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FRET imaging of *Teen* sensor captures ERK activation changes preceding morphological defects in a RASopathy zebrafish model and phenotypic rescue by MEK inhibitor

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Abstract

Background
RASopathies are genetic syndromes affecting development and having variable cancer predisposition. These disorders are clinically related, and are caused by germline mutations affecting key players and regulators of the RAS-MAPK signaling pathway generally leading to an upregulated ERK activity. Gain-of-function (GOF) mutations in \textit{PTPN11}, encoding SHP2, a cytosolic protein tyrosine phosphatase positively controlling RAS function, underlie approximately 50% of Noonan syndromes (NS), the most common RASopathy. A different class of these activating mutations occur as somatic events in childhood leukemias.

Method
Here, we evaluated the application of a FRET-based zebrafish ERK reporter, \textit{Teen}, and use of fast and quantitative FRET protocols to monitor non-physiological RASopathy-associated changes in ERK activation. In a multi-level experimental workflow, we tested the suitability of the \textit{Teen} reporter to detect \textit{pan}-embryo ERK activity correlates of morphometric alterations driven by the NS-causing \textit{Shp2}^{D61G} allele.

Results
Spectral unmixing- and acceptor photobleaching (AB)-FRET analyses captured pathological ERK activity preceding the manifestation of quantifiable body axes defects, a morphological pillar used to test the strength of SHP2 GoF mutations. Last, the work shows that by multi-modal FRET analysis we can quantitatively trace back to early development the modulation of ERK phosphorylation obtained by low-dose MEK inhibitor treatment, occurring before the onset of morphological defects.

Conclusion
This work proves the usefulness of FRET imaging protocols on both live and fixed \textit{Teen} ERK reporter fish to readily monitoring and quantifying pharmacologically- and genetically-induced ERK activity modulations in early embryos, representing a useful tool in pre-clinical applications targeting RAS-MAPK signaling.

Keywords: RASopathies, ERK, zebrafish embryos, FRET
Introduction

Extracellular signal-regulated kinases (ERK) 1 and 2 are the last tiers of the mitogen-activated protein kinase (MAPK) signaling cascade, a major evolutionary conserved effector pathway of RAS proteins. The cascade translates a wide array of morphogens’ inputs, thereby controlling several cellular processes, such as proliferation, survival, migration, and differentiation, during critical developmental windows (Gotoh et al., 1991; Krens et al., 2006; Shaul and Seger, 2007; Krens et al., 2008a). Upon growth factor stimulation and autophosphorylation of activated cell surface receptor tyrosine kinases, RAS proteins are activated by guanine nucleotide exchange factors that are recruited to the membrane. RAS activation also requires proper function of SHP2, a cytoplasmic protein tyrosine phosphatase acting as positive regulator of the pathway (Tartaglia et al., 2004b; Tajan et al., 2015) by dephosphorylating regulatory tyrosine residues representing docking sites for proteins negatively controlling RAS function (Dance et al., 2008).

Somatic mutations in several genes encoding the three members of the RAS subfamily, their effectors, and a number of positive and negative modulators of RAS function represent the most common event driving oncogenesis (COSMIC database, https://cancer.sanger.ac.uk/cosmic). Mounting genetic evidence of the last two decades has also showed that germline mutations in the same genes underlie a family of developmental disorders collectively called “RASopathies” (Tartaglia and Gelb, 2010; Rauen, 2013; Tartaglia et al., 2022). Despite the different genes and mutations involved, these disorders are clinically related, the majority sharing upregulation of signal flow through the RAS-MAPK pathway during development. Major shared features include post-natal short stature and distinctive craniofacial gestalt, developmental delay, variable cognitive deficits, congenital heart defects and hypertrophic cardiomyopathy, skeletal defects and ectodermal anomalies (Tidyman and Rauen, 2009; Tartaglia et al., 2011; Rauen, 2013; Jindal et al., 2017), testifying the importance of a correct RAS-MAPK signaling for proper development.

Germline gain-of-function (GoF) mutations in PTPN11, encoding SHP2, account for 50% of Noonan syndrome (NS), (Tartaglia et al., 2001; 2002), the most common and clinically variable among RASopathies (Tartaglia et al., 2011). Mutations are almost always missense changes and perturb SHP2’s function through distinct mechanisms, with the majority enhancing SHP2’s function by impairing the switch between the active and inactive states, favoring a shift in the equilibrium toward the former (Tartaglia et al., 2004b). Mutations are more prevalent among subjects with pulmonic stenosis and short stature, while are less common in individuals with hypertrophic cardiomyopathy and/or severe cognitive deficits. Of note, SHP2 activity during neurodevelopment
is particularly important for a correct corticogenesis (Yamamoto et al., 2005). Consistently, functional data in mice demonstrate that the NS-associated \textit{SHP2}^{D61G} mutation causing GoF of SHP2 and enhanced RAS-MAPK signaling, impairs the balance between gliogenesis and neurogenesis in the developing cortex (Gauthier et al., 2007; Ehrman et al., 2014). Children with NS are predisposed to a spectrum of hematologic abnormalities and malignancies, including juvenile myelomonocytic leukemia (JMML) (Niemeyer, 2014), and a distinct class of mutations in this gene are acquired as somatic events and occur in approximately one-third of children with isolated JMML as well as variable proportions of other childhood myeloid and lymphoid malignancies (Tartaglia et al., 2003, 2004a). These mutations alter residues located at the interface between the N-SH2 and PTP domains but are more activating compared to germline variants and are not compatible with embryonic/fetal development (Keilhack et al., 2005; Tartaglia et al., 2006).

During the last two decades, >20 genes have been causally linked to RASopathies (Tartaglia et al., 2022). We now know that in these disorders, increased RAS-MAPK signaling can result from the upregulated activity of various GTPases of the RAS family, increased function of signal transducers positively controlling RAS activity or favoring RAS interactions with RAF kinases, functional upregulation of the three tiers of the MAPK cascade, or inefficient signaling switch-off operating at different levels. Notably, most RASopathy-causing mutations show functional convergence operating at the level of RAS and RAF proteins (Tartaglia et al., 2022). Given the rarity of these conditions, poor therapeutic options are currently available for patients. Our understanding of the molecular genetics of RASopathies, however, has favored to more accurately appreciate the molecular mechanisms impacting RAS signaling, which is a prerequisite to approach targeted therapies to ameliorate or treat the evolutive complications of these disorders (Gelb et al., 2022; Hebron et al., 2022). MEK inhibitors have been proposed as possible therapeutic intervention (Andelfinger et al., 2019), and have been successfully applied to treat RASopathy-associated cardiomyopathy and lymphedema (Gelb et al., 2022).

Evidence of possible efficacy of early MEKi administration to prevent RASopathy-associated developmental defects begins to accumulate in mice (Chen et al., 2010; Hernández-Porras et al., 2014; Inoue et al., 2014) and fish (Runtuwene et al., 2011; Anastasaki et al., 2012; Bonetti et al., 2014) models. Similarly, these model systems have been used to validate also targeted therapeutic approaches (Lee et al., 2014; Bobone et al., 2021; Das et al., 2021). However, further pre-clinical studies to clarify the current uncertainty about treatment windows and specific phenotypic rescue
correlated to ERK activity modulation in developing tissues are necessary for an effective use of the available MEKi in the clinical practice.

The use of zebrafish as a vertebrate model offers the possibility of settings parallel morphological and molecular pre-clinical readouts in vivo. A number of assays can be performed in embryos for carrying out a rapid functional classification of variants of unknown significance (VoUS), and for a detailed study of mutations’ impact on embryogenesis. Zebrafish can be also an informative experimental model system to validate potential therapeutic solutions in terms of dosage and effective treatment windows (Patton et al., 2021).

Zebrafish represents a solid model to investigate the differential impact of both GoF and loss-of-function (LoF) RASopathies-causing mutations (Runtuwene et al., 2011; Bonetti et al., 2014; Nakagama et al., 2020; Motta et al., 2021). Clear diseases features that are translatable in scorable morphological parameters are conveniently recapitulated in young fish mutants can demonstrate variants strength and mechanism of action underlying variable clinical severity. Convergence extension impairment impacting early axes morphogenesis are often an early disease hallmark scored in RASopathies models in both fish and insects (oval embryo assay), consistently with the activity of growth factor-stimulated RAS-MAPK signaling during gastrulation (Delfini et al., 2005; Anastasaki et al., 2012; Gervaise and Arur, 2016; Patel et al., 2019; Hayashi and Ogura, 2020).

Spatio-temporal ERK phosphorylation dynamics driven by RASopathies-associated variants and modulated by drug treatments in developing embryos can now be studied live thanks to various FRET-based ERK activity sensors developed and tested in vivo (Kamioka et al., 2012; Lauri et al., 2021). In a FRET-based extracellular-regulated kinase reporter (EKAR) type, active ERK (pERK) phosphorylates a substrate within the sensor, which is then translated in a conformational change bringing together a donor (D)-acceptor (A) pair (such as CFP and YFP). A FRET phenomenon can then be registered in vivo within tissues and quantified, which is proportional to the distance between D and A and therefore the level of pERK in the cells, a proxy for activated RAS/MAPK-ERK signaling (Komatsu et al., 2011). While quantitative determination of RAS-MAPK signaling during mice embryogenesis is challenging, FRET-based sensors in vivo in zebrafish, such as the recently developed EKAR-type Teen biosensor (Tg[ef1a:ERK biosensor-nes] Teen) (Wong et al., 2019), are very useful.

Teen reporter recapitulates high ERK activity in known domains of the developing embryos and enables a continuous readout and spatial mapping of ERK dynamics during gastrulation and segmentation stages and upon pharmacological modulation (Wong et al., 2019). A complementary
A morpho-molecular approach with spatio-temporal resolution at pan-embryo level is expected to contribute to faster and precise mutants’ strength testing – and thereby disease sub-type classification – as well as to cogent verification of drug efficacy in vivo. Indeed, a proof of principle approach was obtained by ERK actuators and shown by Patel et al., 2019 (Patel et al., 2019), exploring early real-time see-through examination of morphological changes and co-occurring ERK activity fluctuations in early insect and fish embryos. Here, by using complementary quantitative FRET imaging protocols both in live embryos and on fixed specimens, we tested the potential of Teen biosensor to capture as early as possible spatially-restricted pathogenic ERK activity events (molecular level) correlating with the onset of known axes defects (morphological level) in whole developing fish mutants expressing the NS-causing Asp61Gly (D61G) substitution in Shp2. We examine the effectiveness of low- and high-dose treatment with the MEKi, PD0325901 with respect to both the morphological and ERK activity rescue and tested the usefulness of two quantitative FRET protocols to assess early drug effect on pERK.

Materials and Methods

Zebrafish husbandry

Wild-type zebrafish (NHGRI) (LaFave et al., 2014) were obtained from EZRC (European Zebrafish Resource Center), and Tg[ef1α:ERK biosensor-nes] (Teen) (Wong et al., 2019) were obtained from (National BioResource Project of Japan for Zebrafish, RIKEN) (Urasaki et al., 2006; Okamoto and Ishioka, 2010). Fish were cultured following standard protocols. Zebrafish lines were housed in a water-circulating system (Tecniplast©) under controlled conditions (light/dark 14:10, 28 °C, 350–400 μS, pH 6.8–7.2) and fed daily with dry and live food (freshly hatched nauplii of Artemia salina). All animal experiments were performed according to standard breeding and ARRIVE guidelines (https://arriveguidelines.org) with the approval of the Italian Ministry of Health (DGSA -Direzione generale della sanità animale e dei farmaci veterinari, code: 23/2019-PR).

Embryos treatment with SHP099

A pool of Tg[ef1α:ERK biosensor-nes] (Teen) zebrafish embryos were collected at 4 hpf and treated until 24 hpf with SHP099 inhibitor (MedChem Express, HY-100388, 10 - 15 μM) in 200 μl per well in a 24 well plate (with circa 20 per well). Embryos were kept in the incubator at 28°C during the time of treatment, fixed at 24 hpf in dry ice. All experimental conditions received the same concentrations of DMSO (vehicle control, 0.01%).
Embryos treatment with recombinant EGF

For western blot analysis and standard FRET imaging using “Calcium Calculator” module of Leica Sp8 Tg[ef1α:ERK biosensor-nes] (Teen) zebrafish embryos at 24 hpf were collected and treated with acute exposure to EGF 1 mg/ml (Gibco, 400-25-1MG) in 200 μl per well in a 24 well plate (with circa 20 per well). For brain EGF delivery in vivo, 1-2 nl of a stock solution of EGF (1 mg/ml) were delivered to the brain ventricle via microcapillary.

Shp2 mRNA injection in zebrafish embryos and treatment with the MEK inhibitor PD0325901

The full coding sequence of the zebrafish shp2WT and mutant (shp2D61G) from the pCS2+_eGFP-2a-Shp2a (Bonetti et al., 2014) were subcloned without GFP tag into pCSDest vector using Gateway cloning (ThermoFisher, 11789020, 11791020). Plasmid linearization was performed with KpnI-HF enzyme (New England Biolabs, #R3142) and capped shp2 mRNAs was transcribed using mMACHINE™ SP6 Transcription Kit Poly A Tailing Kit (ThermoFisher, AM1340, AM1350) following manufacturer’s instructions. 60 pg was capped mRNA was injected in one-cell stage Tg[ef1α:ERK biosensor-nes] (Teen) zebrafish embryos using FemtoJet 4x microinjection system (Eppendorf). Injected embryos were cultured under standard conditions. Body axis and length measurements were conducted in embryos at the end of gastrulation (11/12 hpf) and in hatched larvae, respectively. For FRET experiments, Teen fish were screened for GFP fluorescence at around 4 hpf. For the treatment with PD0325901 MEK inhibitor, embryos were randomly divided in three subgroups of 20 – 25 embryos at 4 hpf and treated with low (0.25 μM) and high (1 μM) doses. Embryos were raised until 5.30/6 hpf (for FRET experiments) and 11/12 hpf (for FRET experiments, body axis analyses and western blots) or until 55/72 hpf (for body elongation measurements). All experimental conditions received the same concentrations of DMSO (vehicle control, 0.01%).

Oval embryo test and body axes ratio measurements

At the end of gastrulation (11/12 hpf), live Shp2-overexpressing zebrafish embryos were imaged using Leica M205 microscope (Leica Microsystems) with a 2x magnification objective to evaluate the occurrence of oval shape (aberration of major vs minor axis). Hatched embryos were laterally sided and imaged with the same conditions to measure overall body elongation. Embryo axes ratio (major-to-minor) and body length were measured using Fiji software.

Fluorescence Resonance Energy Transfer (FRET) by “Acceptor photobleaching” (AB) modality

Tg[ef1α:ERK biosensor-nes] (Teen) positive zebrafish embryos were fixed in 4% PFA/PBS at room temperature for 1 hour and stored in PBS 1X at 4°C. Fish were mounted laterally in 2% low-melting
agarose dissolved in PBS1x (Sigma-Aldrich, A9414). FRET assay experiments were carried out using the FRET-AB module of the LAS X software of the Leica TCS-SP8X confocal microscope (Leica Microsystems) equipped of an Argon laser with 458-476-488-496-514-nm lines (50% of laser power), using a Fluotar Visir 25x/0.95 numerical aperture (NA) water immersion objective, with a 1024x1024 image format, an optical zoom of 1.5x, 400 Hz scan speed, and a 16-bit image resolution. FRET-AB wizard of LAS X software was used for the evaluation of FRET efficiencies. Firstly, 2 pre-bleaching imaging steps were performed in sequential mode using the 458 nm excitation line to acquire the donor CFP signal (465-500 nm capture range of the emitted light), and the 514 nm excitation line to acquire the acceptor YFP signal (520-555 nm capture range). Then specific regions of interest (ROIs) were designed for the next bleaching step. For FRET-AB acquisitions at 24 hpf, all the ROIs were designed with similar sizes for all samples. After 50 bleaching iterations of the acceptor fluorophore using the 514 nm laser line (100% intensity) in each designed ROI, an imaging step of donor and acceptor was performed.

**In vivo spectral FRET imaging**

Spectral FRET imaging was performed on a TCS-SP8X confocal microscope (Leica Microsystems) equipped with a stage incubator (OkoLab, Italy) allowing to maintain stable conditions of temperature (28°C) and humidity during live cell imaging, using the following setting: XYλZt scanning mode with the 458 nm excitation laser line (50% of the Argon laser power), operating in the 460-570 nm of the spectral range with 5 nm of l step size, using a PLapo 10x/0.40 NA dry objective, 512x512 image format and 8 bit image resolution (for 6 hpf samples) or 1024x1024 and 16 bit image resolution (for 24 hpf samples), scan speed of 400 Hz, z-step size of 8 mm. Spectral 3D time series were acquired with a 30 min intervals. CFP and YFP signals were separated by LAS X software using the spectral dye separation tool, using the following settings: CFP (465-500 nm) and YFP (525-570 nm) emission spectra. After the imaging setting definition, a third channel was set up to record the YFP/CFP ratio fluorescence.

**Measurements of in vivo FRET changes in EGF bath-treated embryos**

Live FRET changes induced by bath stimulation with recombinant EGF (1mg/ml) were assessed at TCS-SP8X confocal microscope (Leica Microsystems) equipped of a stage incubator (OkoLab, Italy) allowing maintenance at 28°C and a humidified atmosphere, using the XYZt scanning mode with the activation of the 458 nm laser line (50% of the Argon laser power) to excite the CFP donor only, and
the detection of the two wavelength range for CFP (465-500 nm) and YFP (525-570 nm) emission spectra, respectively.

**FRET data analysis and image rendering**

FRET data of zebrafish live and fixed samples were processed using the Leica LASX v. 3.5 and Fiji. Intensity FRET signal from the spectral unmixing imaging on live samples and FRET efficiency data from the AB experiments on fixed specimens were obtained using the spectral dye separation and the FRET-AB modules, respectively, implemented within Leica LAS X v.3.5. To analyze changes in FRET signal from the spectral unmixing data, analysis of selected ROI was conducted from sum-intensity Z-projections of ratiometric images (FRET, YFP, Channel 2) / donor, CFP, Channel 1) from the entire embryos in 8 or 16-bit format.

For FRET-AB, the mean values of FRET efficiency within the bleached ROI were obtained by the following formula: 
$$E = \frac{(DΩ-DA)}{DΩ},$$
where DA is the donor-intensity before bleaching, and DΩ is the donor-intensity after bleaching. Donor-Acceptor (D-A) fluorophore distance (calculated as RDA value) was obtained using the following formula:
$$R_{DA} = \sqrt{\frac{R_0^2 - R^2}{E}} = \frac{R_0}{\sqrt{E}} - 1.$$

To obtain RDA values, the Foster distance (R₀) for YFP-CFP (A-D) FRET pair was assumed to be 4.7 nm as previously reported (Patterson et al., 2000; Hennigan et al., 2009; Vogel et al., 2014).

For image rendering, confocal sum-intensity z-projections were generated for both the FRET (YFP, channel 1) and donor (CFP,channel 2) using Fiji dedicated plugins. “Smart” LUT was used to show FRET/CFP ratiometric image and brightness, contrast, smoothing and noise correction parameters were adjusted equally within the image and among samples belonging to the same experiment.

**pERK levels detection via immunoblot of embryos protein extracts**

Not injected control fish and fish expressing Shp2 (WT and mutant) treated either with vehicle control or PD0325901 MEK inhibitor were collected at 24 hpf, fixed in dry ice and stored at -80°C. Total protein lysates were obtained by sonication in cold RIPA lysis buffer (150 mM NaCl, 50 mM TrisHCl pH 8.1% Triton x-100, 0.5% Sodium deoxycholate, 0.1% SDS, ddH₂O) containing inhibitor cocktail 2 and 3 protein phosphatases (Sigma-Aldrich, P5726, P0044) and Roche cOmplete Mini EDTA-free protease tablets (Roche, 1183617000). Equal amounts of protein extracts (~ 30 μg) were separated on a 10% Sodium dodecyl sulfate (SDS)-polyacrylamide gel. The total protein concentration was determined by the Bradford assay (Bio-Rad, #5000205) using Infinite M Plex.
After electrophoresis, proteins were transferred to Trans-Blot Turbo Mini 0.2 µm nitrocellulose Transfer Packs (Biorad, #1704158) using Trans-Blot Turbo Transfer System (Biorad). Blots were blocked with 5% non-fat milk powder (diluted in TBST 1X, Santa Cruz Biotechnology, sc-2324) overnight at 4 °C constantly shaking and incubated with the primary antibody in blocking solution (5% non-fat milk in TBST 1X). The following primary antibodies were used: rabbit polyclonal p42/p44 MAPK (Erk1/2) (1:3000, Cell Signaling, #9102) and mouse monoclonal phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (1:1000, Cell Signaling, #9106). Following several washes in TBST 1X, membranes were incubated with anti-rabbit- (1:8000, Thermo Fisher, 31460) and anti-mouse-HRP-conjugated secondary antibodies (1:3000, Thermo Fisher, 31450). Immunoreactive proteins were detected by SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher, 34095) according to the manufacturer’s instructions, and an Alliance Mini HD9 with Q9 Mini 18.02-SN software (Uvitec) was used for chemiluminescence detection. For densitometric analysis of specific protein bands the free Band/Peak Quantification tool of Fiji software was used.

**pERK level detection in embryonic tissues via whole-mount Immunohistochemistry**

24hpf zebrafish embryos (not injected and EGF-treated) were fixed in 4% PFA/PBS (Thermo Fisher, 28908) for 3 h at room temperature (RT), washed in 0.8% PBS-Triton and stored in PBS 1X at -20°C. Immunostaining was adapted from a previously reported protocol (Myklatun, Lauri et al., 2018). Briefly, fixed samples were washed in PBS-Triton 0.8% and incubated with 150 mM Tris–HCl pH 9.0 for 5 min at RT and 15 min at 70 °C for antigen retrieval. Samples were permeabilized with 1 µg/ml proteinase K (Sigma-Aldrich, P2308) for 20 min at RT, followed by post-fixation in 4% PFA/PBS for and incubation for 2 hours in blocking solution in 5% of normal goat serum (NGS), 1% of BSA, 1% DMSO in 0.8% PBS-Triton at RT. The following primary antibodies were used: mouse monoclonal p44/42 MAPK (ERK, 1:250, Cell Signaling, 4696S), rabbit polyclonal phospho-p44/42 MAPK (pERK, 1:250, Cell Signaling, 4695S). The following secondary antibodies were used: goat anti-mouse Alexa Fluor 488 (1:600, Thermo Fisher, A11001) and goat anti-rabbit Alexa Fluor 633 (1:600, Thermo Fisher, A21070). The Stellaris 5 confocal microscope (Leica Microsystems) equipped with LAS X software v. 4.5 and an HC FLUOTAR L VISIR water immersion 25x/0.95 objective were used for z-stack image acquisition. Z-stacks of embryos mounted in 90% glycerol/PBS were acquired sequentially with 499 nm and 631 nm laser lines and emission range of 507-551 nm and 644-740 nm, respectively. Brain acquisition were obtained by scanning with a 1024x1024 format at 200 Hz and 1.5 z-step size. Eye and tail acquisition were obtained by scanning with a 512x512 format at 400 Hz and 5 z-step size.
Statistical assessment and illustrations preparation

Statistical comparisons were performed using unpaired One-tail Student’s t-tests and One-Way ANOVA with Dunnett’s and Holm-Sidak post hoc test as indicated in the relative figure legends using GraphPad Prism software v. 9.4.1. Data are represented as mean ± SEM (for parametric data) or median with interquartile range (for non-parametric data) and statistical significance was assumed by p < 0.05 (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

The schematic illustrations of this paper were generated by researchers using Illustrator (Adobe). Embryos, plate, tubes and western blot schematic images were obtained by BioRender.com.

Results

ERK activity changes are registered live in Teen embryos upon positive and negative pharmacological modulation of RAS/MAPK signaling

RASopathies are largely caused by mutations affecting signal transducers and modulators operating at various levels along the RAS-MAPK signaling pathway resulting in sustained ERK activity. The generalized upregulation of this signaling cascade during embryogenesis reflects the multisystem involvement of these disorders. Therefore, we first set out to assess the possibility to register a non-physiological FRET signal increase obtained by acute stimulation with a growth factor able to activate signaling through this cascade (Figure 1A, upper schematics); then we assessed pathological ERK activity registration in presence and absence of signal modulation driven by a potent MEKi (Figure 1A and B). To obtain such positive control of signal modulation, we treated Teen reporter zebrafish 22-24hpf embryos with high-dose recombinant EGF by microcapillary-mediated delivery to the proximity of the anterior-most ventricles of the forebrain. Indeed, the diamond-shaped ventricles are visibly open at this stage (Lowery and Sive, 2005) and readily accessible for microscope-guided manipulations, such as dye or drug delivery (Figure 2A). Moreover, this is a developmental domain with active RAS-MAPK signaling, as shown both in mouse and in zebrafish embryos (Wong et al., 2019).

To acquire FRET information correlating to ERK activity in the Teen embryos we employed “spectral unmixing” wizard of the confocal microscope used to perform donor and acceptor emission spectra decomposition of the CFP-YFP FRET pair from live acquisitions obtained solely by donor excitation with 458 nm laser and collection of emission spectra between 460-570 nm. We first ensured that the imaging conditions used for spectral unmixing were compatible with correct development of the embryo from gastrulation to pharyngula-stage and that ERK activation observed using this
method reproduced what had previously been reported in embryos and using Teen sensor. As assessed by time lapse from late gastrulation to segmentation, Teen embryo developed normally (Supplementary video 1 and Supplementary Figure 1). As reported (Sari et al., 2018; Wong et al., 2019), Donor (CFP) signal was evidence in the entire embryo and relatively high FRET signal (active ERK) could be confirmed in regions previously reported for with sustained ERK activity (i.e. margin of the animal pole in late gastrula), forming tail bud, forebrain, midbrain-hindbrain and segments in later embryo, (Supplementary Figure 1).

In 22-24hpf embryos, by comparing live spectral recording before (T0) and after (T2 = 30 minutes) live EGF delivery and quantifying the FRET/CFP signal intensity (FRET index), we were able to register clear increased ERK activity in tissue in the closed proximity of the anterior ventricle, especially within the ventral domain (Figure 2B-D). Spectral unmixing applied to a x,y,z acquisition is generally a lengthy procedure, requiring the live collection of many emission spectra, which limits the imaging of fast occurring ERK changes in a 4D (x,y,z,t) imaging setting. To visualized the increased EGF-induced ERK activity in embryos we also employed a simple scanning-based imaging mode using donor excitation at 458 nm and separated single emission windows for donor and acceptor analyzed in post-imaging ratiometric quantification. We observed a global increase of ERK activity in known domains of the embryo occurring already upon 10 min from the start of the EGF treatment, which was obtained by simple bath immersion (Supplementary video 2 and Supplementary figure 2). The increased signal observed in the both live FRET data, including the active ventral forebrain domain, was validated by standard whole-mount immunofluorescence performed against phosphorylated ERK (pERK) and normalized by total ERK (tERK) on siblings treated with the same concentration and timing of EGF as compared to control embryos (Supplementary Figure 3A). The data were consistent also with western blot analysis corroborating increased levels of pERK from whole-embryo protein extracts upon 10 minutes treatment (Supplementary Figure 3B).

Next, calculation of the FRET index by spectral unmixing was also able to capture a strong and global reduction of the signal correlating to ERK activity in live 24hpf embryos obtained by upstream signal inhibition, via the specific SHP2 inhibitor SHP099, compared to siblings treated with DMSO as control vehicle (Figure 1A, lower schematics and Figure 3 A,B). Significantly reduced signal was evident when we performed ratiometric quantification of the signal in spatially distinct regions normally associated with high RAS-MAPK signaling (Figure 3B,B’). Also in this case, we further validated the global reduction of the pERK signal observed by spectral unmixing in Teen embryos by western blot analysis from whole-embryo protein extracts of siblings treated with the same protocol.
The experiments showed that acute treatment of developing embryos with EGF and persistent treatment with the specific SHP2 inhibitor from early gastrula (4hpf) to pharyngula stage (24 hpf) effectively activate and reduce ERK activation in known domains characterized by high MAPK signaling, and that such modulation can be efficiently captured with live FRET imaging.

By use of spectral unmixing and specific modulatory drugs in addition to those previously reported, these results confirmed and broadened existing evidence that the protocols used for live FRET imaging in *Teen* embryos can be used to observe non-physiologically positive and negative modulation of ERK activity acting at various levels of the RAS/MAPK signaling in live fish.

**Acceptor Photobleaching (AB)-FRET can detect decreased ERK activity mediated by pharmacological signal inhibition in fixed Teen fish specimens**

Next, we assessed the versatility of the *Teen* reporter to other FRET imaging protocols, that could broaden the application of this zebrafish ERK biosensor. We asked whether the ability to detect pharmacologically-induced changes of ERK activity observed in live tissue by FRET-dependent YFP acceptor emission measurements could be reproduced by deriving directly the energy transfer efficiency (E) from CFP Donor (D) to the YFP Acceptor (A). This value correlates directly with the distance between D and A (R<sub>DA</sub>) and thereby, in *Teen* sensor, with ERK activity (Algar et al., 2019). To this aim we used the Acceptor photobleaching (AB)- FRET protocol (Figure 1A, lower schematics) where E can be estimated by measuring the fluorescence emitted by D and A before and after performing Acceptor Bleaching (AB). AB in fact impedes FRET phenomenon and consequentially increases D fluorescence emission proportionally to the distance between A and D fluorophores within the reporter pair (here a proxy for limited ERK activity). Because of the bleaching step and hence the incompatibility of AB-FRET for live acquisition, we turned to fixed samples.

We fixed a group of 24hpf *Teen* fish which underwent the same SHP099 treatment protocol that produced potent signal inhibition in the live experiments. We limited the AB procedure mainly to representative regions of interest known for high RAS-MAPK signaling (various brain domains and the presomitic mesoderm, PSM of the growing tail bud region, Tb). In these domains we could effectively derive a decrease in FRET E (%) upon SHP099 chronic treatment, with a stronger effect in the brain (Figure 3 A, C,C'). Increased distance between D and A in SHP099-treated embryo was observed when R<sub>DA</sub> was retrieved from the efficiency data, which was significant for anterior brain regions (Supplementary Figure 4B).
Overall, the data indicate that also AB-FRET protocol can be used in fixed Teen embryos to detect strong modulation of the signal.

**Increased ERK activity can be recorded by FRET in Teen embryos expressing the NS-causing Shp2$^{D61G}$ allele before the onset of quantifiable morphological defects**

Having obtained effective FRET data for pharmacologically-induced positive and negative signal modulation in both live and fixed Teen embryos, we next tested the suitability of the approach to detect early signaling impairment caused by a recurrent pathogenic SHP2 amino acid substitution, D61G, causing NS, which determines recognizable morphological defects occurring during embryogenesis (Bonetti et al., 2014). Therefore, we turned our analysis to an established zebrafish RASopathy model obtained by overexpressing Shp2 carrying the NS-causing D61G substitution in Teen embryos. Overexpression of WT Shp2 can induce a certain degree of phenotype in mice models (Hu et al., 2017). The more severe impact of the mutant form (Shp2$^{D61G}$) on morphological development compared to overexpression of Shp2$^{WT}$ is however clearly visible in zebrafish models (Bonetti et al., 2014; Paardekooper Overman et al., 2014). Therefore, we decided to use embryos overexpressing the WT protein as control, such to be able to assess the sensitivity of the protocol in discriminating pathogenic signaling changes of limited entity, resembling the small activity increase necessary to trigger developmental defects in RASopathies.

First we confirmed the characteristic shortening of the individuals accompanied by edema in hatched Shp2$^{D61G}$ expressing fish (50-72hpf, *morphological level 1*, L1, Figure 4 A), which was anticipated by impaired axes establishment quantifiable at the end of gastrulation, when head and tailbuds begin to be visible and somitogenesis starts (11-12hpf, *morphological level 2*, Figure 4 B). Next, we examined by spectral unmixing one of the Shp2$^{D61G}$ mutant Teen embryos exhibiting an aberrantly elongated major axis at this stage and noted a visible increase in FRET signal compared to the control fish expressing Shp2$^{WT}$ (*molecular level 2*, L2, Figure 4 C). This was especially clear in the tail region (right insets in Figure 4 C).

We set out to assess whether an increased FRET signal could set apart Shp2$^{D61G}$ activity on RAS-MAPK signaling already at around 5 hpf (approximately 40% epiboly, *molecular level 3*). At these early stages, control and mutant embryos usually appear morphologically indistinguishable and therefore early genotype-phenotype correlation which might speed up and simplify validation and drug screening is difficult. We specifically investigated the margin region of the animal pole where high FGF-induced ERK activity controls morphogenic gastrulation movements (Krens et al., 2008b;
Wong et al., 2019). Indeed, despite no morphological changes were visible in early gastrulae, we documented an increased signal in live Shp2\textsuperscript{D61G}-expressing gastrulae by measuring spectral unmixing-derived FRET index (Figure 1B, upper schematics and Figure 4D-D’). The live data obtained offer a first indication that an early molecular alteration at the level of ERK activity due to constitutively active Shp2 could potentially be detected in Teen NS mutant gastrulae by live FRET. Given the success obtained with applying AB-FRET in SHP099-treated embryos, we investigated further this possibility and we set out to examine directly the entity of transfer efficiency (E) by attempting AB in the margin region of the animal pole of fixed gastrulae (Figure 1B, upper schematics). This additional experiment with a complementary FRET technique corroborated the occurrence of detectable and statistically significant signal increase in a different clutch of NS mutant embryos (Figure 4E,E’). The increase in E in NS mutants at 5-6hpf proportionally correlated with a decrease of the derived R\textsubscript{DA} (Figure 4E’’ and Supplementary Figure 5) and therefore with increased ERK activity.

**Dose-dependent reduction of pathogenic ERK activity is captured early in Teen embryos treated with MEK inhibitor and predicts morphological rescue**

Given that short window treatment with low-dose MEKi can rescue some morphological phenotypes in fish models of NS caused by Shp2\textsuperscript{D61G} (Bonetti et al., 2014), we next asked whether spectral unmixing- FRET could detect signs of moderately reduced ERK activity supposedly obtained by MEKi already in early NS gastrulae. Because of successful reports on prolonged treatment specifically with low-dose MEKi PD0325901 in another RASopathy model clinically related to NS, the cardio-facio-cutaneous syndrome (CFCS) caused by BRAF\textsuperscript{Q257R} (Anastasiaki et al., 2012), we first tested whether longer treatments with low-dose MEKi could rescue also body axis defects due to expression of the NS-associated Shp2\textsuperscript{D61G}.

Hence, we treated embryos with 0.25 \textmu M of the specific MEKi PD0325901 from 4hpf to the desired stage, employing long and short treatment windows and e first verified occurrence of morphological rescue of major RASopathy traits, compared to fish treated with control vehicle (DMSO).

Indeed, we found that constant long treatment (between 4hpf and 55/60 hpf) with low-dose MEK inhibitor partially rescued body shortening in hatching embryos (morphological level 1, Figure 5A). Likewise, a shorter treatment (between 4hpf and 11/12hpf) was already able to rescue the aberrant ratio between the major and minor axes examined by “oval embryo assay” at early segmentation stage (morphological level 2, L2 Figure 5B).
Quantification of live FRET revealed a reduced ERK activity in embryos expressing Shp2\textsuperscript{D61G} treated with 0.25 μM PD0325901 that was visible upon 5 hours of treatment (between 4hpf and 9hpf or 11/12hpf, Supplementary Figure 6A). The capacity of the MEKi to significantly block ERK activation in a dose-dependent manner during this crucial developmental window was further demonstrated by assessing and comparing pERK levels from whole-embryos protein extracts of NS fish treated with DMSO (control), 0.25 μM (low-dose) and 1 μM (high-dose) PD0325901 during the same developmental period. In agreement with the live imaging FRET imaging experiment and previous reports (Anastasaki et al., 2009, 2012; Bonetti et al., 2014) treatment with low-dose yielded a significant pERK/tERK reduction (Supplementary Figure 6B).

Last, we assessed the suitability of AB-FRET measurements, which permits testing a larger cohort and yielded convincing results in capturing increased ERK activity already in early NS gastrulae, to record dose-dependent, spatially restricted ERK modulation to our drug treatments in early Shp2\textsuperscript{D61G} fish mutants upon a short treatment window (between 4hpf and 5.30/6hpf) (Figure 5C-D’, molecular level 3, L3). Analysis of the signal registered in the margin region of early gastrulae before performing AB (Pre AB-FRET analysis) showed already a reduced spatial quenching of the Donor in NS mutants treated with 1 μM PD0325901 for a couple of hours, examined by calculating YFP/CFP and CFP signal intensities (Figure 5D). The values show a strong inhibition of the FRET phenomenon occurring within the Teen reporter at the animal pole, demonstrating a low residual ERK activity obtained by relatively short treatment with high-dose PD0325901.

The strong effect of high-dose MEKi in the animal pole margin region was further confirmed by analyzing the signal registered upon AB in the same embryos (Post AB-FRET analysis). In particular, comparing directly the estimated transfer E % and the R\textsubscript{DA} obtained from measuring NS embryos treated with low-dose (0.25 μM) vs. high-dose (1 μM) MEK inhibitor, we documented a milder signal reduction at the margin region obtained by short treatment window (between 4hpf and 5.30/6hpf) with low-dose treatment (Figure 5C,D” and Figure 6A), a dose which was sufficient to induce morphological rescue if embryos were treated till completion of gastrulation. Altogether, the AB-FRET data are consistent with the strong and milder reduction observed from whole-embryo extracts of fish treated with low and high MEKi dose, respectively, for a longer time window (Supplementary Figure 6B).

Overall, when visualizing the whole collected data by plotting the measures of the earliest morphological sign observed in our NS model (body axes defect at 11/12hpf) vs. E values retrieved from early AB-FRET experiments (5.30/6hpf) in a x,y graph, the pathogenic effect of the NS mutation
(Shp$^{D61G}$ vs. Shp$^{WT}$) correlating to aberrant ERK activation and differential impact of low- vs. high-dose MEK treatment on early signaling and embryo morphogenesis was appreciated (Figure 6B).

Last, the strong modulatory effect on ERK activity observed upon short treatment with $1 \mu M$ PD0325901 in gastrulae (Figure 5D-D''), reduced axes defects when treatment till gastrulation was performed (Figure 6B). However, this treatment option negatively impacted embryo development if prolonged till hatching and even worsened body length phenotype of NS mutants (Figure 6C, Supplementary Figure 7), confirming the same toxicity observed upon prolonged treatment of other RASopathies fish models (Grzmil et al., 2007; Anastasaki et al., 2009, 2012).

**Discussion**

The ease of transparent, rapidly developing zebrafish embryos as experimental animal models for rapid pathogenicity testing of new disease genes and variants potentially affecting RAS-MAPK signaling and causing RASopathies is by now established and the model is routinely employed to investigate also disease mechanisms (Runtuwene et al., 2011; Bonetti et al., 2014; Nakagama et al., 2020; Motta et al., 2021). The convenience of zebrafish embryos-based screenings to test off label treatments, new drug concepts, concentrations and developmental windows is unsurpassed among vertebrate models also in the context of RASopathies (Anastasaki et al., 2012; Bonetti et al., 2014; Patton et al., 2021). However, current functional workflows in zebrafish applied to RASopathies are mainly based on morphological readouts, such as the characteristic reduction in body elongation, cardiac and craniofacial defects, which appear only in late embryos. The earliest morphological hallmarks informative to classify pathogenicity of new human variants affecting RAS-MAPK signaling (i.e. impairment of the embryonic axes) are observed only once gastrulation is completed and segmentation stage begins (between 10 and 12 hpf) and monitoring of ERK activation status is determined mainly by static western blot.

The establishment of recent reporter systems supposedly able to capture ERK activity fluctuations during embryogenesis offers the potential to expand this functional approach to RASopathies towards detecting early molecular signs that can speed up variants’ strengths and impact analysis and classification, anticipate genotype-phenotype correlation quantifiable at segmentation stage, following Patel et al., 2019 (Patel et al., 2019), and serve as fast molecular readout for drug testing in vivo.

With such potentials in mind, the pilot workflow presented here supported the suitability of one of the latest FRET-based EKAR-type ERK reporter system (Teen sensor) developed by Sari et al. (2018)
to register positive and negative modulation of ERK activity in vivo, report early molecular correlates (ERK activity changes), anticipating phenotype occurrence and low-dose pharmacological correction in a well-established RASopathy model caused by constitutive SHP2 activation.

First, we showed that a “spectral unmixing”-FRET protocol similar to the one originally employed for Teen sensor, can capture a strong ERK activation in WT embryos upon upstream signal stimulation (embryo acute EGF exposure). From our ventral confocal scans of developing brain, increased live ERK activity was particularly clear in the ventral forebrain, consistently with data in mice, which indicated the high susceptibility of this domain to RAS-MAPK signaling activation, likely under the local control of SHP2 (Gauthier et al., 2007; Ehrman et al., 2014). As demonstrated by mutant mice expressing constitutively active NS-causing SHP2D61G, this regulatory phosphatase probably plays a crucial role in balancing progenitors’ differentiation during cortex development, controlling gliogenesis and neurogenesis (Gauthier et al., 2007). Further studies would be necessary to establish whether a similar control is established in zebrafish anterior brain and whether this is an ancestral vertebrate trait.

Employment of a different FRET protocol applicable to fixed specimens (AB-FRET) allowed us also to register potent ERK signal inhibition, obtained by acting upstream, directly on zebrafish SHP2 ortholog in vivo, via the specific SHP2 inhibitor, SHP099. These pilot data prove that both positive and negative pharmacologically-induced ERK modulation can be recorded in Teen embryos, broadening previous reports, in which FRET signal registration was performed in Teen expressing zebrafish embryos only via spectral unmixing and upon signal modulation obtained by different downstream activators and inhibitors (Wong et al., 2019).

Demonstrating the relevance of this FRET-based ERK activity readout in whole embryo for human diseases, by using the validated live FRET and AB-FRET protocols here, we were able to register increased ERK activity in a transient NS zebrafish model generated by over-expression of the mRNA encoding Shp2D61G. Specifically, in a first step of our experimental workflow, we verified the occurrence of a subset of the morphological hallmarks normally used to score NS (and other RASopathies) from late to early embryos. Body elongation and axes morphogenesis defects (morphological level 1 and level 2) were confirmed in our model, consistently with the known role of FGF-stimulated RAS-MAPK signaling in paraxial mesoderm and gastrulation in various species (Delfini et al., 2005; Gervaise and Arur, 2016; Hayashi and Ogura, 2020).

During early segmentation stages in which axes defects begin to be quantifiable, in vivo FRET imaging by “spectral unmixing” showed encouraging results in capturing spatially-restricted
diseased NS-associated ERK activation (*molecular level 1*). Furthermore, the *in vivo* FRET index calculated from signal detected already in early gastrulae demonstrated that the technique might be sensitive to moderate/mild signs of ERK activity increases occurring in NS mutant embryos ahead of start of head and tail morphogenesis (*molecular level 2*). Moreover, we additionally assessed and reported the positive performance of AB-FRET in documenting early ERK activity increase occurring in morphologically indistinguishable NS gastrulae (expressing Shp2^{D61G}) compared to WT animals (expressing Shp2^{WT}). The effect was clear when examining the margin of the animal pole, where active RAS-MAPK signaling is crucial during embryogenesis for epiboly and gastrulation movements (Krens et al., 2008a; Wong et al., 2019) (*molecular level 3*).

Altogether the experiments show the suitability of both live- and AB-based FRET for RASopathy signal investigation *in vivo* in fish models and demonstrate that Shp2^{D61G} causes an increased ERK activity early in the animal pole margin of gastrulating embryos, preceding the onset of discernable morphological defects. It is worth stressing that, given the GoF mechanism underlying the observed defects, we expected that overexpression of the WT form of Shp2 could already lead to aberrantly increased signaling and developmental defects, as reported in mice models (Gauthier et al., 2007). Nevertheless, paralleling the performance of macro/morphological analysis, our FRET protocols were sensitive enough to capture subtle ERK signal differences between the overexpression of WT Shp2 or the disease-associated Shp2^{D61G} mutant.

Last, expanding on various reports of successful employment of MEKi in mice and fish models (Anastasaki et al., 2009, 2012; Chen et al., 2010; Bonetti et al., 2014; Hernández-Porras et al., 2014; Inoue et al., 2014; Gelb et al., 2022; Hebron et al., 2022) and together with the work by Sari et al. (2018) and Wong et al (2019) we provide a ground assessment for the possible application of various FRET protocols in RASopathies fish models expressing *Teen* biosensor as molecular spatio-temporal readout *in vivo* to assess early effects of pharmacological modulation of aberrant ERK signaling. Indeed, to this aim, definition of effective concentrations and critical developmental window via establishment of quantitative, fast readouts matched by spatial and temporal molecular information in animal models is essential.

We used low-dose potent MEKi developed as targeted pharmacological molecule for cancer treatment (Cheng and Tian, 2017) that, given the mechanistic overlap has also been proposed as possible treatment also in RASopathies (Andelfinger et al., 2019; Gelb et al., 2022) and were already successfully used in fish RASopathies models (Anastasaki et al., 2009, 2012; Bonetti et al., 2014).
The results obtained by interrogating the morphological and molecular levels in our workflow showed that chronic treatment with low-dose PD0325901 before gastrulation till hatching of fully developed embryos with functional nervous system and swimming behavior can rescue body elongation defects caused by \textit{Shp2}$^{D61G}$. Of note, the morphological rescue obtained is matched by a moderate reduction in ERK activity registered in late (9-11/12hpf) and very early (5.30/6hpf) stages preceding the morphological rescue as observed respectively via live- and AB-FRET in \textit{Teen} embryos and confirmed by pERK immunoblots on whole-embryos extracts. Together with additional evidence in other mice and fish RASopathies models, including a RAF and SOS1-dependent NS mice models (Pagani et al., 2009; Chen et al., 2010; Holter et al., 2019), BRAF-dependent CFC and NS fish models (Anastasaki et al., 2009, 2012; Bonetti et al., 2014) as well as a NS model in \textit{Drosophila} (Oishi et al., 2006), and recent developments in FRET-based \textit{in vivo} applications (Sari et al., 2018; Patel et al., 2019; Wong et al., 2019; Wilcockson et al., 2022), our data support the usefulness of animal models as well as reporter systems to advance pre-clinical applications in the field of RASopathies. Furthermore, when comparing directly fixed NS mutant gastrulae treated with low- vs. high-dose of MEK inhibitor by calculating efficiency of energy transfer (E) and deriving the estimated relative distance between donor and acceptor (R$_{DA}$), AB-FRET is able to show a dose-dependent spatially-restricted response in terms of ERK activity in very early (asymptomatic) fish. However, despite the specificity of the PD0325901, the morphological analysis presented here also confirms the toxicity previously observed in WT fish and CFCs models with prolonged high-dose treatment (Grzmił et al., 2007; Anastasaki et al., 2009, 2012) pointing to the importance of the developmental time window and to restore a proper equilibrium of the RAS-MAPK pathway levels during embryogenesis.

Altogether the experiments presented here expand on the potential previously introduced for \textit{Teen} sensor fish, which proved the ability to register spatially active pERK level changes occurring in zebrafish embryos by means only of “spectral unmixing” protocol and only under physiological conditions or upon potent signal modulation in wild-type embryos (Sari et al., 2018; Wong et al., 2019). Specifically, by examining the utility of \textit{Teen} sensor fish in the context of RASopathies, this work further demonstrates that early ERK activity fluctuations at the animal pole caused by genetic mutations involved in NS, as well as a gradual signal modulation on diseased fish (potentially correlating to “correction”) via increasing doses of MEK inhibitors, precede and correlate with morphological changes and can be registered by using different FRET protocols already in early gastrulae just few hours from birth. This is possible in both live and fixed specimens.
Certainly, the use of zebrafish model comes with the unique benefit of performing live recordings of molecular signaling events (i.e., ERK activity) with the aim to map developmental fluctuations and immediate whole-animal in vivo responses. Nevertheless, the possibility to additionally test a larger cohort of fixed embryos in a relatively fast manner and at an informative stage without the variability of the dynamically changing live response, as we have done here with AB-FRET, could be advantageous when scaling up drug testing in large molecules screening studies.

**Conclusion**

In conclusion, our work supports the use of FRET on live and fixed Teen ERK reporter fish as a possible whole-embryo molecular readout to map disease-associated as well as pharmacological-induced modulation of ERK correlates in RASopathy research. Future studies will be needed to confront dynamic and spatially-resolved fluctuations of ERK signaling in different RASopathies models and to expand critically on our pilot assessment on the suitability of the proposed protocol to examine alternative drugs targeting RAS-MAPK signaling now increasingly available.

**Figure legends**

**Figure 1:** Schematics of the study design and outcome summarizing the main steps of pharmacologically- and genetically induced RAS-MAPK pathway modulation assessed in Teen ERK reporter embryos. (A) Positive and negative modulation of RAS-MAPK signaling through pharmacological approach (by acute stimulation with Epidermal growth factor, EGF, or prolonged exposure to SPH2 inhibitor SHP099, respectively) are assessed by spectral unmixing (obtaining FRET index value for EGF and SHP099 treatment) as well as AB-FRET (obtaining the efficiency, E % and R_dA values for SHP099 treatment) in live and fixed samples, respectively. (B) Genetic modulation of RAS-MAPK signaling in early embryo of a well-established Shp2_D61G-NS zebrafish model and the rescue with low- (0.25 μM) and high-dose (1 μM) MEK inhibitor PD0325901 are assessed by both spectral unmixing- and AB-FRET in live and fixed samples for short (4 hpf – 11/12 hpf) and long (4 hpf – 55/60 hpf) treatment windows, respectively and precede onset of classical RASopathy morphological hallmark validated in later developmental stages (body axis establishment and body length defects occurring from 11-12hpf and 50-72hpf, respectively).

**Figure 2:** Increase in ERK activity is observed by spectral unmixing-FRET in the forebrain of live Teen embryos upon EGF ventricle delivery. (A) The schematics depict the experimental approach
used to stimulate local pERK increase (increased ERK activity) within the anterior brain upon direct delivery of rat EGF via forebrain ventricle delivery in live 24hpf fish. FRET imaging using Spectral unmixing mode was performed before (T0) and after T2 (30') delivery (T1). The lower schematics indicate the FRET signal analysis performed in various regions around the ventricle, as indicated in the legend. (B) Representative sum-intensity projection of confocal x,y,z live scans obtained by spectral unmixing from two embryos showing signal relative to FRET (red) and CFP (donor, green) before (pre) and 30 minutes after EGF delivery (pre and post EGF, respectively.) FRET-based pERK signal increase is marked by red arrows. D: dorsal, V: ventral, ML: medio-lateral domains. (C) Close ups of the sum intensity projection of superficial z-layers of the ratiometric image (FRET/CFP) rendered with the “smart” LUT intensity scale showing high FRET signal correlating to ERK activity (white arrows). (D) Bar graphs reporting the quantification and statistical support for the increased ERK activity signal (FRET/CFP fluorescent intensity, in arbitrary units) before and after EGF delivery. Fold change (FC) data are expressed as mean ± SEM of two independent biological replicates, n = 2 embryos. Paired one-tailed Student t-test is used to assess statistical significance (* p< 0.05).

Figure 3: Decreased ERK activity in live and fixed Teen embryos upon prolonged SHP099 exposure is reported by spectral unmixing- and AB-FRET (A) Schematics depicting the continual treatment with the Shp2 inhibitor SHP099 during zebrafish development from late blastula till 24hpf stage (red square), stage employed in FRET imaging. (B) Representative sum-intensity projection of confocal x,y,z live scans obtained by spectral unmixing from two embryos (treated either with DMSO vehicle control or with SHP099) showing FRET (YFP acceptor, red) and CFP (donor, green) signal. FRET decrease quantified in the different brain regions (Fb: forebrain, Mb: midbrain, Hb: hindbrain) is observed by a dashed white circle and indicated by red arrow. The insets show close ups of the sum intensity projection of the ratiometric image (FRET/CFP) rendered with the “smart” LUT intensity scale showing FRET signal correlating to ERK activity. (B') Bar graph reporting the quantification and statistical support for FRET/CFP fluorescent intensity (arbitrary units, a.u.) expressed as fold change (FC) of treated vs. control fish. Data are expressed as mean ± SEM of two independent biological replicates, n of embryos = 2. (C) Representative AB-FRET images before (Pre-AB panel) and after (Post-AB panel) AB-FRET. Acceptor bleaching in various regions (ROI 1, forebrain, Fb; ROI2, midbrain, Mb) is outlined by white dotted ellipse and indicated with a schematic on the left by a black ROI and shown on the right panel (Acceptor YFP, white arrows). Fb and Mb regions are sampled from the
same embryo. (C') The graph reports the FRET efficiency (E%) expressed as median with interquartile range from AB-FRET data for Fb and Mb (left panel) and Hb and Tb (PSM, right panel). In B' and C' one-tail Student t-test is used to assess statistical significance (*p < 0.05, ** p < 0.01). For Control fish n = 6, 2, 4, 6 (Fb, Mb, Hb and Tb, respectively). For SHP099-treated fish n = 7, 4, 7, 7 (Fb, Mb, Hb and Tb, respectively).

**Figure 4: Increased ERK signal measured by spectral unmixing and AB-FRET in Shp2<sup>D61G</sup> zebrafish mutants showing morphological defects.** (A) Representative bright-field micrographs of hatched zebrafish embryos overexpressing WT and mutant (D61G) form of Shp2. Bar graph showing the penetrance of mild and severe body length defects in Shp2<sup>D61G</sup> expressing embryos expressed as fold change (FC) relative to shp2<sup>WT</sup> embryos. One-sided Chi-square’s test in a 2 × 2 contingency table (Shp2<sup>WT</sup> vs. Shp2<sup>D61G</sup> *** p < 0.001) is used to assess statistical significance. N= 20 and 10 (Shp2<sup>WT</sup> and Shp2<sup>D61G</sup> respectively). (B) Representative bright-field micrographs of mutant embryos at early segmentation stage compared to control fish (expressing Shp2<sup>WT</sup>). Major and minor axes defects are visible, outlined by a dashed orange line and quantification of major/minor axis ratio (B'). One-tail Student t-test is used to assess the statistical significance (**** p < 0.0001). Data are expressed as mean ± SEM of three independent biological replicates. N = 77 and 64 (Shp2<sup>WT</sup> and Shp2<sup>D61G</sup> respectively). (C, D) Single plane images of confocal x,y,z,t and sum-intensity projection of confocal x,y,z live scans (C and D, respectively) obtained by spectral unmixing-FRET of two embryos at around 11-12 hpf (C) and 5-6hpf (D) expressing Shp2<sup>WT</sup> and Shp2<sup>D61G</sup> showing FRET (YFP, red) and CFP (donor, green) signal. pERK signal increase (FRET channel) in the tail bud (PSM) (C) and in the margin (D) of zebrafish embryos is indicated by white arrows. A dashed white line outlines the developing embryo (C) and the margin (D). Close ups on the right rendered with “Smart” LUT in Fiji show increased signal in the tail (C) and animal pole margin (D) region. (D', D'') Bar graphs reporting the quantification of ERK signal relative to panel D (FRET index) calculated on FRET/CFP ratiometric image as raw integrated density (arbitrary units, a.u.) expressed as raw values (D') and fold change, FC (D'’). Data are expressed as mean ± SEM. One-tail Student’s test is used to assess the statistical significance (ns = not statistically significant). N= 4 and 3 (Shp2<sup>WT</sup> and Shp2<sup>D61G</sup> respectively). (E) Representative confocal images (single plane) showing donor (CFP, green) before (Donor-Pre) and after (Donor –Post) AB-FRET for embryos expressing Shp2<sup>WT</sup> and mutants expressing Shp2<sup>D61G</sup>. A dashed white line indicates the animal pole margin targeted for Acceptor Bleaching (AB). For each
condition, insets of the right show close ups on the donor (CFP) at the margin region before and after AB (pre- and post-) rendered with “Smart” LUT in Fiji. Bright pixels showing FRET signal increase upon AB are indicated by white arrows. Embryos are outlined by a continuous white line. (E’-E’’) Box plot with median (middle line), 25th–75th percentiles (box), and min–max values (whiskers) showing the quantification of AB-FRET efficiency (E %, E’) and R DA values (nm, E’’) in the margin of zebrafish embryos overexpressing WT and mutant (D61G) Shp2. One-tailed T Student’s test is used to assess the statistical significance (* p < 0.05, ** p < 0.01). N = 8 and 5 (Shp2WT and Shp2D61G respectively).

L1-L2: different analysis levels (morphological and molecular).

Figure 5: Reduced ERK activity measured by spectral unmixing and AB-FRET in zebrafish Shp2D61G NS-causing mutants exhibiting morphological defects upon low-dose MEK inhibitor treatment.
(A) Representative bright-field micrographs of hatched zebrafish embryos expressing Shp2WT, Shp2D61G treated with DMSO vehicle control (Shp2D61G) or with 0.25 μM PD0325901 (PD) since 4hpf. The bar graph on the right shows the penetrance of mild and severe body length defects in Shp2D61G and the rescue obtained by PD0325901 treatment as fold change (FC) relative to shp2WT embryos. One-sided Chi-square’s test in a 2 × 2 contingency table (Shp2WT vs. Shp2D61G **** p < 0.0001, Shp2D61G vs. Shp2D61G + 0.25 μM PD and Shp2WT vs. Shp2D61G + 0.25 μM PD ** p < 0.01) is used to assess statistical significance. N= 15, 23 and 21 (Shp2WT, Shp2D61G - or + 0.25 μM PD, respectively).
(B) Representative bright-field micrographs of mutant embryos at early segmentation stage treated either with DMSO vehicle control (Shp2D61G) or with 0.25 μM PD compared to control fish (expressing Shp2WT). Major and minor axes defects are visible, outlined by a dashed red line, embryos are outlined by a dashed black line. Quantification of major/minor axis ratio is shown in the scatter plot. One-way ANOVA with Dunnett’s post hoc tec is used to assess the statistical significance (* p < 0.05, **** p < 0.0001) after outliers’ removal (ROUT method Q = 1%). Data are expressed as mean ± SEM of two independent biological replicates. N= 16, 19 and 25 (Shp2WT, Shp2D61G - or + 0.25 μM PD, respectively). (C) Representative confocal images (single plane) showing donor (CFP, green) before (Donor-Pre) and after (Donor –Post) AB-FRET for mutants treated with DMSO vehicle control (Shp2D61G) or with the PD0325901 (Shp2D61G + 0.25 and 1 μM PD). A dashed white line indicates the animal pole margin targeted for Acceptor Bleaching (AB). For each condition, insets of the right show close ups on the donor (CFP) at the margin region before and after AB (pre- and post-) rendered with “Smart” LUT in Fiji. The acceptor (YFP, blue) is shown in the small inset below before and after bleaching of the margin region (dashed white line and arrow). Embryos are
outlined by a continuous white line. (D) Quantification of signal intensity before AB-FRET. The upper and lower scatter plots (Median and interquartile range) show Acceptor/Donor (YFP, CFP) and CFP signal intensity, respectively. N= 18, 9 and 12 (shp2\textsuperscript{D61G}, Shp2\textsuperscript{D61G} + 0.25 µM PD and Shp2\textsuperscript{D61G} + 1 µM, respectively). A marked reduction in the FRET (and reduced Donor quenching) is observed in NS mutants treated with 1 µM PD. Krustal-Wallis with Dunn’s post hoc test is used to assess statistical significance (* p < 0.05). (D') AB-FRET data quantification Box plot with median (middle line), 25th–75th percentiles (box), and min–max values (whiskers) shows the quantification of AB-FRET efficiency (E %) and R\textsubscript{DA} values (nm, right inset) in the margin of mutant embryos treated with DMSO vehicle control (Shp2\textsuperscript{D61G}) low and high-dose of PD0325901 (Shp2\textsuperscript{D61G} + 0.25 µM PD or 1 µM PD, respectively). One-way ANOVA with Dunnett’s post hoc test is used to assess the statistical significance (* p < 0.05). For FRET efficiency (E) dataset, n= 18, 9 and 12 (Shp2\textsuperscript{D61G}, Shp2\textsuperscript{D61G} + 0.25 µM PD and Shp2\textsuperscript{D61G} + 1 µM, respectively). For R\textsubscript{DA} dataset E % values = 0 were excluded from the analysis, resulting in n = 17, 9 and 8 (Shp2\textsuperscript{D61G}, Shp2\textsuperscript{D61G} + 0.25 µM PD and Shp2\textsuperscript{D61G} + 1 µM, respectively). The lower graph shows the percentage of embryos classified based on high or low values of AB-FRET-derived E (> or < 7.53, respectively). One-sided Chi-square’s test in a 2 × 2 contingency table (shp2\textsuperscript{D61G} vs. Shp2\textsuperscript{D61G} + 0.25 µM PD ns = not statistically significant, Shp2\textsuperscript{D61G} vs. Shp2\textsuperscript{D61G} + 1 µM PD * p < 0.05) is used to assess statistical significance. N = 18, 9, and 12 (Shp2\textsuperscript{D61G}, Shp2\textsuperscript{D61G} + 0.25 µM PD and Shp2\textsuperscript{D61G} + 1 µM, respectively). L1-L2: different analysis levels (morphological and molecular).

Figure 6: Molecular and morphological effect of the treatment with MEK inhibitor PD0325901 on zebrafish embryos overexpressing Shp2\textsuperscript{D61G} and showing developmental features modeling NS. (A) AB-FRET efficiency (E %, x) and R\textsubscript{DA} values (nm, y) calculated from AB-FRET on 5-6 hpf zebrafish embryos overexpressing mutant (D61G, red) Shp2 - and + treatment with low- (0.25 µM, light green) and high- (1 µM, dark green) dose of the MEK inhibitor PD0325901. High E values correlating with low RDA values are schematically indicated by a red bar as “genetic effect” of the Shp2\textsuperscript{D61G} allele. On the contrary low E values correlating with high RDA values are indicated by a green bar as “treatment effect” due to short PD exposure. (B) schematics and x,y graph showing the correlation between ERK activation (measured as AB-FRET E FC, x axis) and body axes (measured as ratio between major and minor axis, y axis) in zebrafish embryos expressing Shp2\textsuperscript{D61G} compared to control (Shp2\textsuperscript{WT}) or Shp2\textsuperscript{D61G} treated with DMSO vehicle control or low and high PD dose (right). Modulation of the oval shape of the embryo measured at 11-12hpf (y axis) and of ERK activity
measured by FRET already at 5-6hpf (x axis) are depicted by a schematic illustration. For Shp2\textsuperscript{WT} and Shp2\textsuperscript{D61G} colors from white (normal) to dark bronze (severe) indicate the severity of body shortening. (C) Heat maps with relative color scale bars showing the correlation between ERK activity (orange, bottom graph, AB-FRET measurement), body axes morphology (violet, middle graph, major/minor axis ratio measurement) and embryo elongation (bronze, upper graph, body length measurement) in NS mutants expressing Shp2\textsuperscript{D61G} treated with DMSO vehicle control or with low- (0.25 \( \mu \)M) and high- (1 \( \mu \)M) PD0325901 dose. High ERK activity in NS mutants (Shp2\textsuperscript{D61G} expressing zebrafish embryos) decreases in a dose-dependent manner upon short-time treatment with low and high PD0325901 dose in gastrulae. Accordingly, a dose-dependent effect (morphological correction) is observed at the level of body axis measured at 11-12hpf. However, toxicity with prolonged 1 \( \mu \)M PD0325901 treatment is shown (worsening of the body length phenotype upon 1 \( \mu \)M treatment, measured in hatched embryos).

Declaration

Ethics approval and consent to participate
Animal experiments were approved by the Italian Ministry of Health (DGSA - Direzione generale della sanità animale e dei farmaci veterinary).

Consent for publication
Not applicable.

Availability of data and materials
Raw uncropped blots and measurement data used for generating graphs in this study are provided as Supplementary Information 2 (Source data file). Confocal scans as well as the constructs generated in this study are made available by corresponding authors upon request.

Competing interests
The authors declare that they have no competing interests.

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Authors' contributions
G.F. performed and analyzed the in vivo experiments; S.P. conducted the FRET imaging sessions; V.B. performed and analyzed the immunoblots experiments and performed the immunohistochemistry with technical support from C.P.; G.P. acquired and assessed confocal images from pERK/tERK stained embryos; M.T. co-supervised the work with A.L. and provided critical feedback on the experimental strategy. A.L. conceived the study, designed the experiments and wrote the manuscript. All the authors contributed to the final version of the manuscript and to the figures and provided feedback.

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References


Figures

Figure 1

Schematics of the study design and outcome summarizing the main steps of pharmacologically- and genetically induced RAS-MAPK pathway modulation assessed in Teen ERK reporter embryos. (A) Positive and negative modulation of RAS-MAPK signaling through pharmacological approach (by acute stimulation with Epidermal growth factor, EGF, or prolonged exposure to SPH2 inhibitor SHP099, respectively) are assessed by spectral unmixing (obtaining FRET index value for EGF and SHP099 treatment) as well as AB-FRET (obtaining the efficiency, E % and RDA values for SHP099 treatment) in
live and fixed samples, respectively. (B) Genetic modulation of RAS- MAPK signaling in early embryo of a well-established Shp2D61G-NS zebrafish model and the rescue with low- (0.25 μM) and high-dose (1 μM) MEK inhibitor PD0325901 are assessed by both spectral unmixing- and AB-FRET in live and fixed samples for short (4 hpf – 11/12 hpf) and long (4 hpf – 55/60 hpf) treatment windows, respectively and precede onset of classical RASopathy morphological hallmark validated in later developmental stages (body axis establishment and body length defects occurring from 11-12hpf and 50-72hpf, respectively).

Figure 2

Increase in ERK activity is observed by spectral unmixing-FRET in the forebrain of live Teen embryos upon EGF ventricle delivery. (A) The schematics depict the experimental approach used to stimulate local pERK
increase (increased ERK activity) within the anterior brain upon direct delivery of rat EGF via forebrain ventricle delivery in live 24hpf fish. FRET imaging using Spectral unmixing mode was performed before (T0) and after T2 (30') delivery (T1). The lower schematics indicate the FRET signal analysis performed in various regions around the ventricle, as indicated in the legend. (B) Representative sum-intensity projection of confocal x,y,z live scans obtained by spectral unmixing from two embryos showing signal relative to FRET (red) and CFP (donor, green) before (pre) and 30 minutes after EGF delivery (pre and post EGF, respectively.) FRET-based pERK signal increase is marked by red arrows. D: dorsal, V: ventral, ML: medio-lateral domains. (C) Close ups of the sum intensity projection of superficial z-layers of the ratiometric image (FRET/CFP) rendered with the “smart” LUT intensity scale showing high FRET signal correlating to ERK activity (white arrows). (D) Bar graphs reporting the quantification and statistical support for the increased ERK activity signal (FRET/CFP fluorescent intensity, in arbitrary units) before and after EGF delivery. Fold change (FC) data are expressed as mean ± SEM of two independent biological replicates, n = 2 embryos. Paired one-tailed Student t-test is used to assess statistical significance (* p< 0.05).
Decreased ERK activity in live and fixed Teen embryos upon prolonged SHP099 exposure is reported by spectral unmixing- and AB-FRET (A) Schematics depicting the continual treatment with the Shp2 inhibitor SHP099 during zebrafish development from late blastula till 24hpf stage (red square), stage employed in FRET imaging. (B) Representative sum-intensity projection of confocal x,y,z live scans obtained by spectral unmixing from two embryos (treated either with DMSO vehicle control or with SHP099) showing
FRET (YFP acceptor, red) and CFP (donor, green) signal. FRET decrease quantified in the different brain regions (Fb: forebrain, Mb: midbrain, Hb: hindbrain) is observed by a dashed white circle and indicated by red arrow. The insets show close ups of the sum intensity projection of the ratiometric image (FRET/CFP) rendered with the “smart” LUT intensity scale showing FRET signal correlating to ERK activity. (B’) Bar graph reporting the quantification and statistical support for FRET/CFP fluorescent intensity (arbitrary units, a.u.) expressed as fold change (FC) of treated vs. control fish. Data are expressed as mean ± SEM of two independent biological replicates, n of embryos = 2. (C) Representative AB-FRET images before (Pre-AB panel) and after (Post-AB panel) AB-FRET. Acceptor bleaching in various regions (ROI 1, forebrain, Fb; ROI2, midbrain, Mb) is outlined by white dotted ellipse and indicated with a schematic on the left by a black ROI and shown on the right panel (Acceptor YFP, white arrows). Fb and Mb regions are sampled from the same embryo. (C’) The graph reports the FRET efficiency (E%) expressed as median with interquartile range from AB-FRET data for Fb and Mb (left panel) and Hb and Tb (PSM, right panel). In B’ and C’ one-tail Student t-test is used to assess statistical significance (*p < 0.05, ** p < 0.01). For Control fish n = 6, 2, 4, 6 (Fb, Mb, Hb and Tb, respectively). For SHP099-treated fish n = 7, 4, 7, 7 (Fb, Mb, Hb and Tb, respectively).
Increased ERK signal measured by spectral unmixing and AB-FRET in Shp2D61G zebrafish mutants showing morphological defects. (A) Representative bright-field micrographs of hatched zebrafish embryos overexpressing WT and mutant (D61G) form of Shp2. Bar graph showing the penetrance of mild and severe body length defects in Shp2D61G expressing embryos expressed as fold change (FC) relative to shp2WT embryos. One-sided Chi-square's test in a 2 × 2 contingency table (Shp2WT vs. Shp2D61G ***
(B) Representative bright-field micrographs of mutant embryos at early segmentation stage compared to control fish (expressing Shp2WT). Major and minor axes defects are visible, outlined by a dashed orange line and quantification of major/minor axis ratio (B’). One-tail Student t-test is used to assess the statistical significance (**** p < 0.0001). Data are expressed as mean ± SEM of three independent biological replicates. N = 77 and 64 (Shp2WT and Shp2D61G respectively). (C, D) Single plane images of confocal x,y,z,t and sum-intensity projection of confocal x,y,z live scans (C and D, respectively) obtained by spectral unmixing-FRET of two embryos at around 11-12 hpf (C) and 5-6hpf (D) expressing Shp2WT and Shp2D61G showing FRET (YFP, red) and CFP (donor, green) signal. pERK signal increase (FRET channel) in the tail bud (PSM) (C) and in the margin (D) of zebrafish embryos is indicated by white arrows. A dashed white line outlines the developing embryo (C) and the margin (D). Close ups on the right rendered with “Smart” LUT in Fiji show increased signal in the tail (C) and animal pole margin (D) region. (D’, D”) Bar graphs reporting the quantification of ERK signal relative to panel D (FRET index) calculated on FRET/CFP ratiometric image as raw integrated density (arbitrary units, a.u.) expressed as raw values (D’) and fold change, FC (D”). Data are expressed as mean ± SEM. One-tail Student’s test is used to assess the statistical significance (ns = not statistically significant). N= 4 and 3 (Shp2WT and Shp2D61G respectively). (E) Representative confocal images (single plane) showing donor (CFP, green) before (Donor-Pre) and after (Donor –Post) AB-FRET for embryos expressing Shp2WT and mutants expressing Shp2D61G. A dashed white line indicates the animal pole margin targeted for Acceptor Bleaching (AB). For each condition, insets of the right show close ups on the donor (CFP) at the margin region before and after AB (pre- and post-) rendered with “Smart” LUT in Fiji. Bright pixels showing FRET signal increase upon AB are indicated by white arrows. Embryos are outlined by a continuous white line. (E’-E”) Box plot with median (middle line), 25th–75th percentiles (box), and min–max values (whiskers) showing the quantification of AB-FRET efficiency (E %, E’) and RDA values (nm, E”) in the margin of zebrafish embryos overexpressing WT and mutant (D61G) Shp2. One-tailed T Student’s test is used to assess the statistical significance (* p < 0.05, ** p < 0.01). N = 8 and 5 (Shp2WT and Shp2D61G respectively). L1-L2: different analysis levels (morphological and molecular).
Figure 5

Reduced ERK activity measured by spectral unmixing and AB-FRET in zebrafish Shp2D61G NS-causing mutants exhibiting morphological defects upon low-dose MEK inhibitor treatment. (A) Representative bright-field micrographs of hatched zebrafish embryos expressing Shp2WT, Shp2D61G treated with DMSO vehicle control (Shp2D61G) or with 0.25 μM PD0325901 (PD) since 4hpf. The bar graph on the right shows the penetrance of mild and severe body length defects in Shp2D61G and the rescue obtained...
by PD0325901 treatment as fold change (FC) relative to shp2WT embryos. One-sided Chi-square’s test in a 2 × 2 contingency table (Shp2WT vs. Shp2D61G **** p < 0.0001, Shp2D61G vs. Shp2D61G + 0.25 μM PD and Shp2WT vs. Shp2D61G + 0.25 μM PD ** p < 0.01) is used to assess statistical significance. N= 15, 23 and 21 (Shp2WT, Shp2D61G - or + 0.25 μM PD, respectively). (B) Representative bright-field micrographs of mutant embryos at early segmentation stage treated either with DMSO vehicle control (Shp2D61G) or with 0.25 μM PD compared to control fish (expressing Shp2WT). Major and minor axes defects are visible, outlined by a dashed red line, embryos are outlined by a dashed black line. Quantification of major/minor axis ratio is shown in the scatter plot. One-way ANOVA with Dunnett’s post hoc test is used to assess the statistical significance (* p < 0.05, **** p < 0.0001) after outliers’ removal (ROUT method Q = 1%). Data are expressed as mean ± SEM of two independent biological replicates. N= 16, 19 and 25 (Shp2WT, Shp2D61G- or + 0.25 μM PD, respectively). (C) Representative confocal images (single plane) showing donor (CFP, green) before (Donor-pre) and after (Donor-post) AB-FRET for mutants treated with DMSO vehicle control (Shp2D61G) or with the PD0325901 (Shp2D61G + 0.25 and 1 μM PD). A dashed white line indicates the animal pole margin targeted for Acceptor Bleaching (AB). For each condition, insets of the right show close ups on the donor (CFP) at the margin region before and after AB (pre- and post-) rendered with “Smart” LUT in Fiji. The acceptor (YFP, blue) is shown in the small inset below before and after bleaching of the margin region (dashed white line and arrow). Embryos are outlined by a continuous white line. (D) Quantification of signal intensity before AB-FRET. The upper and lower scatter plots (Median and interquartile range) show Acceptor/Donor (YFP, CFP) and CFP signal intensity, respectively. N= 18, 9 and 12 (shp2D61G, Shp2D61G + 0.25 μM PD and Shp2D61G + 1 μM, respectively). A marked reduction in the FRET (and reduced Donor quenching) is observed in NS mutants treated with 1 μM PD. Krustal-Wallis with Dunn’s post hoc test is used to assess statistical significance (* p < 0.05). (D’) AB-FRET data quantification Box plot with median (middle line), 25th–75th percentiles (box), and min–max values (whiskers) shows the quantification of AB-FRET efficiency (E %) and RDA values (nm, right inset) in the margin of mutant embryos treated with DMSO vehicle control (Shp2D61G) low and high-dose of PD0325901 (Shp2D61G + 0.25 μM PD or 1 μM PD, respectively). One-way ANOVA with Dunnett’s post hoc test is used to assess the statistical significance (* p < 0.05). For FRET efficiency (E) dataset, n = 18, 9 and 12 (Shp2D61G, Shp2D61G + 0.25 μM PD and Shp2D61G + 1 μM, respectively). For RDA dataset E % values = 0 were excluded from the analysis, resulting in n = 17, 9 and 8 (Shp2D61G, Shp2D61G + 0.25 μM PD and Shp2D61G + 1 μM, respectively). The lower graph shows the percentage of embryos classified based on high or low values of AB-FRET-derived E (> or < 7.53, respectively). One-sided Chi-square’s test in a 2 × 2 contingency table (shp2D61G vs. Shp2D61G + 0.25 μM PD ns = not statistically significant, Shp2D61G vs. Shp2D61G + 1 μM PD * p < 0.05) is used to assess statistical significance. N = 18, 9, and 12 (Shp2D61G, Shp2D61G + 0.25 μM PD and Shp2D61G + 1 μM, respectively). L1-L2: different analysis levels (morphological and molecular).
Molecular and morphological effect of the treatment with MEK inhibitor PD0325901 on zebrafish embryos overexpressing Shp2D61G and showing developmental features modeling NS. (A) AB-FRET efficiency (E %, x) and RDA values (nm, y) calculated from AB-FRET on 5-6 hpf zebrafish embryos overexpressing mutant (D61G, red) Shp2 - and + treatment with low- (0.25 μM, light green) and high- (1 μM, dark green) dose of the MEK inhibitor PD0325901. High E values correlating with low RDA values are
schematically indicated by a red bar as “genetic effect” of the Shp2D61G allele. On the contrary, low E values correlating with high RDA values are indicated by a green bar as “treatment effect” due to short PD exposure. (B) Schematics and x,y graph showing the correlation between ERK activation (measured as AB-FRET E FC, x axis) and body axes (measured as ratio between major and minor axis, y axis) in zebrafish embryos expressing Shp2D61G compared to control (Shp2WT) or Shp2D61G treated with DMSO vehicle control or low and high PD dose (right). Modulation of the oval shape of the embryo measured at 11-12hpf (y axis) and of ERK activity measured by FRET already at 5-6hpf (x axis) are depicted by a schematic illustration. For Shp2WT and Shp2D61G colors from white (normal) to dark bronze (severe) indicate the severity of body shortening. (C) Heat maps with relative color scale bars showing the correlation between ERK activity (orange, bottom graph, AB-FRET measurement), body axes morphology (violet, middle graph, major/minor axis ratio measurement) and embryo elongation (bronze, upper graph, body length measurement) in NS mutants expressing Shp2D61G treated with DMSO vehicle control or with low- (0.25 μM) and high- (1 μM) PD0325901 dose. High ERK activity in NS mutants (Shp2D61Gerexpressing zebrafish embryos) decreases in a dose-dependent manner upon short-time treatment with low and high PD0325901 dose in gastrulae. Accordingly, a dose-dependent effect (morphological correction) is observed at the level of body axis measured at 11-12hpf. However, toxicity with prolonged 1 μM PD0325901 treatment is shown (worsening of the body length phenotype upon 1 μM treatment, measured in hatched embryos).

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