



ANTIBODY VALIDATION ARTICLE

**REVISED** Validation of commercial ERK antibodies against the ERK orthologue of the scleractinian coral *Stylophora pistillata* [version 2; referees: 3 approved]

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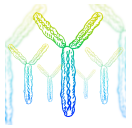
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**Abstract**

The extracellular signal-regulated protein kinase (ERK) signalling pathway controls key cellular processes, such as cell cycle regulation, cell fate determination and the response to external stressors. Although ERK functions are well studied in a variety of living organisms ranging from yeast to mammals, its functions in corals are still poorly known. The present work aims to give practical tools to study the expression level of ERK protein and the activity of the ERK signalling pathway in corals. The antibody characterisation experiment was performed five times and identical results were obtained. The present study validated the immune-reactivity of commercially available antibodies directed against ERK and its phosphorylated/activated forms on protein extracts of the reef-building coral *Stylophora pistillata*.



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	Invited Referees		
	1	2	3
<b>REVISED</b>			
<b>version 2</b>	report		report
published 03 Jul 2017	↑		↑
<b>version 1</b>	?		?
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**REVISED** Amendments from Version 1

In order to comply with the referees' requests, we added an experiment consisting of inducing ERK activity by exposing corals to UV radiation, thermal stress or a combination of both.

We added new figures as well as their corresponding text in the Methods and the Results sections. Specifically, in order to answer one of the referees' concerns, we have slightly modified Figure 2 and Supplementary Figure S1 by adding the amido black total protein stainings of the western blot membranes as a loading control. We have also added a new Figure 3 and the corresponding Supplementary Figure S2 (full length images of the western blot).

See referee reports

## Introduction

Mitogen activated protein kinases (MAPKs) are highly conserved proteins involved in signalling pathways and control key cellular processes such as proliferation, differentiation, migration, survival and apoptosis (Dhillon *et al.*, 2007). The MAPK gene family encompasses three major subfamilies: the extracellular signal-regulated kinase (ERK), p38/HOG and c-Jun N-terminal kinase (JNK) groups. The ERK family is the most studied in mammals (Boulton *et al.*, 1990; Dhillon *et al.*, 2007) because it is involved in meiosis, mitosis and post mitotic functions in differentiated cells, as well as in the oxidative stress response and wound healing (Castellano *et al.*, 2014; Johnson & Lapidat, 2002; Matsubayashi *et al.*, 2004; Runchel *et al.*, 2011). The ERK gene family is evolutionarily conserved and is found in all eukaryotes, including yeasts, plants, vertebrates and invertebrates (Chen *et al.*, 2001; Widmann *et al.*, 1999). Although recent molecular studies have shown the existence of ERK genes in different coral species (Mayfield *et al.*, 2010; Siboni *et al.*, 2012; van de Water *et al.*, 2015), ERK activity and specific functions are not yet clearly defined. ERK activation occurs through phosphorylation of the Threonine and Tyrosine residues of an

ERK-specific TEY motif by the upstream kinases of ERK, the mitogen-activated protein kinase kinase (MAPKK or MEK). ERK phosphorylation on these residues is classically considered the most appropriate readout for the activity of the ERK signalling pathway. However, it has never been monitored in corals. Overall, MAPK activities in corals have only been investigated once, in a study focusing on the JNK subfamily (Courtial *et al.*, 2017).

In this work, we used the scleractinian coral *Stylophora pistillata*, a very abundant species in most tropical reefs (Veron & Stafford-Smith, 2000). We applied the same protocol as in Courtial *et al.* (2017) to demonstrate the efficiency of antibodies directed against the mammalian phosphorylated forms of ERK (pERK) and total ERK to detect the ERK orthologs in *S. pistillata* (Table 1). According to the manufacturer's instructions, the antibody used in this study and directed against the Thr202/Tyr204 di-phosphorylated active ERK (Thermo Scientific Pierce; MA5-15174) showed reactivity with fruit fly, human, mink, mouse, non-human primate, pig, rat and zebrafish. The immunogen used to generate this rabbit IgG monoclonal antibody was a synthetic phosphopeptide corresponding to residues surrounding the phospho-Thr202/Tyr204 of the human p44/ERK1 MAP kinase. This antibody is not cross-reactive with the corresponding phosphorylated residues of either JNK/SAPK or p38. The ERK1/ERK2 antibody (Thermo Scientific Pierce; MA5-15605) used in the study previously showed reactivity with human and mouse samples. The immunogen used to generate this mouse IgG2b monoclonal antibody was a purified recombinant fragment of human MAPK.

## Methods

### Maintenance of *Stylophora pistillata* nubbins and human fibroblasts

Nubbins of *Stylophora pistillata* were collected from five mother colonies maintained in the aquaria facilities of the Centre Scientifique de Monaco. Two small nubbins (3–5 cm long) were

**Table 1. Primary and secondary antibodies.**

Antibody	Manufacturer	Catalogue number	RRID	Concentration
p44+42 MAPK (Erk1,2) Antibody (3F8)	Thermo Fisher Scientific	MA5-15605	AB_10983247	1µg/mL (1/1000)
Phospho-p44 MAPK + p42 MAPK pTyr204 Antibody (B.742.5)	Thermo Fisher Scientific	MA5-15174	AB_10980347	1µg/mL (1/1000)
Peroxidase-AffiniPure Goat Anti-Mouse IgG (H+L) antibody	Jackson ImmunoResearch Labs	115-035-003	AB_10015289	1µg/mL (1/10000)
Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L) antibody	Jackson ImmunoResearch Labs	111-035-003	AB_2313567	1µg/mL (1/10000)
IRDye 800CW Goat Anti-Rabbit IgG (H+L)	LI-COR Biosciences	926-32211	AB_621843	0.1µg/mL (1/10000)
IRDye 680RD Goat anti-Mouse IgG (H + L)	LI-COR Biosciences	926-68070	AB_10956588	0.1µg/mL (1/10000)

cut off from each mother colony, and were allowed to heal for four weeks in 15 L open system tanks before the experiments. Corals were maintained in the same conditions as the mother colonies, *i.e.* at 25°C, under a photosynthetic active radiation of 200  $\mu\text{mol photon.m}^{-2}.\text{s}^{-1}$  provided by 400 W metal halide lamps (HPIT, Philips) and were fed twice a week with *Artemia salina nauplii*. Seawater in the tanks was continuously renewed at a rate of 10 L.h<sup>-1</sup>.

Immortalized skin fibroblasts (BJ-EHLT cells) were kindly provided by E. Gilson's lab (IRCAN) and cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Villebon-sur-Yvette, France) supplemented with 10% heat-inactivated fetal calf serum (Dutscher, Brumath, France) at 37°C in an atmosphere of 5% CO<sub>2</sub>, as previously described (Biroccio *et al.*, 2013).

#### UO126 treatment of coral nubbins

Incubations were performed in 100 mL beakers containing one coral nubbin each, and filled with 40 mL of 0.45  $\mu\text{m}$  filtered seawater. They were placed in the dark for one hour in either a control condition containing 0.005% DMSO (vehicle) or a condition with 5  $\mu\text{mol.L}^{-1}$  UO126 (Selleck Chemicals), a MEK inhibitor (Tang *et al.*, 2003). The incubation medium was continuously stirred using magnetic stirrers at a constant temperature of 25°C. At the end of the incubation, nubbins were frozen and kept at -80°C prior to western blot analysis.

#### UVR and temperature treatment of coral nubbins

Incubations were performed in 100 mL beakers containing one coral nubbin each and filled with 40 mL of 0.45  $\mu\text{m}$  filtered seawater and continuously stirred using magnetic stirrers. High temperature or/and ultraviolet radiation (UVR) stresses (*i.e.* four environmental conditions: control (at 25°C and without UVR), thermal stress (30°C without UVR), UVR stress (25°C under UVR), thermal and UVR stresses (30°C and under UVR)) were applied to corals and ERK activation was monitored after 30 minutes of stress. Thermal stress corresponded to an increase in temperature from the normal culture condition of 25°C to 30°C. The UVR stress corresponded to an increase in UVR from 0 (HQI lamps in the culture conditions) to a radiation intensity of about 3 W.m<sup>-2</sup> UVB and 30 W.m<sup>-2</sup> UVA (Q-Panel UVA 340 lamps). At the end of the incubation, nubbins were frozen and kept at -80°C prior to western blot analysis.

#### Western blot analysis

Immuno-detections were performed as in Courtial *et al.* (2017; Table 2 and Table 3). Briefly, nubbins were airbrushed in 1 mL Laemmli buffer (2% SDS, 10% glycerol, 50mM Tris HCL pH7), (Laemmli, 1970) using an air-pick (5 bars) to remove the totality of the tissues surrounding the skeleton was removed from coral. Samples were then sonicated for 30 seconds, and centrifuged (3 × 5 minutes at 15 000 g) to remove the lipid

**Table 2. Tissue extraction and western blot protocol.**

Process	Reagent	Manufacturer	Catalogue number	Concentration/Composition
Tissue extraction	Laemmli buffer 1.5X	Homemade		150 mM Tris-HCl pH 7, 25% glycerol, 2% SDS
Sample preparation	Laemmli - 1,4 Dithiothreitol - bromophenol blue solution	Homemade		1.5 X- 50 mM - 0.1%
Electrophoresis	ECL gradient gel 8-16%	GE Healthcare Lifesciences	29-9901-58	
	TG-SDS 10X running buffer	EUROMEDEX	EU0510	1X
Protein transfer	DUNN transfer buffer	Homemade		10 mM NaHCO <sub>3</sub> - 3 mM Na <sub>2</sub> CO <sub>3</sub> - 10% Ethanol (pH 9.9)
Blocking	Blocking reagent	Homemade		PBS + milk (3%)
Washes	Wash buffer 10X	Homemade		PBS 10X Tween 20 1N
Membrane coloration	Coloration buffer	Homemade		Isopropanol (25%) + acetic acid (10%) + amido black (0.1%)
Membrane destain	Destain buffer	Homemade		Isopropanol (25%) + acetic acid (10%)
Target detection	Immobilon Western HRP Substrate	Millipore	WBKLS0500	
Reagents	BCA QuantiPro BCA Assay Kit	Sigma-Aldrich	QPBCA-1KT	
	Milk	Itambe ®		
	Methanol	Sigma-Aldrich		

**Table 3. Reagents for tissue extraction and western blots.**

Protocol steps	Reagent	Time	Temperature
Tissue extraction	Laemmli 1.5 X (1mL)		RT
Sonication	Laemmli 1.5 X	30 sec	RT
Centrifugation (x3 15000 g)	Laemmli 1.5 X	3 × 5 min	RT
Addition of 1,4 Dithiothreitol - bromophenol blue solution	Laemmli 1.5 X		RT
Heat up		5 min	95°C
Electrophoresis (100 V)	Running buffer	variable	RT
Transfer (200 mA)	Transfer buffer	overnight	4°C
Coloration	Isopropanol (25%) + acetic acid (10%) + amido black (0.1%)	5 min	RT
Destain	Isopropanol (25%) + acetic acid (10%)	3 × 5 min	RT
Blocking	PBS + milk (3%)	30 min	RT
Primary antibodies	PBS + milk (1%) + ab (1/1000)	overnight	4°C
Washes (3 times)	Wash buffer 1X	3 × 15 min	RT
Secondary antibody	PBS + milk (1%) + ab (1/10000)	2h	RT
Washes (5 times)	Wash buffer 1X	5 × 15 min	RT
Detection	Immobilon Western HRP Substrate	30 sec – 10 min	RT

supernatant and debris. Fibroblasts were washed twice in phosphate buffered saline solution (PBS), lysed in Laemmli buffer directly in the dishes and sonicated for 30 seconds. The total protein concentration of all samples was determined using a BCA protein Assay Kit (Thermo Fisher Scientific), according to the manufacturer's recommendation. 1,4 Dithiothreitol (1 mM) and bromophenol blue (0.1%) were added to the samples, which were then heated for 5 minutes at 95°C.

60 µg of proteins were separated on 10% polyacrylamide gels at 300 mA and 110 V at room temperature. Proteins were then transferred on a PVDF membrane at 4°C overnight in Dunn's transfer buffer at 200 mA. After a rinse in distilled water, membranes were saturated for 30 minutes in PBS - 3% low fat milk, rinsed in PBS and incubated with primary antibodies diluted in PBS - 1% low fat milk at 4°C overnight. The antibody directed against Thr202/Tyr204 di-phosphorylated active ERK was from Thermo Scientific Pierce (rabbit monoclonal; MA5-15174; batch no. OC1680806); the anti-ERK1/2 antibody was from Thermo Scientific Pierce (mouse monoclonal; MA5-15605; batch no. PH1895491). After extensive washing (4×30 minutes) in PBS – 0.1% Tween 20, membranes were incubated for 2 hours at room temperature in the simultaneous presence of IRDye 680RD goat anti-mouse (925-68070) and IRDye 800CW goat anti-rabbit (925-32211; Li-COR Biotechnology GmbH, Bad Homburg, Germany) secondary antibodies, or with anti-mouse and anti-rabbit

HRP-conjugated antibody. Another set of extensive rinsing (4×30 minutes) in PBS – 0.1% Tween 20 was performed before membranes were imaged with an Odyssey device (LI-COR Biosciences, Lincoln, Nebraska) to detect fluorescence and HRP activity using Millipore ECL.

Densitometric analysis of the western blots was performed using Image Studio v2.1 software (Li-COR Biosciences). Intensity of the pERK signal was normalized to the intensity of ERK signal. The relative intensities between control and inhibitor conditions were compared using a t-test. Statistical analysis was done using the software Graphpad Prism v5.03.

## Results and discussion

In order to confirm the presence of an ERK ortholog in corals, the human protein sequence of ERK1 (NP\_001035145) was compared to the transcriptome database of *Stylophora pistillata* using the BLAST software (Altschul *et al.*, 1990; Karako-Lampert *et al.*, 2014). An open reading frame was retrieved from the best hit sequence with a predicted molecular weight of 42 kDa (Spi\_isotig05348). This sequence (hereafter referred to as Spi-ERK for *S. pistillata* ERK) is the only one that shows an homology as high as 81%, 80% and 78% with the protein sequences of the cnidarians *Nematostella vectensis* ERK (Nv-ERK; XP\_001629498.1), *Hydra vulgaris* ERK (Hv-ERK; XP\_002154499.3) and the human MAPK3/ERK1 (Hs-ERK1), respectively (Figure 1)

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                                                                    D I
Spi_ERK -----MAAPPPPEVVRGQAFDVGPRYTNSYIGEGAYGMVCSAL
Nv_ERK -----MAASTGPEVVRGQVFDVAPRYTNLNYIGEGAYGMVVAGV
Hv_ERK -----MASAEIVRGQVFDVGPRYKDLSYIGEGAYGMVCCAT
Hs_ERK1 MAAAAAQGGGGGPEPRRTEGVGPGVPEVEMVKGQPFDVGPRYTQLQYIGEGAYGMVSSAY
                                                *:*:** ***.***.:*.***** **

                D II                D III                D IV                D V
Spi_ERK DNATGQRVAIKKISPFEHQTYCQRTLREIKILRRFKHENIIGIMDIIRAPNMDDMKDVYI
Nv_ERK DNTTGERIAIKKISPFEHQTYCQRTLREIKILSRFKHENIIGILDIIRAPRAEDMKDYI
Hv_ERK DIYTGNKVAIKKISPFEHQTYCQRTLREIKILSRFNHENIIINIQDIKRSPTIDDMKDVYI
Hs_ERK1 DHVRKTRVAIKKISPFEHQTYCQRTLREIQILLRFRHENVIGIRDILRASTLEAMRDVYI
*      :*:*****:*** **.***:*.* ** *:      :*:**

                                D VIA                D VIB
Spi_ERK VQSLMETDLYKLLKTQKLSNDHICYFLYQILRGLKYIHSANVLHRDLKPSNLLLNTTCDL
Nv_ERK VQPLMETDMYKLLKTQRLSNDHICYFLYQILRGLKYIHSANVLHRDLKPSNLLLNTTCDL
Hv_ERK VQTLMETDLYKLLKTQKLSNDHICYFLYQILRGLKYIHSANVLHRDLKPSNLLLNTTCDL
Hs_ERK1 VQDLMETDLYKLLKSQQLSNDHICYFLYQILRGLKYIHSANVLHRDLKPSNLLINTTCDL
* * *****:*****:*.*****:*****:*****:*****:*****:*****

                D VII                D VIII                D IX
Spi_ERK KICDFGLARVADPEHDHTGFLTEYVATRWRRAPEIMLNSKGYSKAIDIWSVGCILAEMLS
Nv_ERK KICDFGLARIADPDHDHTGFLTEYVATRWRRAPEIMLNSKGYSKAIDIWSVGCILAEMLA
Hv_ERK KICDFGLARVADPEHDHTGMLTEYVATRWRRAPEIMLNSKGYTKSIDIWSVGCILAEMLS
Hs_ERK1 KICDFGLARIADPEHDHTGFLTEYVATRWRRAPEIMLNSKGYTKSIDIWSVGCILAEMLS
*****:*****:*****:*****:*****:*****:*****:*****:*****:

                                D X
Spi_ERK NRPLFPGKHYLDQLNRILEVLGSPSQDDLQCIMNDKARSYIQSLAYKPRVPWQRYFGGAD
Nv_ERK NRPLFPGKHYLDQLNLIILNVLGSPIADLLC IKNEKARSYLQSLPYKPKTPFIKLFSNAD
Hv_ERK NRPLFPGKHYLDQLNLIILNVLGSPDKESLEFIRNLKAKSYLQGLPIKERTPWKTLFPEAD
Hs_ERK1 NRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINMKARNYLQSLPSKTKVAWAKLFPKSD
***:***** ** :**** :.* ** ***:.*:.* * * :. : * :*

                                                                    D XI
Spi_ERK NKAIELLDKMLTFNPNKRITVEEALAHPYLEQYYDPQDEPVAEEPFRFETELDDLPKERL
Nv_ERK AKALDLIENLLAFNPNKRITVEEALAHPYLEQYYDPADEPVAETPFKFQMELDLPKEKL
Hv_ERK PKALDLLDKLLAFNPNKRVTVEEALAHEYLEQYYDPNDEPVAEEPFTFDTELDDLPKEKL
Hs_ERK1 SKALDLLDRMLTFNPNKRITVEEALAHPYLEQYYDPTDEPVAEEPFTFAMELDLPKERL
* * :. :. :. :*:**:*:*:***** ***** ***** ** * *****:*

Spi_ERK KELIFKETASYPGSPYLGDFHLGQQ
Nv_ERK KGKLNTKF-----
Hv_ERK KEMIFESTHMYGSA-----
Hs_ERK1 KELIFQETARFQPGVLEAP-----
* : .

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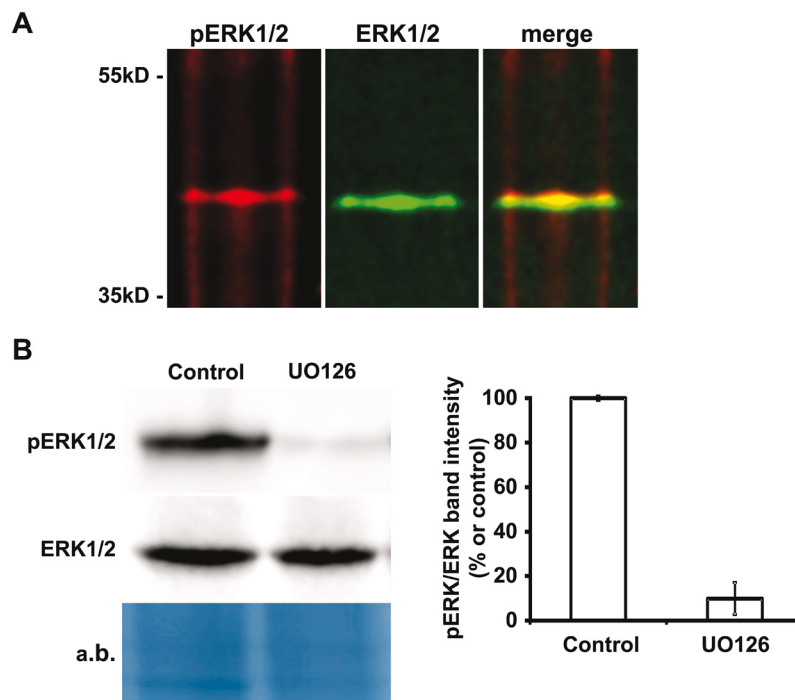
**Figure 1. Sequence alignment of MAPK orthologs.** The ERK orthologs of *Stylphora pistillata* (Spi-ERK), *Nematostella vectensis* (Nv-ERK), *Hydra vulgaris* (Hv-ERK), and the human ERK1 (Hs-ERK1) protein sequences are shown. The ERK-specific TEY motif is highlighted in red. The eleven conserved kinase domains are underlined.

(Krishna *et al.*, 2013; Putnam *et al.*, 2007). These sequences all contain both the conserved kinase domains (Hanks & Hunter, 1995) and the TEY motif of the catalytic domain, which is unique for ERK orthologs (Davis, 2000; Figure 1). An interesting point to note is that a unique sequence showing these features is present in *N. vectensis* and *H. vulgaris* genomes, as well as in the *S. pistillata* transcriptome database. This result suggests that a single ortholog of ERK is present in these cnidarians, consistently with previous work where only one ERK ortholog was found (Castellano *et al.*, 2014; Russo *et al.*, 2004) but as opposed to the two genes encoding ERKs in most mammalian genomes (Ip & Davis, 1998). Furthermore, based on the high level of sequence conservation between distant species (Hanks & Hunter, 1995), antibodies directed against portions of the ERK human proteins may recognize ERKs from other species. Accordingly, we detected a single immune-reactive band with the total-ERK antibody by western blot on *S. pistillata* extracts (Figure 2A and Supplementary Figure S1). Spi-ERK should retain the mechanism of activation by phosphorylation of the Threonine and the Tyrosine residues of the ERK-specific TEY motif. Hence, the MA5-15174 antibody directed against the phosphorylated Thr202 and the Tyr204 (*i.e.* the phosphorylated TEY motif) should detect a phosphorylated TEY motif of Spi-ERK (phospho-ERK). This is consistent with what we observed, as we detected a unique

immune-reactive band of approximately 40 kDa with both antibodies (Figure 2A).

Interestingly, the fluorescent immunoblot technique showed that the bands detected with the phosphorylated- and the total-ERK antibodies mostly co-migrate, suggesting that the same protein is detected (Figure 2A). The slight electrophoretic migration shift of the band detected with the anti-phosphorylated ERK antibody would be consistent with the phosphorylation of the threonine and tyrosine residues of the TEY motif as previously described (Aoki *et al.*, 2011). These results suggest that ERK and its phosphorylated form are correctly recognized by the antibodies.

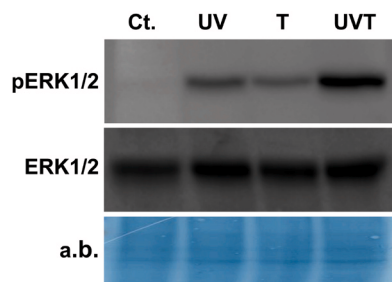
RNAi interference techniques are not available in coral, and the confirmation that the immune reactive bands observed here specifically correspond to ERK could not be obtained through this method. In order to test the specificity of the antibodies, we therefore used U0126, a very potent and selective inhibitor of MEK (Bain *et al.*, 2007). The limited thickness of the animal tissue covering the skeleton and the very large surface of contact of both ectoderm and endoderm with the seawater render *S. pistillata* suitable for treatment with drugs directly diluted in the seawater as we previously showed (Courtial *et al.*, 2017). U0126 was previously



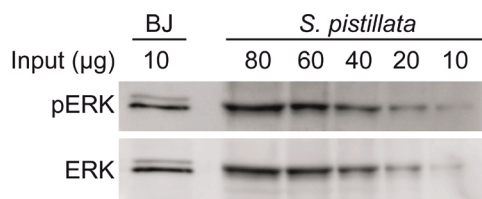
**Figure 2. Detection of ERK activity in corals.** (A) Fluorescent immunoblot revealing activated (pERK) and total forms of ERK (ERK) present in *Styphora pistillata* nubbins. Molecular weight standards in kilo Daltons (kDa) are indicated on the left side of the figure. (B) Immunoblot performed with ERK and pERK antibodies on protein extracts from coral nubbins incubated in the absence (Control) or presence of the MEK inhibitor U0126. Densitometric analysis of activated ERK intensities is presented on the right of the figure. The amido black total protein staining of the western blot membrane is shown as a loading control. The medians and standard deviations of three independent experiments are presented (\*\*\*,  $p < 0.01$ , t-test).

shown to efficiently block MEK activity in a wide variety of organisms, including cnidarians (Hasse *et al.*, 2014; Picco *et al.*, 2007; Röttinger *et al.*, 2004). When the inhibitor was added to the seawater, the intensity of the band detected by the anti-total ERK did not vary, while the intensity of the band detected with the anti-phosphorylated ERK antibody was significantly reduced (Figure 2B and Supplementary Figure S1). Altogether, our results strongly suggest that the proteins detected with the two antibodies were ERK and pERK.

To confirm that Spi-ERK activity can dynamically respond to changes in experimental conditions, we performed an induction experiment by modifying culture conditions of the corals. Courtial *et al.* (2017) showed that thermal and UVR stresses induced the formation of reactive oxygen species which are known to trigger ERK phosphorylation (McCubrey *et al.*, 2006). ERK phosphorylation was enhanced in corals exposed to UVR, high temperature or a combination of both (Figure 3 and Supplementary Figure S2). These results confirm that the antibodies characterized herein can be used to monitor ERK activity in corals.



**Figure 3. Induction of Spi-ERK phosphorylation by thermal and UV stresses.** Immunoblot performed with ERK and pERK antibodies on protein extracts from coral nubbins incubated for 30 minutes in control (Cont.), thermal stress (T), UV stress (UV) or a combination of thermal and UV stresses (UV + T) conditions. The amido black total protein staining of the western blot membrane is shown as a loading control.



**Figure 4. Relative sensitivities of ERK antibodies toward the human and coral proteins.** Immunoblot performed with anti-ERK and anti-phospho-ERK on total protein extracts of human fibroblasts (BJ) and *Stylophora pistillata*. The amount of protein loaded in each lane is indicated on the Supplementary Figure S4.

Finally, to assess the performance of these antibodies, we compared the signal obtained on *S. pistillata* and human fibroblasts protein extracts (Figure 4 and Supplementary Figure S3). We loaded on the same gel 10 µg of fibroblast total protein extract and different amounts of *S. pistillata* extracts (ranging from 80 to 10 µg). A signal comparable to the one obtained with the fibroblast extract was observed using 40 µg of coral proteins for both antibodies. This suggests that the affinity of the antibodies towards the coral proteins may be lower than for their human counterparts.

#### Dataset 1. Supplementary Figure S1. Uncropped blot images for Figure 2 and supplementary replicates

<http://dx.doi.org/10.5256/f1000research.11365.d159188>

(A) Biological replicates of fluorescent immunoblots performed in control conditions (Ct) are shown (Replicates 1 and 2). The portions of the images used in the main text are outlined. (B) Biological replicates of immunoblots performed on protein extracts from coral nubbins incubated in the absence (Control) or presence of the MEK inhibitor U0126 (UO) (Replicates 1 to 5). The amido black total protein staining of the western blot membrane is shown as a loading control. The portions of the images used in the main text are outlined.

#### Dataset 2. Supplementary Figure S2. Uncropped blot images for Figure 3 and supplementary replicates

<http://dx.doi.org/10.5256/f1000research.11365.d166821>

The portions of the images used in the main text are outlined.

#### Dataset 3. Supplementary Figure S3. Uncropped blot images for Figure 4 and supplementary replicates

<http://dx.doi.org/10.5256/f1000research.11365.d166825>

The portions of the images used in the main text are outlined.

## Conclusion

This work showed that MA5-15174 and MA5-15605 are two specific antibodies that can be used to quantitatively assess *Stylophora pistillata* ERK phosphorylation/activity in different experimental or environmental conditions. We demonstrated the specificity of these antibodies and their good affinity towards their coral targets. It therefore provides the coral research community with a potent tool for the analysis of the activity of a signalling pathway involved in a wide variety of biological processes.

## Data availability

**Supplementary Figure S1. Uncropped blot images for Figure 2 and supplementary replicates.** (A) Biological replicates of fluorescent immunoblots performed in control conditions (Ct) are shown (Replicates 1 and 2). The portions of the images used in the main text are outlined. (B) Biological replicates of



immunoblots performed on protein extracts from coral nubbins incubated in the absence (Control) or presence of the MEK inhibitor U0126 (UO) (Replicates 1 to 5). The portions of the images used in the main text are outlined. doi, [10.5256/f1000research.11365.d159188](https://doi.org/10.5256/f1000research.11365.d159188) (Courtial *et al.*, 2017a).

**Supplementary Figure S2. Uncropped blot images for Figure 3 and supplementary replicates.** The portions of the images used in the main text are outlined. doi, [10.5256/f1000research.11365.d166821](https://doi.org/10.5256/f1000research.11365.d166821) (Courtial *et al.*, 2017b).

**Supplementary Figure S3. Uncropped blot images for Figure 4 and supplementary replicates.** The portions of the images used in the main text are outlined. doi, [10.5256/f1000research.11365.d166825](https://doi.org/10.5256/f1000research.11365.d166825) (Courtial *et al.*, 2017c).

### Author contributions

CFP, GP, LC and VP conceived and designed the experiments. LC and VP performed the experiments. CFP, GP, LC and VP

analyzed the data. CFP and GP contributed reagents/materials/analysis tools. CFP, GP, LC and VP wrote the paper.

### Competing interests

No competing interests were disclosed.

### Grant information

Financial support to CFP, GP, LC and VP was provided by the Centre Scientifique de Monaco and Pierre and Marie Curie University.

*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

### Acknowledgements

The authors thank Y. Cormerais for his help in the experimental setup, N. Caminiti-Seconds for the first coral extracts and assays as well as Pr. Denis Allemand, Director of the Centre Scientifique de Monaco for scientific support.

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# Open Peer Review

Current Referee Status:   

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## Version 2

Referee Report 21 July 2017

doi:[10.5256/f1000research.13040.r23990](https://doi.org/10.5256/f1000research.13040.r23990)



**María L. Parages**

Department of Ecology, Faculty of Sciences, University of Málaga, Málaga, Spain

After author response, I recommend the manuscript for indexing. So I suggest the new status as Approved.

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Referee Report 04 July 2017

doi:[10.5256/f1000research.13040.r24020](https://doi.org/10.5256/f1000research.13040.r24020)



**Andrea Pitzschke** 

Department of Cell Biology and Physiology, University of Salzburg, Salzburg, Austria

In the revised version Courtial et al. have addressed my concerns and considered my suggestions in sufficient detail. I therefore recommend the manuscript for indexing.

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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## Version 1

Referee Report 22 June 2017

doi:[10.5256/f1000research.12269.r23149](https://doi.org/10.5256/f1000research.12269.r23149)



**María L. Parages**

Department of Ecology, Faculty of Sciences, University of Málaga, Málaga, Spain

In this manuscript the authors Courtial et al. validate the use of commercial ERK antibodies for the detection of MAPK-like proteins in Coral. Although the paper is well written and has the quality to be indexed, a few issues should be figure out before its final acceptance.

In terms of samples preparation and to allow future replication by others researchers would be appropriate to give more information about how to prepare the samples. For example, "Briefly, coral tissue was removed from the skeleton in 1 mL Laemmli buffer" so, how much coral tissue will be dissolved and resuspended it in 1 mL of Laemmli Buffer? Less than 0.5 gr? More?

While it is true that the authors note that immune-detection were performed as in Courtial *et al.* (2017), and that DTT (Dithiothreitol) and BFB (Bromophenol blue) were added to the samples and heated (5 minutes at 95°) before loading the gels, it is not specified how tissue extraction was performed. I must assume that the Lysis Buffer used was Laemmli Buffer? And in this case, how they have been unable to detect phosphorylated ERK? As far as I know lysis buffer for phosphorylated proteins usually contents EDTA or EGTA to chelate Mg<sup>2+</sup>/Ca<sup>2+</sup>, DTT for reduction of disulfide bonds, serine protease inhibitor (Aprotinin/Leupeptine), phosphatase inhibitors to block dephosphorylation like Na orthovanadate, or Beta-glycerophosphate (false substrate for phosphatase, between others... They also keep everything ice cold? Also, it surprises me that they used 3% low fat milk for membranes blocking, due to also screws up phosphor-tyrosine detection.

Regarding the presence of an ERK ortholog in coral, the authors show the sequence with code Spi\_isotig05348 (Spi\_ERK) has the best hit with the human ERK protein (NP\_0011035145) and they make reference to Liew *et al.*, 2014. I was looking for this sequence into this paper, and I could not find it. The authors should provide in which database is the transcriptome, as well as, the Spi\_ERK sequence itself.

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Are sufficient details of materials, methods and analysis provided to allow replication by others?**

Partly

**Are all the source data underlying the results available to ensure full reproducibility?**

Partly

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** Molecular Ecology

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 28 Jun 2017

**Lucile Courtial**, Centre Scientifique de Monaco, Monaco

***In this manuscript the authors Courtial et al. validate the use of commercial ERK antibodies for the detection of MAPK-like proteins in Coral. Although the paper is well written and has the quality to be indexed, a few issues should be figure out before its final acceptance.***

We thank Dr. Parages for her comments and suggestions. To answer her concerns, we have added information and replaced a citation in the manuscript as detailed bellow.

***In terms of samples preparation and to allow future replication by others researchers would be appropriate to give more information about how to prepare the samples. For example, "Briefly, coral tissue was removed from the skeleton in 1 mL Laemmli buffer" so, how much coral tissue will be dissolved and resuspended it in 1 mL of Laemmli Buffer? Less than 0.5 gr? More?***

All of the tissue from 3-5cm long *S. pistillata* nubbins was used. We added the precision in the materials and methods:

P4: "Two small nubbins (3-5 cm long) were cut off from each mother colony and were allowed to heal for four weeks in 15 L open system tanks before the experiments."

P5: "Briefly, nubbins were airbrushed in 1 mL Laemmli buffer (2% SDS, 10% glycerol, 50mM Tris HCL pH7) ( Laemmli 1970) using an air-pick (5 bars) to remove the totality of the tissues surrounding the skeleton was removed from coral."

***While it is true that the authors note that immune-detection were performed as in Courtial et al. (2017), and that DTT (Dithiothreitol) and BFB (Bromophenol blue) were added to the samples and heated (5 minutes at 95°) before loading the gels, it is not specified how tissue extraction was performed. I must assume that the Lysis Buffer used was Laemmli Buffer?***

***And in this case, how they have been unable to detect phosphorylated ERK? As far as I know lysis buffer for phosphorylated proteins usually contents EDTA or EGTA to chelate Mg<sup>2+</sup>/Ca<sup>2+</sup>, DTT for reduction of disulfide bonds, serine protease inhibitor (Aprotinin/Leupeptine), phosphatase inhibitors to block dephosphorylation like Na orthovanadate, or Beta-glycerophosphate (false substrate for phosphatase, between others...***

Coral tissues were indeed lyzed in laemmli buffer. To prevent any doubt when reading the methods, we added a precision in the text:

P3: "Briefly, nubbins were airbrushed in 1 mL Laemmli buffer (2% SDS, 10% glycerol, 50mM Tris HCL pH7) (Laemmli 1970) using an air-pick (5 bars) to remove the totality of the tissues surrounding the skeleton."

To answer the concern of Dr. Parages about the possible phosphatase activity in the samples, we would like to specify the fact that, due to a high concentration of SDS, the Laemmli buffer is a strongly denaturing buffer for proteins, including phosphatases. Therefore, this buffer, commonly used at room temperature, preserves phosphorylation of the proteins without the need to add phosphatases inhibitors (see Picco et al. 2016 for example).

***They also keep everything ice cold? Also, it surprises me that they used 3% low fat milk for membranes blocking, due to also screws up phosphor-tyrosine detection.***

We fully agree with Dr. Parages, the use of milk as a blocking buffer is usually not recommended for the detection of phospho-proteins as it may contain phospho-proteins that can interact with the anti-phospho primary antibodies. However, we have successfully used this blocking buffer in diverse experimental setups, including human cultured cells, ascidian embryos as well as coral

lysates. We used this buffer in the course of this study because it gives far less background noise than any other blocking buffers tested.

**Regarding the presence of an ERK ortholog in coral, the authors show the sequence with code Spi\_isotig05348 (Spi\_ERK) has the best hit with the human ERK protein (NP\_0011035145) and they make reference to Liew et al., 2014. I was looking for this sequence into this paper, and I could not find it. The authors should provide in which database is the transcriptome, as well as, the Spi\_ERK sequence itself.**

We are grateful to the reviewer for questioning this particular point as it allowed us to uncover a significant error in the citation we used.

The reference for the Spi EST containing the ERK orthologue open reading frame was obtained from the database generated during the study by Karako-Lampert, Zoccola et al. (Plos One 2014) and not the one by Liew et al (p.7) . The database containing the sequence can be downloaded from this address: <http://data.centrescientifique.mc/CSMdata-stylodata.html>. As mentioned in the manuscript, the ERK1 human protein sequence was blasted (tblastn) against the Karako-Lampert database using the blast tool hosted on a publically accessible local server of the Centre Scientifique de Monaco (<http://data.centrescientifique.mc/blast/blast.php>). The correct reference has been added to the manuscript.

Due to current major security concerns for our servers, we have chosen not to include the aforementioned URL in the present manuscript. However, this URL is openly disclosed in the Karako-Lampert et al. paper, which should allow readers to access the database without the needing to contact corresponding authors.

**Competing Interests:** No competing interests were disclosed.

Referee Report 16 June 2017

doi:10.5256/f1000research.12269.r23532



**Immacolata Castellano**

Department of Biology and Evolution of Marine Organisms, Stazione Zoologica Anton Dohrn, Naples, Italy

The paper by Courtial et al. describes the cross-reactivity of two commercial antibodies produced against the mammalian forms of ERK for the scleractinian coral *Stylophora pistillata*. This should open new perspective for the study of ERK signalling in response to different environmental cues.

The paper is clear and well written, however it lacks of some references.

In the Introduction, the authors should cite other invertebrates where ERK signalling is known to be conserved and regulated by environmental cues, for example *Ciona intestinalis* (Castellano et al, PLOS One 2014, Castellano et al, Open Biology 2015). Similarly, in Results and Discussion, when the authors say that .. "a single orthologue of ERK is present in these cnidarians, as opposed to the two genes encoding ERKs in most mammalian genomes", they should specify that also in other invertebrates, only one ERK form was found (Russo et al, JBC 2004; Castellano et al, PloS One 2014). Also the use of the MEK inhibitor U0126 was assessed in *C. intestinalis* (Castellano et al, Open Biology 2015).

Finally the authors pay attention along the text to some errors, i.e. change “Thr2020/204” with Thr202/204, and “through this medium” with “through this method”.

**Is the work clearly and accurately presented and does it cite the current literature?**

Partly

**Are sufficient details of materials, methods and analysis provided to allow replication by others?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** Biochemistry

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Author Response 28 Jun 2017

**Lucile Courtial**, Centre Scientifique de Monaco, Monaco

***The paper by Courtial et al. describes the cross-reactivity of two commercial antibodies produced against the mammalian forms of ERK for the scleractinian coral Stylophora pistillata. This should open new perspective for the study of ERK signalling in response to different environmental cues.***

***The paper is clear and well written, however it lacks of some references.***

We thank Dr. Castellano for her comments and suggestions. We have added information to the manuscript to answer her concerns as detailed bellow.

***In the Introduction, the authors should cite other invertebrates where ERK signalling is known to be conserved and regulated by environmental cues, for example Ciona intestinalis (Castellano et al, PLOS One 2014, Castellano et al, Open Biology 2015).***

We added a precision and the reference in the text: “The ERK gene family is evolutionnarily conserved and is found in all eukaryotes, including yeasts, plants, vertebrates and invertebrates (Widmann et al. 1999; Chen et al. 2001; Castellano et al. 2014).”

***Similarly, in Results and Discussion, when the authors say that .. "a single orthologue of ERK is present in these cnidarians, as opposed to the two genes encoding ERKs in most mammalian genomes", they should specify that also in other invertebrates, only one ERK form was found (Russo et al, JBC 2004; Castellano et al, PloS One 2014).***

We added the precision in the text:P4 “This result suggests that a single ortholog of ERK is present in these cnidarians, consistently with previous work where only one ERK ortholog was found

(Russo et al. 2004; Castellano et al. 2014) but as opposed to the two genes encoding ERKs in most mammalian genomes (Ip and Davis 1998).”

**Also the use of the MEK inhibitor U0126 was assessed in *C. intestinalis* (Castellano et al, Open Biology 2015).**

Despite extensive search into the reference cited by Dr. Castellano, we could not find any experiment using U0126 in this paper. The aforementioned reference instead reports the use of a dual specificity phosphatase inhibitor. Moreover, the work of Picco et al. (2007) cited in the manuscript already reports the use of U0126 in *Ciona* embryos. We therefore did not include the suggested citation in the text.

**Finally the authors pay attention along the text to some errors, i.e. change “Thr2020/204” with Thr202/204, and “through this medium” with “through this method”.**

We changed the errors in the revised manuscript.

**Competing Interests:** No competing interests were disclosed.

Referee Report 30 May 2017

doi:10.5256/f1000research.12269.r22707



**Andrea Pitzschke** 

Department of Cell Biology and Physiology, University of Salzburg, Salzburg, Austria

The manuscript provides first insight into a putative MAPK in the coral *Stylophora pistillata*.

Experiments include protein extraction and immunoblot analysis.

Overall: The experiments that had been performed are properly designed. However, the manuscript lacks sufficiently-detailed information, as well as controls (protein loading). Conclusions are premature or should be re-phrased.

Detailed points of criticism:

Title “orthologue” is inappropriate. Should be “homologue”.

## Methods

P3 “small rubbings selected” – please be more specific about size and sampling:

“tissue removed from coral”: be more specific. Tissue primarily from the surface, how deep was the cut into the material? (I suggest to include a schematic figure incl. scale-bar). This information is important because inhibitors (e.g. U0126) will only diffuse over a short distance, i.e. not reach deeper layers.

P4: “extensive washing”: duration and number of solution changes missing



Fig.2B: “% or control” rather OF control. The error bar in the control sample is irrelevant, as it is defined as strictly 100%.

There is no documentation of protein loading (e.g. Coomassie-stained membrane after immunodetection). The U126-independent intensity of the ERK-Signal is insufficient as control.

Conclusions: “...antibody can be used...in different experimental or environmental conditions”

This conclusion is premature. As a minimum, the authors should perform an induction experiment. The inhibitory approach (U126) only evidences that a MAPKK is the upstream regulator. Coral research community will only benefit from the antibody and the current study if dynamic ERK activity responses can be monitored.

**Is the work clearly and accurately presented and does it cite the current literature?**

Partly

**Are sufficient details of materials, methods and analysis provided to allow replication by others?**

Partly

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 28 Jun 2017

**Lucile Courtial**, Centre Scientifique de Monaco, Monaco

***Overall: The experiments that had been performed are properly designed. However, the manuscript lacks sufficiently-detailed information, as well as controls (protein loading). Conclusions are premature or should be re-phrased.***

We thank Dr. Pitzschke for her comments and suggestions. We have added information to the manuscript and performed an additional experiment to answer her concerns as detailed bellow.

***Detailed points of criticism:***

***Title “orthologue” is inappropriate. Should be “homologue”.***

We have used the term “orthologue” in our manuscript in its definition notably given by Walter Fitch (Fitch 1970, 2000), that is: “Orthology is that relationship where sequence divergence follows speciation, that is, where the common ancestor of the two genes lies in the common ancestor of the taxa from which the two sequences were obtained”.

Dr. Pitzschke is right, the Spi- and Hs-ERK proteins are homologues but, on top of that, they also

are orthologues. We therefore still think that the term “orthologue” is more accurate in our case.

### **Methods**

**P3 “small nubbins selected” –please be more specific about size and sampling: “tissue removed from coral”: be more specific. Tissue primarily from the surface, how deep was the cut into the material?**

To be more specific, we added the following sentences in the revised manuscript:

P2: “Two small nubbins (3-5 cm long) were cut off from each mother colony and were allowed to heal for four weeks in 15 L open system tanks before the experiments.”

P3: “Briefly, nubbins were airbrushed in 1 mL Laemmli buffer (*i.e.* lysing buffer, 1.5 X, Laemmli 1970) using an air-pick (5 bars) to remove the totality of the tissues surrounding the skeleton was removed from coral.”

**This information is important because inhibitors (e.g. UO126) will only diffuse over a short distance, i.e. not reach deeper layers.**

We added a sentence to prevent further doubts concerning the bioavailability of UO126.

P6: “In order to test the specificity of the antibodies, we therefore used UO126, a very potent and selective inhibitor of MEK (Bain et al. 2007). The limited thickness of the animal tissue covering the skeleton and the very large surface of contact of both ectoderm and endoderm with the seawater render *S. pistillata* suitable for treatment with drugs directly diluted in the seawater as we previously showed (Courtial et al. 2017) ...”

**P4: “extensive washing”: duration and number of solution changes missing**

We added precisions in the materials and methods: P4 : “4 x 30 minutes”

**Fig.2B: “% or control” rather OF control. The error bar in the control sample is irrelevant, as it is defined as strictly 100%.**

**There is no documentation of protein loading (e.g. Coomassie-stained membrane after immunodetection). The U126-independent intensity of the ERK-Signal is insufficient as control.**

We added the amido black colored membranes in Figure 2 and Figure S1 as a loading control.

**Conclusions: “...antibody can be used...in different experimental or environmental conditions”**

**This conclusion is premature. As a minimum, the authors should perform an induction experiment. The inhibitory approach (U126) only evidences that a MAPKK is the upstream regulator. Coral research community will only benefit from the antibody and the current study if dynamic ERK activity responses can be monitored.**

We performed an additional experiment and added a figure and related text in the manuscript to justify our statement (P9).

**Competing Interests:** No competing interests were disclosed.