Supplemental Information:

Table of content:

Experimental Procedures:

Western Blotting: Cells were lysed Cells were lysed using NP buffer supplemented with protease inhibitors. Typically, 50–100μg proteins were subjected to SDS-PAGE (7.5–10%). Proteins from gels were then electro-transferred onto PVDF membranes. After blocking with 5% nonfat dry milk (NFDM) dissolved in Tris-buffered saline containing 0.1% Tween 20 (TTBS), blots were probed overnight at 4 °C, with specific primary antibodies in TTBS containing 2% NFDM. The primary antibodies used were typically procured from Abcam and were used in 1:500 to 1:2000 dilutions. Next day, membranes were incubated for 1 h at room temperature with a horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG in TTBS containing 2% NFDM. Detection was performed using the enhanced chemi-luminescence reagent (ECL Western blotting detection reagents; Amersham Biosciences). Quantification of bands was performed by densitometry using the ImageJ software. For ADCYs blots blocking and antibody treatment were performed in bovine serum albumin (BSA), as NFDM

was not working effectively with these antibodies. For co-IPs with STIM1 ΔS/P, STIM1/GOK antibody from BD biosciences was used as it targets N-terminal of STIM1 protein.

qRT-PCR: Cultured cells were grown to 70-80% confluence before RNA extraction. Total RNA was extracted using Qiagen RNeasy Mini kit following the manufacturer's protocol. cDNA was made from 500ηg to 1μg of RNA reverse transcribed using oligo (dT) primers and Super Script III reverse transcriptase. Real-time PCR analysis was performed using Roche LightCycler 480 and Roche Software. PCR reactions were performed using Kapa SYBR Green Supermix. The PCR reaction started with 2 min at 50 °C, then 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 1 min at 55 °C, 45 s at 65 °C. Quantification was measured as sample fluorescence crossed a predetermined threshold value that was just above the background. Expressions of genes of interest were compared with those of the housekeeping gene GAPDH and were measured using comparative threshold cycle values. The list of primer sequences used in this study is provided in **Table S2**.

Plasmids and cloning: ADCY6 was amplified from the commercial plasmid (DNASU; plasmid id: MmCD00297230) and was cloned in in pBluescript SK(+) vector. It was then sub cloned in pmECFP-1 vector (Addgene; plasmid #38772) by inserting it between EcoRI and BamHI restriction sites to get ADCY6-CFP. zSTIM1a and zSTIM1b were amplified from zebrafish embryos and were cloned in mcherry-C1 (Addgene; plasmid #54563) between HindIII and BamHI restriction sites. The primer sequences used for amplifying ADCY6 and zSTIM1 are listed in the **Table S3**. YFP-STIM1, STIM1 D76A and STIM1 ΔK were procured from Addgene (Plasmid #

18857, 18859 and 18861 respectively). STIM1 ΔS/P was gifted by Jonathan Soboloff (Temple University, PA, USA). mCherry-STIM1 and STIM1 truncations were generously provided by Rainer Schindl and Christoph Romanin (Institute of Biophysics, Johannes Kepler University, Linz, Austria). Yandong Zhou and Donald Gill (Penn State University, Hershey, USA) shared STIM1 F394H construct.

AB FRET: Acceptor bleaching FRET based on donor de-quenching was performed using Leica SP-8 confocal microscope as recommended by the Leica Microsystems, Germany. Image acquisition was performed with open pinhole and analysis was performed using Leica LAS X 2.0.1.14392 software. Data was collected using 63X oil immersion objective with NA 1.2. Cells were transfected with STIM1-YFP or STIM1 ΔS/P-YFP along with ADCY6-CFP. These cells were treated with either DMSO control or Tg for 4min before acceptor bleaching for 1min. The changes in the emission intensities of CFP (donor) before and after bleaching were recorded. FRET efficiencies from several ROIs arising from 8-12 cells/condition were evaluated using the Leica LAS X software wherein AB FRET efficiency was calculated using the below equation:

$$\mathbf{FRET_{eff} = (D_{post}-D_{pre})/D_{post}}$$

where D_{pos} is the fluorescence intensity of the Donor after acceptor photo bleaching, and D_{pre} the fluorescence intensity of the Donor before acceptor photo bleaching.

siRNA/shRNA transfections: siRNA transfections were with done at a final concentration of 100ηM using Dharmafect transfection reagent whereas shRNA

transfections were using Lipofectamine 2000 reagent. In case of transfections in melanogenesis assay, cells were transfected on day3 of the assay and experiment was terminated on day6 for αMSH treatment and on day7 for non-treated experiments.

Tyrosinase activity/DOPA assay: The activity of tyrosinase enzyme was checked in cell lysates by performing DOPA assay as reported earlier (*1*). Briefly, cell lysates were prepared in NP-40 lysis buffer and equal amount of protein was run on gel in non-reducing/native condition. Gel was then immersed in phosphate buffers supplemented with tyrosinase substrate L-DOPA (Sigma chemicals). The activity of tyrosinase enzyme corresponded to formation of black color pigment and was evaluated by taking images.

Confocal Microscopy: Live cell microscopy was performed on B16 cells transfected with eYFP-STIM1 construct using Leica SP-8 confocal microscope (Leica Microsystems, Germany). Image acquisition and analysis was performed using Leica LAS X 2.0.1.14392 software. Data was collected in xyt format at 600hertz using 63X water immersion objective with NA 1.2. Excitation was done at 488nm using argon laser and emission was between 500-550nm. Tg or αMSH addition was done after 1-2min of resting cell image acquisition. Sub-plasmalemmal STIM1 oligomerization/puncta formation was quantified by selecting multiple sub-plasmalemmal ROIs in the same cell before and after addition of αMSH. Data presented in the Figure 1 comes from more than 40 cells used for live cell confocal imaging.

cAMP measurements: cAMP measurements were performed using competitive ELISA based kit from Sigma as per manufacturer's protocol. Briefly, cells were pre-incubated with IBMX for 30 minutes before inducing cAMP generation for inhibiting PDEs activity. Post treatments, cells were lysed using 0.1M HCl again for keeping PDEs activity under check. Subsequently, samples were used for performing ELISA as per manufacturer's instructions (Sigma-Aldrich; Catlog: CA201).

Immunoprecipitation assays: Co-IPs were performed using Millipore's Catch and Release Kit as per manufacture's protocol. Briefly, cells were pre-treated with either vehicle control (DMSO) or Tg for 15 min for inducing STIM1 oligomerization before preparing cell lysates. 500µg of protein lysates were subjected to IPs using either control Rb IgG or m-Cherry Ab in case of STIM1 IPs and ADCY6 Ab for ADCY6 IPs. Finally, STIM1 and ADCY6 IP elutes were immune-probed for ADCYs and STIM1 respectively. For STIM1 ΔS/P co-IPs, STIM1/GOK Ab from BD Biosciences was used for IPs and blotting was performed with ADCY6 Ab.

Zebrafish Maintenance: Zebrafish used in this study were housed at the CSIR-Institute of Genomics and Integrative Biology following standard husbandry practices. ftyrp1:GFP1 plasmid was a kind gift from Dr. Xiangyun Wei (University of Pittsburg, USA) and the transgenic line was generated in house. Widtype and mitfa:GFP embryos were obtained from pairwise mating of adults.

Morpholino Injections: Morpholino (MO) oligonucleotides (Gene Tools, USA) were dissolved in nuclease free water (Ambion, USA) at a concentration of 1 mM according to the protocols recommended by Gene Tools. Glass capillary (World

Precision) micropipettes were pulled using Sutter Instrument (USA) and clipped appropriately to deliver 1–3nl solution into 1–2 cell zebrafish embryos. Eppendorf Femtojet and Narshige micromanipulator were used for injection. Post microinjection, larvae were raised in E3 in 100 mm Petri dishes at a density 100 larvae per dish. The larvae were incubated at 28^0C. Details regarding the morpholino sequences are provided in **Table S1**.

Phenotype analysis: The Morphants were raised at 28^0C for the experiment till respective time points. The larvae were then embedded in 2% methylcellulose for imaging. The Imaging was performed using Zeiss stemi 2000-C microscope equipped with axiocamICc1 camera. Fluorescence images were acquired by Zeiss Axioscope microscope.

Cell count - Imaging FACS analysis: Cell counts for mitfa:GFP and ftyrp1:GFP positive cells from whole embryos were performed using AMNIS imaging FACS. Briefly, the embryos were deyolked in ringer's solution and then trypsinized using TryLExpress (Thermoscientific). The cell suspension was passed through 70uM cell strainer and analysis was performed using IDEAS (AMNIS) software.

Whole embryo Insitu Hybridization (WISH): WISH was performed as described earlier (*2*) and Imaging was done using Zeiss Stemi-2000 C microscope.

Supplemental Tables:

Table S1: Morpholino sequences used for Zebrafish experiments.

Gene	Sequence(5'---3')
Control_MO	CCTCTTACCTCAGTTACAATTTATA
STIM1a_MO	AAGTCACCAATCCGCTGAACTCCAT
STIM1b_MO	GTCTGGCAAATCTCAACAGAGCCAT

Table S2: Primer sequences used for qRT-PCR studies

GENE NAME	FORWARD PRIMER	REVERSE PRIMER
TYR	CGTAATCCTGGAAACCATGACA	GTCAAACTCAGACAAAATTCCACATC
TYRP1	GATCCGTTCTAGAAGCACCAAGA	CCTCAGCATAGCGTTGATAGTGA
DCT	TAATTGTGGAGGCTGCAAGTTC	AGGATGGCCGGCTTCTTC
GAPDH	AACTGCTTAGCACCCCTGGC	ATGACCTTGCCCACAGCCTT
hSTIM1	GCGGGAGGGTACTGAG	TCCATGTCATCCACGTCGTCA
mSTIM1	CAATAACCCCGGCATCCACT	AACTGGAGATGGCGTGTCTG

ORAI1	AGGTGATGAGCCTCAACGAG	CTGATCATGAGCGCAAACAG
ORAI2	GCAGCTACCTGGAACTGGTC	CGGGTACTGGTACTGCGTCT
ORAI3	GAGTGACCACGAGTACCCACC	GGGTACCATGATGGCTGTGG
ADCY1	GGTAGCTTCATCGTCCTCCG	CCTTCAGGCCTACCTACCCT
ADCY2	TAAACCGAGTGCTGCTGGAG	GCTGCCATGTATGTGCTACC
ADCY3	TGCAGAGCCCGAGATGTTCG	GGGAGCAGCCCCTTTTTACA
ADCY4	CTGGAGCCTAGCTTTGCTGA	GGATGAGATGACTCCCGCAG
ADCY5	AGGAGCACAGCATTGAGACC	CTCTGGGTTCGCACTTTCCT
ADCY6	GGGTTTGACGACACTGAGGT	AGTGATTCTCCCTCACCGC
ADCY7	GCAGGTAACAGGGTCGGAG	AGGGCCTTATCACGAGGAGT
ADCY8	GCACAAGCTTTGCATGTCCA	AGTACTCTGGGTAGGAGCAGA
ADCY9	GGTGTGTGTGGGCTTTTTCC	AATGCAGCAGTAGGCATGGT
ADCY10	ACATGGCACTTCTGCTGTCT	CACCAGGAAACTGTCTCCCC

Table S3: Primer sequences used for cloning

GENE	FORWARD PRIMER (5'--->3')	REVERSE PRIMER (5'--->3')
zSTIM1a	CTCGAGcgATGGAGTTCAGCGGATTGGT	GGATCCTTATTTCTTCTGTTTTTTGA
zSTIM1b	AAGCTTcgATGGCTCTGTTGAGATTTGC	GGATCCTTATTTCTTAGGCCTCCGGA
ADCY6	GAATTCATGTCATGGTTTAGTGGCCT	GGATCCcgACTGCTGGGGCCCCCGT

References

1. B. B. Fuller, M. A. Drake, D. T. Spaulding, F. Chaudhry, Downregulation of tyrosinase activity in human melanocyte cell cultures by yohimbine. *J Invest Dermatol* 114, 268-276 (2000).
2. B. Aerne, D. Ish-Horowicz, Receptor tyrosine phosphatase psi is required for Delta/Notch signalling and cyclic gene expression in the presomitic mesoderm. *Development* 131, 3391-3399 (2004).

Appendix Figure Legends

Appendix Figure S1. STIM 1/2 and Orai1/2/3 knockdown do not alter B16 ER Ca^{2+} release.

A. qRT-PCR analysis upon siRNAs targeting of STIM1, STIM2, Orai1, Orai2 and Orai3 for evaluating the efficiency of these siRNAs.

B. Amplitude of Tg stimulated Ca^{2+} release upon transfections with siNT control and siRNAs targeting five STIM and Orai homologs.

C. Amplitude of Tg stimulated Ca^{2+} release in shluciferase control, shSTIM1 and shOrai1 stable cells.

Data information: Data represented are Mean ± S.E.M. (**P*<0.05; ***P*<0.01, ****P*<0.001, ****P<0.0001; Unpaired Student's *t*-test).

Appendix Figure S2. Characterization of lentiviral transduced B16 stable cell lines with STIM1 or Orai1 knockdown.

A. Representative GFP and bright field images showing lentiviral transduced B16 stable cells with control shRNA targeting luciferase or shSTIM1 or shOrai1.

B. qRT-PCR analysis of STIM1 expression in shSTIM1 stables in comparison to control stables.

C. qRT-PCR analysis of Orai1 expression in shOrai1 stables in comparison to control stables.

D. Western blot analysis showing significant decrease in STIM1 and Orai1 expression in respective B16 stable cell lines.

Data information: Data represented are Mean ± S.E.M.

Appendix Figure S3. STIM1 regulates pigmentation *in vivo* not by inducing growth arrest.

A. Images of transgenic mitfa:GFP zebrafish embryos at 32hpf; injected with either control morpholino or morpholino targeting STIM1a showing comparable number of GFP positive melanoblasts.

B. Imaging FACS based analysis for quantification of melanoblasts number in control and zSTIM1a morphants.

Data information: Data represented in the bar graphs are Mean ± S.E.M. (**P*<0.05; ***P*<0.01, ****P*<0.001, ****P<0.0001; Unpaired Student's *t*-test).

Appendix Figure S4. ADCYs inhibition decreases αMSH induced Ca^{2+} release.

A. Representative control B16 trace with αMSH stimulus.

B. Representative B16 trace with 30' pre-incubation with 100μM SQ22536 followed by αMSH stimulus.

Appendix Figure S5. Characterization of siRNAs targeting ADCYs.

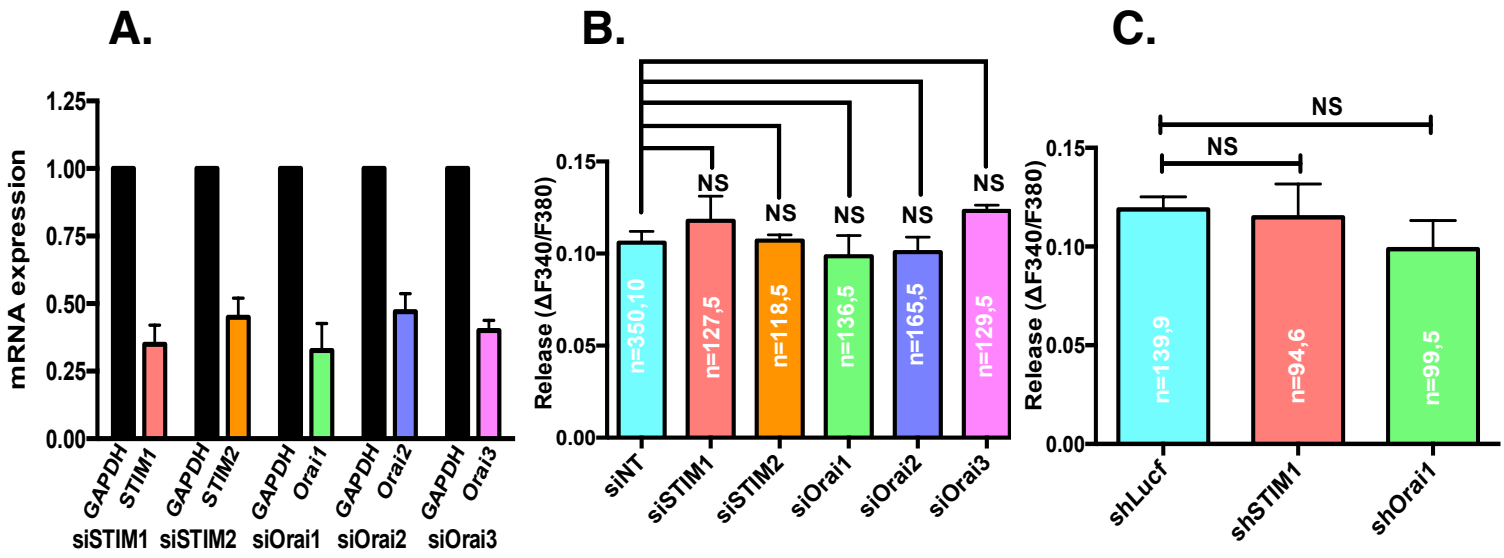
qRT-PCR analysis showing that the ADCYs were silenced at mRNA levels by their respective siRNAs.

Data information: Data represented are Mean ± S.E.M.

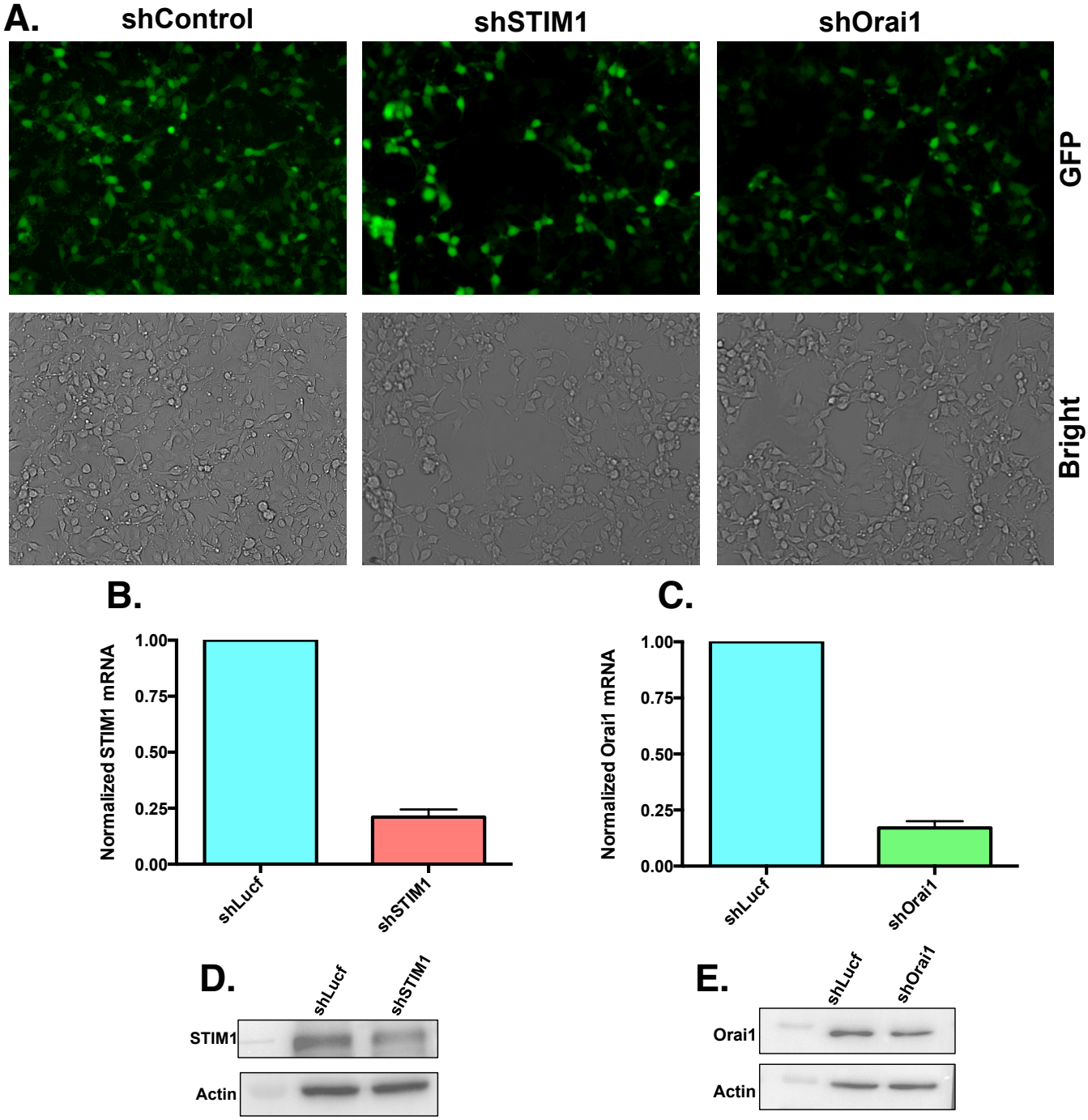
Appendix Figure S6. STIM1 co-IPs with ADCYs.

Immuno-blots showing that STIM1 does not co-IPs with ADCY4, 5, 7 and 9 in B16 cells.

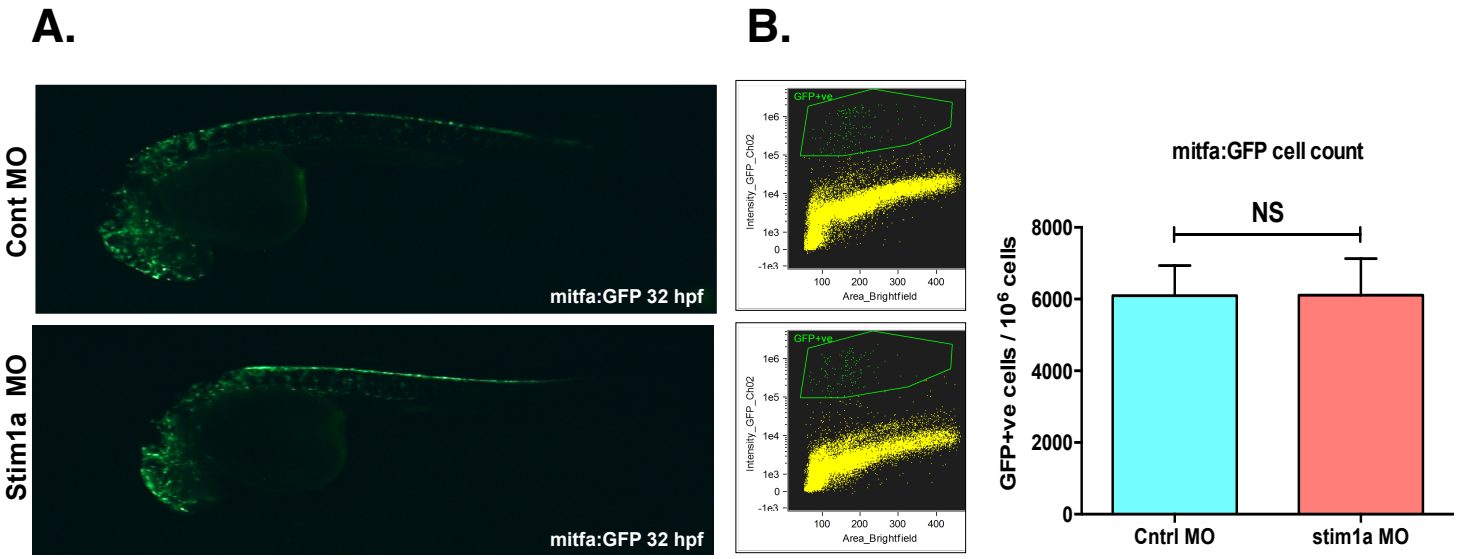
Appendix Fig S1.



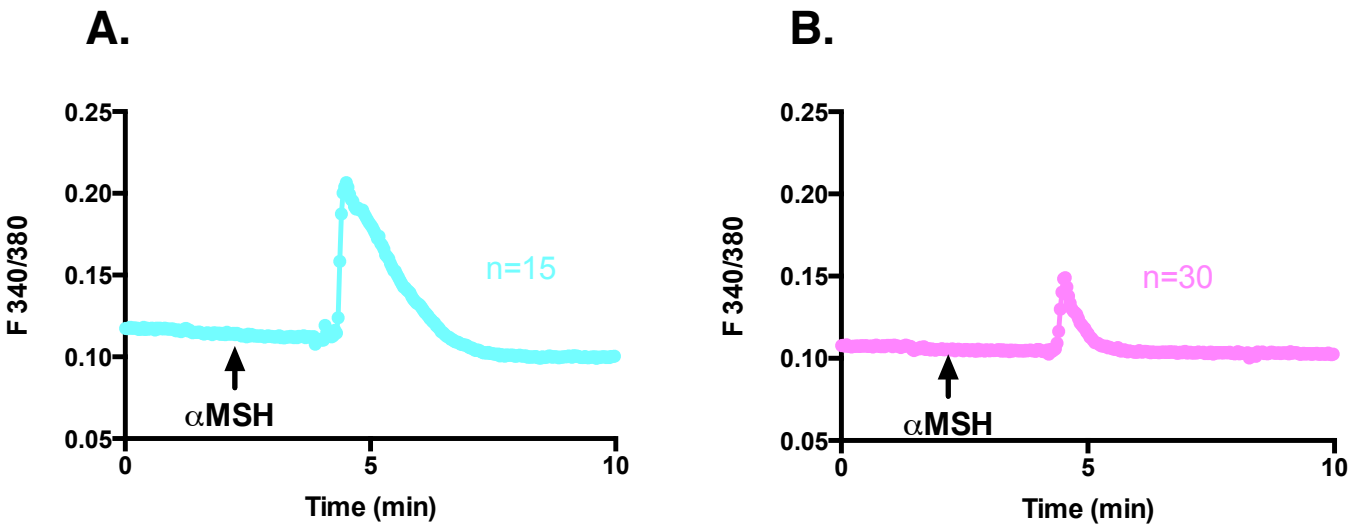
Appendix Fig S2.



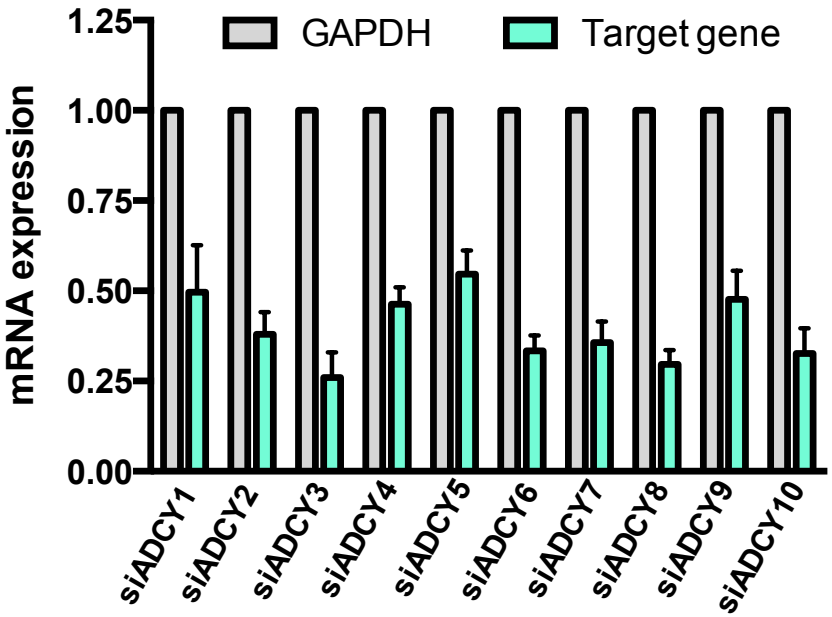
Appendix Fig S3.



Appendix Fig S4.



Appendix Fig S5.



Appendix Fig S6.