: The EC-EKC transgenic line reports tip cell enriched and cell-state dependent Erk-signalling during primary angiogenesis.

(A-A'') Lateral spinning disc confocal images of budding ISVs in a 22 hpf EC-EKC embryo show high Erk activity in ISV tip ECs. Image A shows the fli1aep:EKC expression, image A' shows both the fli1aep:EKC and the fli1a:H2B-mCherry expression, while image A'' shows the nuclear fli1aep:EKC expression intensity. Yellow arrows point to DA ECs with nuclear depleted EKC localisation.

(B) Quantification of the nucleus/cytoplasm EKC intensity ratio in sprouting ISV tip ECs of 22 hpf embryos (0.803, 133 ECs, n=37 embryos) showing consistently higher levels in cytoplasm.

(C) Quantification of the sprouting ISV tip EC/DA stalk EC nuclear EKC intensity ratio with two different methods in 22 hpf embryos (109 ECs, n=37 embryos). DA ECs closest to the sprouting ISV ECs were quantified. Ratios were calculated using a value of nuclear EKC/H2B-mCherry intensity in tip cells (0.777) or using a raw nuclear EKC intensity measurement alone in tip cells (0.817), both showed higher Erk activity in sprouting ISV tip ECs when compared to DA stalk ECs.

(D-E'') Nuclear ellipticity and Erk-activity correlate. Lateral spinning disc confocal images of either an ISV with “migrating EC” (D) or an ISV with “non-migrating EC” (E) in 28 hpf EC-EKC embryos. Migrating or non-migrating determined by position relative to the DLLV. D and E; fli1aep:EKC expression, D' and E'; fli1aep:EKC and fli1a:H2B-mCherry, D'' and E''; nuclear fli1aep:EKC expression intensity. Light blue arrow shows ISV stalk ECs with nuclear depleted EKC localisation.

(F) Quantification of EC ellipticity in “migrating” (125 ECs, n=45 embryos) and “non-migrating” ISV leading ECs based on position relative to DLLV (63 ECs, n=35 embryos) at 28 hpf.

(G) More migratory ECs, with more elliptical nuclei, show higher Erk activity. Quantification of tip/stalk ISV EC nuclear EKC intensity for the most elliptic (47 ECs, n=30 embryos) or less elliptical (oblate) nuclei (47 ECs, n=29 embryos) in 28 hpf embryos. Most elliptic (upper quartile of all migrating ECs in F) and oblate (lower quartile of all non-migrating ECs in F) nuclei were quantified.
ISV: intersegmental vessel; DA: dorsal aorta. Statistical test: Mann-Whitney test was conducted for graphs C, F and G. Error bars represent standard deviation. Scale bars: 25 μm for image A, 15 μm for image D.
**Figure 3 - figure supplement 1: Rapid Erk activation is largely restricted to wounded and adjacent ISV ECs.**

(A-B') Lateral confocal images of 4 dpf EC-EKC larvae pre-ablation (A,A') and 15 mpa (B,B'). Immediately adjacent ISV ECs show rapid Erk activation, while Erk activity in 2nd and 3rd adjacent ISV ECs are largely unchanged. White dotted line shows the wounded site.

(C) Quantification of post/pre-ablation nuclear EKC intensity at 15 mpa in ECs of non-ablated control ISVs (30 ECs, n=10 larvae), and adjacent (27 ECs, n=9 larvae), 2nd adjacent (27 ECs, n=9 larvae), and 3rd adjacent ISVs (27 ECs, n=9 larvae).

DA, dorsal aorta; ISV: intersegmental vessel. Statistical test: Kruskal Wallis test was conducted for graph C. Error bars represent standard deviation. Scale bar: 50 μm for image A.
Figure 3 - figure supplement 2: Macrophages are not required for rapid Erk activation following vessel wounding.

(A-C) Lateral spinning disc confocal images of 4 dpf Tg(kdrl:EGFP);Tg(mpeg1:mCherry) larvae pre-ablation (A), 15 mpa (B), or 3 hpa (C). Macrophages are recruited to the wounded site by 3 hpa but not by 15 mpa.

(D) Quantification of macrophage number recruited to the wounded site pre-ablation (n=25 embryos), 15 mpa (n=14 embryos) or 3 hpa (n=24 embryos).

(E,F) Lateral spinning disc confocal images of 3 dpf Tg(mpeg1:mCherry) uninjected control (E) or spi1/csf3r morphants (F).

(G) Quantification of macrophage number within the trunk spanning 3 somites length in 3 dpf Tg(mpeg1:mCherry) uninjected control (n=29 embryos) or spi1/csf3r morphants (n=25 embryos).

(H,I) Macrophages are required for vessel regeneration. Lateral confocal images of 24 hpa, 4 dpf, Tg(fli1aep:EKC) uninjected control (H) or spi1/csf3r morphants (I).

(J) Quantification of ISV horizontal length (as percentage of control) for ablated ISVs in 24 hpa, 4 dpf, EC-EKC uninjected control (n=11 larvae) or spi1/csf3r morphants (n=13 larvae).

(K-V') Lateral spinning disc confocal images of ISV ECs in 3 dpf EC-EKC uninjected control (K-L', O-P', S-T') and spi/csf3r morphants (M-N', Q-R', U-V'). Erk-signalling is rapidly activated in both ablated and adjacent ISV ECs in larvae with reduced macrophage number. Images K-N' show non ablated control ISV ECs, images O-R' show ablated ISV ECs, images S-V' show adjacent ISV ECs. Images O,Q,S,U were taken pre-ablation, images P,R,T,V were taken 15 mpa. Images K-V show the fli1aep:EKC expression, and images K'-V' shows the nuclear fli1aep:EKC intensity.

ISV: intersegmental vessel; Statistical test: Kruskal Wallis test was conducted for graph D and Mann-Whitney test was conducted for graph G and J. Error bars represent standard deviation. White dotted lines/circles show the wounded sites of each embryos/larvae. Scale bars: 20 \(\mu\)m for image A, 50 \(\mu\)m for image E and H, 15 \(\mu\)m for image K.
**Figure 3 - figure supplement 3: Vegfr-signalling is not required for rapid Erk activation following vessel wounding.**

**(A-B'**) Lateral spinning disc confocal images of ISV ECs in 28 hpf EC-EKC embryos treated for an hour with either 0.5% DMSO (A-A''), with active EC Erk-signalling; or 500 nM AV951 (B-B''), with inactive EC Erk-signalling. Images A and B show the fliaep:EKC expression, while images A' and B' show both the fliaep:EKC and the flia1a:H2B-mCherry expression. Images A'' and B'' show the nuclear fliaep:EKC intensity.

**(C)** Quantification of nucleus/cytoplasm EKC intensity in ISV tip ECs of 28 hpf embryos treated with either 0.5% DMSO (0.849, 65 ECs, n=14 embryos) or 500 nM AV951 (1.423, 53 ECs, n=12 embryos).

**(D-Z')** Vegfr-signalling inhibitors do not block rapid Erk-signalling activation in ablated and adjacent ISVs following vessel wounding. Lateral spinning disc confocal images of ISV ECs in 4 dpf EC-EKC larvae treated with either 0.5% DMSO (D-I'), 15 μM SL327 (J-M'), 4 μM SU5416 (O-R'), 10 μM SU5416 (S-V'), or 500 nM AV951 (W-Z'). Images D-E' show non-ablated control ISV ECs. Images F-G', J-K', O-P', S-T' and W-X' show ablated ISV ECs. Images H-I', L-M', Q-R', U-V' and Y-Z' show adjacent ISV ECs. Images F,H,J,L,O,Q,S,U,W,Y were taken pre-ablation and images G,I,K,M,P,R,T,V,X,Z were taken 15 mpa. Images D-Z show the fliaep:EKC expression, and images D'-Z' show the nuclear fliaep:EKC intensity. White dotted lines show the wounded sites of each larva. ISV: intersegmental vessel; Statistical test: Mann-Whitney test was conducted for graph C. Error bars represent standard deviation. Scale bars: 25 μm for image A, 15 μm for image D.
Figure 4 - figure supplement 1: Distinct Erk activity between ablated and adjacent ISV ECs 3 hpa.

(A-I') Lateral spinning disc confocal images of ISV ECs in 4 dpf EC-EKC larvae at indicated timepoints. Images A-I show the fli1aep:EKC expression, while images A'-I' show the nuclear fli1aep:EKC intensity.

(J-M') Erk signalling is activated in ablated, but not adjacent ISV ECs at 3 hpa. Lateral spinning disc confocal images of ablated and adjacent ISV ECs in 4 dpf EC-EKC larvae before (J-K'), and 3 hours following vessel wounding (L-M'). Images J-M show the fli1aep:EKC expression, while images J'-M' show the nuclear fli1aep:EKC intensity. Images K and M are higher magnification images of the yellow boxes in images J and L. White circle in image L shows the wounded site. Arrows indicate first (white), second (yellow), third (green), fourth (red), and fifth (orange) ECs from the wounded site.

(N) Quantification of post/pre-ablation nuclear EKC intensity of ECs in non-ablated control ISVs (27 ECs, n=9 larvae), ablated ISVs (42 ECs, n=14 larvae), and adjacent ISVs (42 ECs, n=14 larvae) 3 hpa.

ISV: intersegmental vessel; Statistical test: Kruskal Wallis test was conducted for graph N. Error bars represent standard deviation. Scale bars: 20 µm for image A, 20 µm for images J and K.
**Figure 4 - figure supplement 2: Vessel wounding is required for sustained Erk activity in ablated ISV ECs.**

(A,B) Vessels that are not wounded do not sustain Erk activity. Lateral spinning disc confocal images of ISV ECs in 4 dpf EC-EKC larvae at 0 min/pre-ablation (left), 15 minutes/15 mpa (middle), or 3 hours/3 hpa (right). Images A show ISVs in non-ablated control larvae, and images B show ISVs in larvae with tissue ablated in between two ISVs (Control ablation). Images A and B show the *fli1aep:EKC* expression, while images A’ and B’ show the nuclear *fli1aep:EKC* intensity. White dotted lines show the wounded site.

(C) Quantification of post/pre-ablation nuclear EKC intensity of ECs in either non-ablated control ISVs or control ablation ISVs at 15 mpa (control, 39 ECs, n=13 larvae; control ablation, 48 ECs, n=16 larvae) or 3 hpa (control, 18 ECs, n=6 larvae; control ablation, 24 ECs, n=8 larvae).

ISV: intersegmental vessel; Statistical test: Kruskal Wallis test was conducted for graph C. Error bars represent standard deviation. Scale bar: 15 μm
**Figure 5 - figure supplement 1: Vegfr-signalling is required to sustain Erk activity in ablated ISV ECs following vessel wounding.**

(A-J') Lateral spinning disc confocal images of ISV ECs in 4 dpf EC-EKC larvae treated with either 0.5% DMSO (A-D'), 15 μM SL327 (E-F'), 4 μM SU5416 (G-H'), or 10 μM SU5416 (I-J'). A higher concentration of SU5416 (10 μM) is required to block the Erk activity in ablated ISV ECs 3 hpa immediately adjacent to the wound. Images A-B' show non-ablated control ISV ECs. Images C,E,G,I were taken pre-ablation and images D,F,H,J were taken 3 hpa. Images A-J show the fli1aep:EKC expression, and images A'-J' show the nuclear fli1aep:EKC intensity.

(K,L) Lateral confocal images of 4 dpf EC-EKC uninjected control (n=100/100) (K) and kdrl crispant (n=98/103 larvae displayed phenotype indicated) (L). kdrl crispants phenocopy previously published kdrl mutant/morphant vascular phenotypes.

(M-T') High Erk activity is not maintained in kdrl crispants 3 hpa. Lateral confocal images of ISV ECs in 4 dpf EC-EKC uninjected control (M-N', Q-R') and kdrl crispants (O-P', S-T'). Images M-P' show non ablated control ISV ECs, images Q-T' show ablated ISV ECs. Images Q and S were taken pre-ablation, images R and T were taken 3 hpa. Images M-T show the fli1aep:EKC expression, and images M'-T' show the nuclear fli1aep:EKC intensity.

(U-X) Erk-signalling is required for vessel regeneration. Lateral spinning disc confocal images of 24 hpa 5 dpf EC-EKC larvae treated with either: 0.5% DMSO (U), showing a regenerated ISV; or 4 μM SU5416 (V), 15 μM SL327 (W), or 1 μM Trametinib (X); all of which blocked ISV regeneration.

DA, dorsal aorta; ISV: intersegmental vessel. White dotted lines/circles show the wounded site of each larvae. Scale bars: 15 μm for image A, 100 μm for image K, 20 μm for image A, 50 μm for image U.
Figure 5 - figure supplement 2: Macrophages are not required to sustain Erk activity in ablated ISV ECs following vessel wounding.

(A-H') Lateral confocal images of ISV ECs in 3 dpf EC-EKC uninjected control (A-B', E-F') and *spi1/csf3r* morphants (C-D', G-H'). Images A-D' show non-ablated control ISV ECs. Images E and G were taken pre-ablation and images F and H were taken 3 hpa. Images A-H show the *fli1aep:EKC* expression, and images A'-H' show the nuclear *fli1aep:EKC* intensity. White dotted lines show the wounded site of each embryo.

ISV: intersegmental vessel. Scale bar: 20 μm.
Figure 6 - figure supplement 1: Ca^{2+} signalling is required for rapid Erk activation in ablated ISV ECs.

(A-T') Ca^{2+} signalling is required for rapid Erk activation. Lateral spinning disc confocal images of ISV ECs in 4 dpf EC-EKC larvae treated with either 1% DMSO (A-D',G-H',K-N',Q-R'), 50 μM Nifedipine (E-F',I-J'), or 100 μM Amlopidine (O-P',S-T'). Images A-B' and K-L' show non-ablated control ISV ECs. Images C,E,G,I,M,O,Q,S were taken pre-ablation and images D,F,H,J,N,P,R,T were taken 15 mpa. Images A-T show the fli1aep:EKC expression, and images A-T' show the nuclear fli1aep:EKC intensity. White dotted lines show the wounded site of each larvae.

ISV: intersegmental vessel. Scale bar: 15 μm
Figure 6 - figure supplement 2: Ca\textsuperscript{2+} signalling is not required for sustained Erk activation in ablated ISV ECs.

(A) Schematic representation of imaging schedule for larvae in images B-G.

(B-G') Ca\textsuperscript{2+} signalling is required not required for sustaining Erk activation in ablated ISV ECs. Lateral spinning disc confocal images of ISV ECs in 4 dpf EC-EKC larvae treated with either 1% DMSO (A-E') or 50 \(\mu\)M Nifedipine (F-G'). Images B-C' show non-ablated control ISV ECs. Images D and F were taken pre-ablation and images E and G were taken 3 hpa. Images B-G show the \textit{fli1aep:EKC} expression, and images B-G' show the nuclear \textit{fli1aep:EKC} intensity.

(H) Schematic representation of imaging schedule for larvae in images I-N.

(I-N') Lateral confocal images of ISV ECs in 4 dpf EC-EKC larvae treated with either 1% DMSO (I-L') or 50 \(\mu\)M Nifedipine (M-N'). Images I-J' show non-ablated control ISV ECs. Images K and M were taken pre-ablation and images L and N were taken 3 hpa. Images I-N show the \textit{fli1aep:EKC} expression, and images I-N' show the nuclear \textit{fli1aep:EKC} intensity.

ISV: intersegmental vessel. White dotted lines show the wounded site of each larvae.

Scale bar: 15 \(\mu\)m
**Videos**

**Video 1: ISV daughter ECs show asymmetric Erk activity following cytokinesis.**

Time-lapse video of an ISV tip EC undergoing mitosis in a 24-25 hpf EC-EKC embryo. Left panel shows the *fli1aep:EKC* expression, middle panel shows the *fli1a:H2B-mCherry* expression, and the right panel shows the nuclear *fli1aep:EKC* intensity. Z stacks were acquired every 15.5 seconds for 40 minutes using an Andor Dragonfly Spinning Disc Confocal microscope. Photobleaching was minimised using the bleach correction tool (correction method: Histogram Matching) in FIJI.

ISV: intersegmental vessel; DA: dorsal aorta. Scale bar: 25 μm.
**Video 2: ISV ECs in 4 dpf larvae have minimal Erk activity.**

Time-lapse video of the trunk vessels in a 4 dpf EC-EKC larva at indicated timepoints. ECs in functional vessels at 4 dpf have low Erk activity. Left panel shows the fli1aep:EKC expression, middle panel shows both fli1aep:EKC and fli1a:H2B-mCherry expression, and the right panel shows the nuclear fli1aep:EKC intensity. Z stacks were acquired every minute for 41 minutes using an Andor Dragonfly Spinning Disc Confocal microscope. Photobleaching was minimised using the bleach correction tool (correction method: Histogram Matching) in FIJI.

ISV: intersegmental vessel; DA: dorsal aorta. Scale bar: 20 μm.
Video 3: Both ablated and adjacent ISV ECs rapidly activate Erk-signalling following vessel wounding.

Time-lapse video of the trunk vessels in a 4 dpf EC-EKC larva before (pre-ablation) and after (post-ablation) vessel wounding at indicated timepoints. Vessel wounding rapidly activates Erk-signalling in both ablated and adjacent ISV ECs. Post-ablation video starts at 2 minutes post-ablation due to the time taken to transfer the larvae between microscopes and for preparation of imaging. Left panel shows the fli1aep:EKC expression, middle panel shows both fli1aep:EKC and fli1a:H2B-mCherry expression, and the right panel shows the nuclear fli1aep:EKC intensity. Z stacks were acquired every 1 minute for 20 minutes before and after vessel wounding using an Andor Dragonfly Spinning Disc Confocal microscope. Photobleaching was minimised using the bleach correction tool (correction method: Histogram Matching) in FIJI. ISV: intersegmental vessel; DA: dorsal aorta. Scale bar: 20 μm.
Video 4: Ablated ISV ECs rapidly activate Erk-signalling following vessel wounding.

Time-lapse video of the ablated ISV in a 4 dpf EC-EKC larva before (pre-ablation) and after (post-ablation) vessel wounding at indicated timepoints. Post-ablation video starts at 2 minutes post-ablation due to the time taken to transfer the larvae between microscopes and for preparation of imaging. Left panel shows the fli1aep:EKC expression and the right panel shows the nuclear fli1aep:EKC intensity. Z stacks were acquired every 1 minute for 20 minutes before and after vessel wounding using an Andor Dragonfly Spinning Disc Confocal microscope. Photobleaching was minimised using the bleach correction tool (correction method: Histogram Matching) in FIJI.

ISV: intersegmental vessel. Scale bar: 20 μm.
Video 5: Adjacent ISV ECs rapidly activate Erk-signalling following vessel wounding.

Time-lapse video of the adjacent ISV in a 4 dpf EC-EKC larva before (pre-ablation) and after (post-ablation) vessel wounding at indicated timepoints. Post-ablation video starts at 2 minutes post-ablation due to the time taken to transfer the larvae between microscopes and for preparation of imaging. Left panel shows the *fli1aep:EKC* expression and the right panel shows the nuclear *fli1aep:EKC* intensity. Z stacks were acquired every 1 minute for 20 minutes before and after vessel wounding using an Andor Dragonfly Spinning Disc Confocal microscope. Photobleaching was minimised using the bleach correction tool (correction method: Histogram Matching) in FIJI.

ISV: intersegmental vessel. Scale bar: 20 μm.
Video 6: Skin epithelial and muscle cells do not maintain high Erk activity for 3 hours following muscle wounding.

Time-lapse video of the trunk in a 30 hpf $Tg(ubb: Mmu.Elk1-KTR-mCherry)$ embryo following muscle wounding. The white circle shows the wounded site. Skin epithelial and muscle cells surrounding the wounded site do not sustain Erk activity (examples of Erk active cells, with nuclear excluded EKC expression indicated with white arrows). Z stacks were acquired every 21 minutes from 5 mpa until 3 hpa using a Leica SP8 X WLL confocal microscope (n=6 embryos).

Scale bar: 20 μm.
Video 7: ISVs in 4 dpf larvae do not have active Ca$^{2+}$ signalling.

Time-lapse video of ISVs in a 4 dpf Tg(actb2:GCaMP6f);Tg(kdrl:mCherry-CAAX) larva. Functional vessels at 4 dpf have low or undetectable Ca$^{2+}$ signalling. Left panel shows both the actb2:GCaMP6f and the kdrl:mCherry-CAAX expression and the right panel shows the actb2:GCaMP6f expression. Z stacks were acquired every minute for 15 minutes using a Leica SP8 confocal microscope.

ISV: intersegmental vessel. Scale bar: 50 μm.
Video 8: ISVs rapidly activate Ca$^{2+}$ signalling following vessel wounding.

Time-lapse video of both ablated and adjacent ISVs in a 4 dpf $Tg(actb2:GCaMP6f);Tg(kdrl:mCherry-CAAX)$ larva following vessel wounding. Only the wounded ISV activates Ca$^{2+}$ signalling. Left panel shows both the $actb2:GCaMP6f$ and the $kdrl:mCherry-CAAX$ expression and the right panel shows the $actb2:GCaMP6f$ expression. Z stacks were acquired every minute from 5 mpa until 20 mpa using a Leica SP8 confocal microscope.

ISV: intersegmental vessel. Scale bar: 50 μm.
Figure source data

Figure 1-source data 1: Nuclear/cytoplasm EKC measurements in leading ISV ECs of DMSO, SL327, and SU5416-treated 28 hpf embryos.

Figure 1-figure supplement 1-source data 1: EKC measurements in ISV ECs at 22 and 28 hpf.

Figure 2-source data 1: Tip/stalk nuclear EKC measurements in ISV ECs following cell division.

Figure 3-source data 1: Post/pre-ablation nuclear EKC measurements in control, ablated, and adjacent ISV ECs.

Figure 3-figure supplement 1-source data 1: Post/pre-ablation nuclear EKC measurements in adjacent, 2nd adjacent and 3rd adjacent ISV ECs 15 mpa

Figure 3-figure supplement 2-source data 1: Measurements of macrophage number and ISV length.

Figure 3-figure supplement 3-source data 1: Nuclear/cytoplasm EKC measurements in leading ISV ECs of DMSO and AV951-treated 28 hpf embryos.

Figure 4-source data 1: Post/pre-ablation nuclear EKC measurements in control, ablated, and adjacent ISV ECs from pre-ablation to 3 hpa.

Figure 4-figure supplement 1-source data 1: Post/pre-ablation nuclear EKC measurements in control, ablated, and adjacent ISV ECs at 3 hpa.

Figure 4-figure supplement 1-source data 1: Post/pre-ablation nuclear EKC measurements in control and control ablated ISV ECs at 15 mpa and 3 hpa.
Figure 5-source data 1: Post/pre-ablation nuclear EKC measurements in control, ablated, and adjacent ISV ECs at 3 hpa.

Figure 6-source data 1: GCaMP6f intensity measurements and post/pre-ablation nuclear EKC measurements in control, ablated, and adjacent ISV ECs.
Figure 1

**A**

fltl1ap:ERK-KTR-Clover (EKC)

---

Fltl1ap | Elk1 | NLS | NES | Clover

Phosphorylation-sites

Erk inactive
Nuclear enriched EKC

Erk active
Nuclear depleted EKC

**B**

24 hpf Tg(fli1aep:EKC)

**C**

24 hpf Tg(fli1a:EGFP)

**D**

5 dpf Tg(fli1aep:EKC)

**E**

5 dpf Tg(fli1a:EGFP)

28 hpl Tg(fli1aep:EK); Tg(fli1a:H2B-mCherry)

0.5% DMSO
15 μM SL327
4 μM SU5416

**F**

ISV

**G**

**H**

**I**

Nucleus/cytoplasm EKC

0.5% DMSO
15 μM SL327
4 μM SU5416

<0.001
Figure 2

24-25 hpf Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry)

A B C D E F G H I J K

DA ISV

00:00 03:37 08:48 13:59 19:10 24:21 29:32 34:43 39:54

Time post-cytokinesis (min)

Tip/stalk nuclear EKC

1.0

0.0

0.5

1.0

0.0

0.5
Figure 3

4 dpf (post-ablation) Tg(kdr:EGFP)

A

adjacent ISV

ablated ISV

B

wounded site

C

20 min

2 min transfer

20 min

Image 4 dpf

Ablate ISV

Image vessel response

D

ISV

0 min

Pre-ablation

10 min

20 min

2 mpa

Post-ablation

12 mpa

22 mpa

D'

ere

dep

F

adjacent ISV ECs

G

H

I

J

Control

Ablated ISV EC

0.0

0.5

1.0

1.5

post/pre-ablation nuclear EKC

Time (min)

Pre-ablation

Post-ablation (+ 2 min)

K

Control

Ablated ISV EC

L

Control

Ablated ISV EC

Uninjected Control

sp/sic99 MO

Uninjected Control

sp/sic99 MO

Control

DMSO

15 uM SU5416

4 uM SU5416

10 uM SU5416

500 uM AV501

post/pre-ablation nuclear EKC

Time (min)

Pre-ablation

Post-ablation (+ 2 min)
Figure 3-figure supplement 1

4 dpf Tg(fli1aep:EKCI);Tg(fli1a:HH2B-mCherry)
Pre-ablation 15 mpa

A
3rd adjacent ISV
2nd adjacent ISV
Adjacent ISV
DA

B

C

post/pre-ablation nuclear EKC

A'

B'

Control
Adjacent ISV
2nd adjacent ISV
3rd adjacent ISV
Figure 3-figure supplement 2

4 dpf Tg(kdr:EGFP);Tg(mpeg1:mCherry)

Pre-ablation 15 mpa 3 hpa

D

<0.001

0.643

3 dpf Tg(mpeg1:mCherry)

Uninjected control spi1/csf3r MO

E F G

G

<0.001

Uninjected Control spi1/csf3r MO

4 dpf 24 hpa Tg(fli1aeap:EKC)

Uninjected control spi1/csf3r MO

H I J

J

0.040

Uninjected Control spi1/csf3r MO

3 dpf Tg(fli1aeap:EKC);Tg(fli1a:H2B-mCherry)

Uninjected control spi1/csf3r MO

K L M N

M

ISV

K’ L’ M’ N’

ISV

3 dpf Tg(fli1aeap:EKC);Tg(fli1a:H2B-mCherry)

3 dpf Tg(fli1aeap:EKC);Tg(fli1a:H2B-mCherry)

Pre-ablation 15 mpa

Pre-ablation 15 mpa

Pre-ablation 15 mpa

Pre-ablation 15 mpa

Ablated ISV ECs

O P Q R

O’ P’ Q’ R’

Adjacent ISV ECs

S T U V

S’ T’ U’ V’
Figure 4

4 dpf Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry)

A.

A'.

B.

B'.

C.

D.

| Pre-ablation | 15 mpa | 30 mpa | 45 mpa | 1 hpa | 1.5 hpa | 2 hpa | 2.5 hpa | 3 hpa |
|-------------|--------|--------|--------|--------|---------|-------|---------|-------|

- Control
- Ablated ISV EC
- Adjacent ISV EC

C.

D.

post/pre-ablation nuclear EKC
Figure 4-figure supplement 1

4 dpf Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry)

0 min/h 15 min 30 min 45 min 1 h 1.5 h 2 h 2.5 h 3 h

Control

A B C D E F G H I

A' B' C' D' E' F' G' H' I'

4 dpf Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry)

Pre-ablation 3 hpa

J K L M

J' K' L' M'

N

post/pre-ablation nuclear EKC

<0.001

<0.001

<0.001

<0.188

Control Ablated ISV Adjacent ISV
Figure 4-figure supplement 2

4 dpf Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry)

A

0 min/h
Control
15 min
3 h
Pre-ablation
Control ablation
15 mpa
3 hpa

A'

ISV

B

B'

enr

dep

C

post/pre-ablation nuclear EKC

Control
Control
Ablation
Control
Control
Ablation

15 mpa
3 hpa

<0.001

0.533

<0.001


Figure 5

4 dpf Tg(fli1aeap:EKC);Tg(fli1a:H2B-mCherry)

3 hpa

0.5% DMSO  4 μM SU5416  10 μM SU5416

dep

E F G

H I

post/pre-ablation nuclear EKC

post/pre-ablation nuclear EKC

ISV horizontal length (%)

post/pre-ablation nuclear EKC

Control  DMSO  4 μM SU5416  10 μM SU5416

First EC from wound  Second EC from wound  Third EC from wound

0.124

<0.001  <0.001  >0.999  >0.999

0.003  0.033  >0.999  >0.999

<0.001  <0.001  >0.999  >0.999

0.14  0.01  0.02

Control  DMSO  4 μM SU5416  10 μM SU5416

1st EC  2nd EC  3rd EC  4th EC  6th EC
Figure 5-figure supplement 2

3 dpf Tg(fli1aep:EKC);Tg(fli1a:H2B-mCherry) sp1/csf3r MO

Uninjected control 0 h 3 h

A

B

C

D

E

F

G

H

Uninjected control Pre- ablation 3 hpa

Control

A’

B’

C’

D’

E’

F’

G’

H’

Ablated ISV ECs

ISV

enr
dep

enr
dep
Figure 6

4 dpf Tg(actb2:GCaMP6f);Tg(kdrl:mCherry-CAAX)

A

5 mpa

10 mpa

15 mpa

20 mpa

B

Control

Ablated ISV EC

Adjacent ISV EC

Time post-ablation (min)

C

post-ablation nuclear EKC

D

post-ablation nuclear EKC

E

post-ablation nuclear EKC

F

post-ablation nuclear EKC

4 dpf Tg(fli1a:eKCl);Tg(fli1a:H2B-mCherry)

2 mpa

5 mpa

8 mpa

11 mpa

14 mpa

G

H

Nuclear EKC

D statistic

0.847

0.812

0.710

0.673

0.693

0.5

1.5

Time post-ablation (min)
Figure 6-figure supplement 2

A

1% DMSO or 50 μM Nifedipine

3 hours

30 mins

Image 4 dpf Ablate ISV

H

1% DMSO or 50 μM Nifedipine

30 mins

3 hours

Image 4 dpf Ablate ISV

Wash with 1% DMSO

Image vessel response 3 hpa

4 dpf Tg(lf1aep:EKC);Tg(lf1a:H2B-mCherry)

Control (1% DMSO)

0 h

3 h

Pre-ablation

1% DMSO

3 hpa

50 μM Nifedipine

Pre-ablation

3 hpa

B

C

D

E

F

G

B'

C'

D'

E'

F'

G'

I

J

K

L

M

N

I'

J'

K'

L'

M'

N'
Figure 7

Pre-ablation

15 minutes post-ablation

3 hours post-ablation

macrophage

Wounded site

Erk inactive
Ca^{2+} signalling inactive EC

Erk active
Ca^{2+} signalling active EC

Order of Erk signalling activation

Vegfa

Vegfr signalling activity

Erk active
Ca^{2+} signalling active EC