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Supplemental Information

Sustained ERK Activation Underlies Reprogramming in Regeneration-Competent Salamander Cells and Distinguishes Them from Their Mammalian Counterparts

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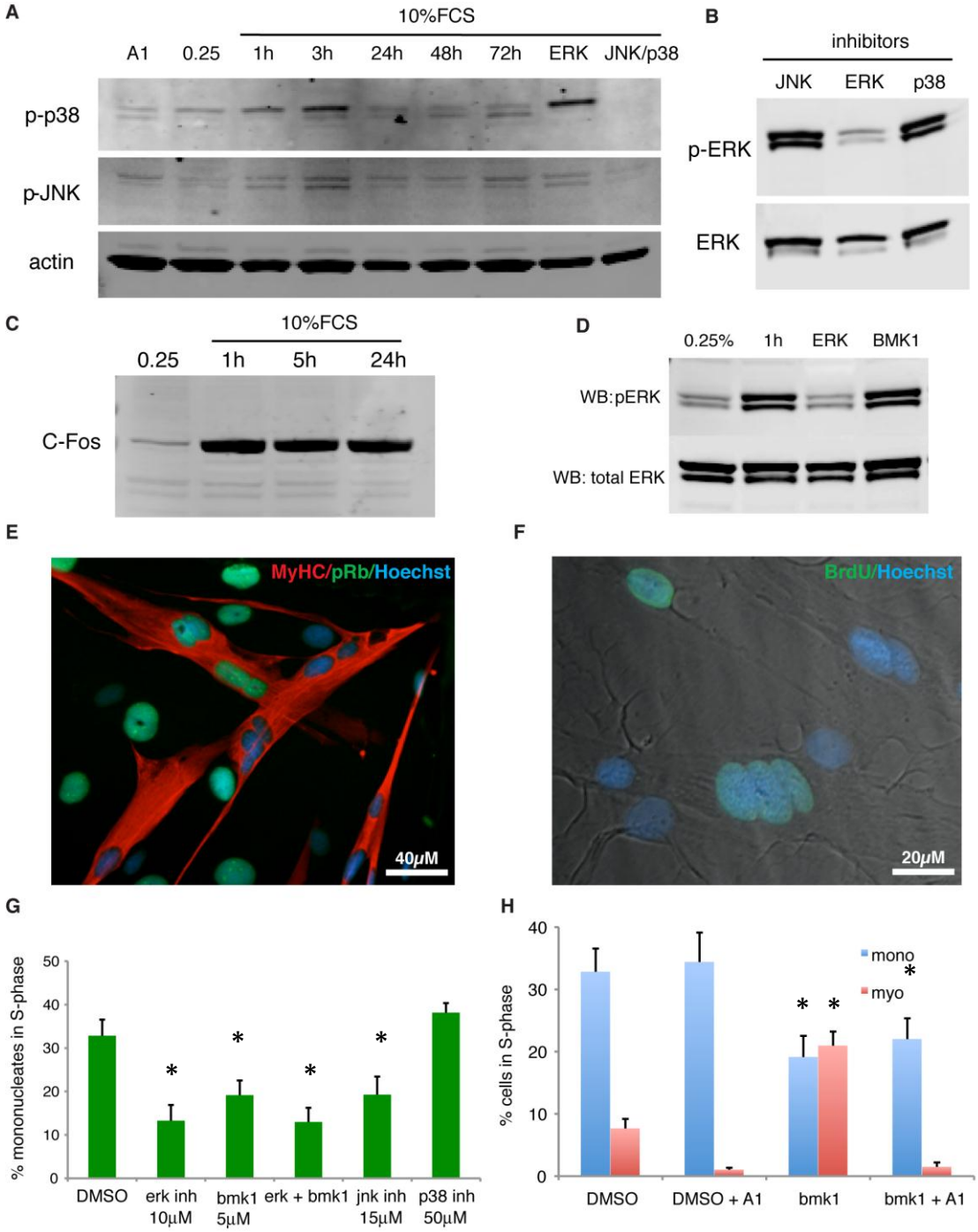

Figure S1. Activation of JNK, p38 and c-Fos during myotube S-phase re-entry (A, C) Western blot analysis of A1 myotube extracts pre (0.25%FCS) or at different times post induction with 10%FCS. ERK indicates treatment with an ERK inhibitor, JNK/p38 denote treatment with either a JNK or p38 inhibitor respectively. Treatments were initiated at 0h post induction. (B) Western blot analysis of A1 myotube extracts 1 hour post serum induction, treated with the indicated inhibitors. Note that the ERK inhibitor specifically abrogates ERK phosphorylation. **Inhibition of BMK1/ERK5 promotes S phase re-entry by decreasing A1 mononucleate proliferation** (D) Western blot analysis of A1 myotube extracts pre (0.25%FCS) or 1 hour post serum induction. (E)

Representative image of A1 myotubes after 2d in high serum stained with antibodies against p-RBS807/811, MyHC and Hoechst 33258. (F) Representative image of A1 myotubes at 3d post-induction in high serum following a BrdU pulse. Myotubes were stained with antibodies against BrdU and Hoechst 33258. (G,H) Quantification of BrdU positive mononucleate cells (G) or combined cultures (H), as measured by immunostaining at 72h post serum induction following a BrdU pulse. Cells were treated with the indicated compounds. In (H), myotubes were induced and 30% confluent A1 proliferating cells were added where indicated (A1). All values represent the mean ± s.e.m (*$p<0.05$). n=3 (A-D), n=4 (G,H), were n indicates the number of independent experiments.

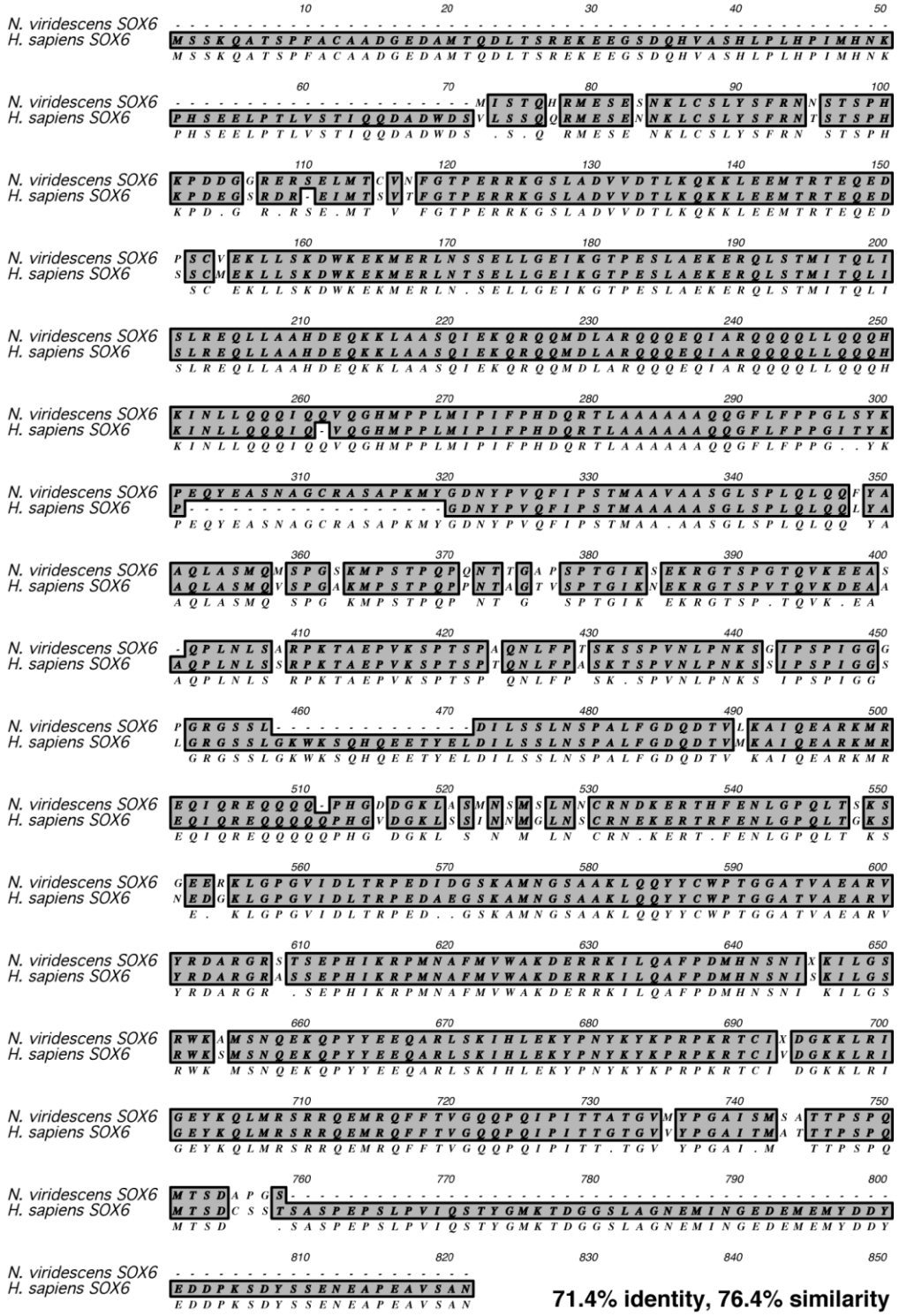

Figure S2. Sequence alignment of human (*Homo sapiens)* and salamander (*Notophthalmus viridescens)* SOX6. MacVector alignment of full-length protein sequences of the muscle-specific gene *Sox6*. The analysis reveals a high degree of evolutionary conservation.

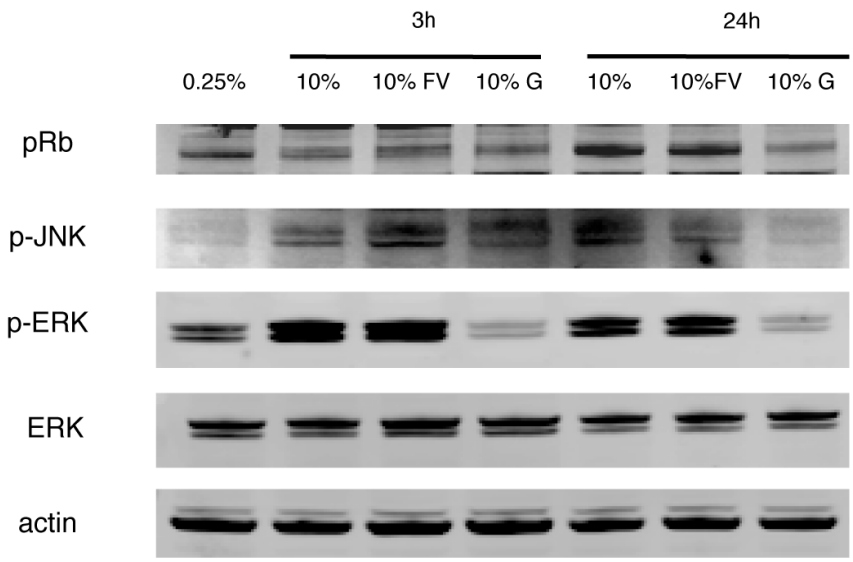

Figure S3. A tyrosine kinase receptor, not responsive to FGF or VEGF, is required for serum-induced sustained ERK activation. (A) Western blot analysis of A1 myotube extracts pre (0.25%FCS), and at 3h and 24h post induction with 10%FCS. Myotubes were treated as indicated. FV indicates treatment with an FGF/VEGF inhibitor, whereas G denotes treatment with a general Receptor Tyrosine Kinase inhibitor. Treatments were initiated at 0h post induction. n=3, were n indicates the number of independent experiments

Supplementary Experimental Procedures

Lipofection

The vector pnGFP-N2 was modified from pEGFP-N2 (Clontech) by addition of a nuclear localization signal to the original EGFP sequence. The MEK1-R4F (plasmid 40810) and MEK1$^{S218E/S222D}$ (plasmid 40809) vectors were obtained from Addgene. These constructs were delivered to Pmi28 cells by lipofection using Lipofectamine 2000 (Invitrogen) as per manufacturer's instructions. Briefly, cells were transfected with 1.5µg of pnGFP-N2, 1.5µg of the indicated vector, and 8µl Lipofectamine 2000 per 3.5cm dish for 4 hours. Cells were then incubated in myotube differentiation media for 3 days in the presence of Ara-C. This method gave a transfection efficiency of 50%, as assessed by immunofluorescence of nuclear GFP. Whole cell extracts were collected at 4 days post lipofection and analysed as described.

Western blot Analysis

Protein extracts were prepared by resuspending cells in 0.02M Hepes (pH 7.9), 0.2mM EDTA, 1.5mM $MgCl_2$, 0.42M NaCl, 25% glycerol, incubating for 30 minutes at 4°C and clearing the debris by centrifugation. The resulting extracts were analysed by SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Whatman), which was incubated in Odyssey blocking buffer (Licor) and incubated with the indicated antibodies overnight (Supplementary Table 3). The membrane was then washed twice in TBS, incubated with IR labelled secondary antibodies, AlexaFluor680 and AlexaFluor800, against the corresponding species (Licor) and analysed with an Odyssey scanner (Licor).

Quantitative RT-PCR

RNA was isolated from purified myotube cell cultures using Tri Reagent (Sigma) and random primed cDNA synthesised using Superscript II (Invitrogen). *Notophthalmus viridescens Sox6* (**GenBank KJ801973**) gene expression was determined by quantitative real time PCR with two sets of primers (*Sox6* 1 fwd: GGCAGTACAGAAACCTGT; *Sox6* 1 rev: CCCCTATTGTAGCATATCTGGC; *Sox6* 2 fwd: GTGCAGTATTGACGTGAGG; *Sox6* 2 rev: GGTTGAAAGGACAGTCTTGAGG ; *Ef1α* fwd: AACATCGTGGTCATCGGCCAT; *Ef1α* rev: GGAGGTGCCAGTGATCATGTT) and iQ SYBR Green supermix (Bio-rad), on a Chromo 4 instrument running Opticon 3 software (Bio-rad). All reactions were run in triplicate and at least 3 independent RNA preparations were analysed for each sample.

In situ hybridization

A 688 kb fragment of axolotl *Gadd45β* (tctcgagGCAAGGATTGGCATATCAC, tctagaGAGACCGAAGGCACCCACGTG) was cloned into pciNEO vector (Promega) and the resulting construct was linearised with either XbaI or XhoI. The respective linearised templates were transcribed with T3 or T7 RNA polymerases to generate digoxigenin-UTP labelled antisense or sense riboprobes following the manufacturer's protocol (Roche). In situ hybridization of cultured

newt A1 cells with the digoxigenin-UTP labelled riboprobes was performed as previously described (Imokawa et al., 2004).

Immunofluorescence staining

Cells were fixed in 2% PFA for 1 minute, followed by a 5-minute incubation in cold 100% methanol and processed as described elsewhere (Duckmanton et al., 2005). Cells were incubated with primary antibodies overnight. In all cases, anti–mouse or anti-rabbit AlexaFluor488 and AlexFluor594 antibodies (Invitrogen) were used for secondary staining. Hoechst 33258 (2µg/ml) was used for nuclei counterstaining. Samples were observed under a Zeiss Axiskop2 microscope and images were acquired with a Hamamatsu Orca camera using Openlab (Improvision) software. Whenever comparative analyses between different cell treatments were performed, all images were acquired with identical camera settings and illumination control. Image processing (contrast enhancement) was equally applied to all matched experimental and control samples using Openlab software.

BrdU Analysis

Cells and myotubes were labeled for 2h and 24h respectively by adding 1 µl/ml 5-bromo-2 deoxyuridine/5-fluoro-2-deoxyuridine (BrdU) to the growth media. Following the corresponding incubation period, cells were fixed in 4% paraformaldehyde for 1 minute followed by 100% methanol for 5 minutes, and stained for bromodeoxyuridine as previously described (Barres et al., 1994; Tanaka et al., 1997).

Supplementary Table 1 – Inhibitors

Inhibitor	Inhibits	Company	Final concentration
U0126	MEK1	Calbiochem	10µM
SP600125	JNK	Calbiochem	15µM
p38 MAP kinase inh	p38	MERK	50µM
BMK1 (XDM8)	ERK5/BMK1	Santa Cruz biotech	5µM
(-)Nutlin3a	MDM2-p53	Cayman	1µM

	interaction		
FGF/VEGF (PD173074)	FGF/VEGF	Insight biotech	5µM
RTK	Receptor tyrosine kinases	MERK	10µM

Supplementary Table 2 – Antibodies

Antibody	Origin	Clonality	Species	Dilution WB	dilution IHC
p-ERK1/2	Sigma	monoclonal	rabbit	1:1000	1:500
Total ERK1/2	Cell signalling	polyclonal	rabbit	1:200	1:100
βactin	Sigma	monoclonal	mouse	1:2000	1:1000
p-JNK	NEB	monoclonal	mouse	1:5000	1:1000
p-p38	ABD Serotec	polyclonal	rabbit	1:500	N/A
Dimethyl H3K9	Millipore	polyclonal	rabbit	1:500	1:300
MyHC	custom made	monoclonal	mouse	1:2000	1:1000
BrdU	Sigma	monoclonal	mouse	N/A	1:3000
pRB S807/811	Cell signalling	polyclonal	rabbit	1:1000	1:500
H3 (D1H2)	Cell signalling	monoclonal	rabbit	1:500	N/A
c-FOS (Ab-1)	Millipore	monoclonal	mouse	1:500	N/A
CCND1-D3	from E. Boucrot	monoclonal	mouse	1:500	N/A
Cyclin E2	Cell signalling	monoclonal	rabbit	1:500	N/A
PCNA (PC10)	Cell signalling	monoclonal	mouse	1:500	N/A

Supplementary References

Barres, B.A., Lazar, M.A., and Raff, M.C. (1994). A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. Development *120*, 1097-1108.

Duckmanton, A., Kumar, A., Chang, Y.T., and Brockes, J.P. (2005). A single-cell analysis of myogenic dedifferentiation induced by small molecules. Chem Biol *12*, 1117-1126.

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