DUSP5 expression in left ventricular cardiomyocytes of young hearts regulates thyroid hormone (T3)-induced proliferative ERK1/2 signaling

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Supplementary Section includes Supplementary Methods and one Supplementary Table.

Supplementary Methods

Cardiomyocyte isolation for immunocytochemistry and immunoblotting

For immunoblotting and immunocytochemistry, hearts were enzymatically digested as described in Materials and Methods section of the manuscript and is adapted from our earlier studies[15]. Before making single cell suspensions, atria were excised, and the LVs were divided into apex, mid and base regions, as described below. Cardiac cells of the apex and base were separately disaggregated into single cell suspension. Cardiomyocytes were purified with 3 low speed centrifugations (18 x g for 4 min at room temperature), which caused cardiomyocytes to settle as a pellet. Supernatant fractions, enriched in non-myocytes, were discarded. These cardiomyocyte preparations were > 95% pure. Aliquots of cardiomyocytes were snap frozen in liquid nitrogen and stored at –80°C for immunoblotting. Additionally, aliquots were fixed with Cytofix (BD Biosciences, 554655) for 5 min and spread on glass slides for immunocytochemistry.

RT-qPCR

RT-qPCR protocol is customized from our earlier studies[15]. Cardiomyocytes in tubes containing RNAlater stabilization solution were removed from –80 °C freezer, thawed on ice and then centrifuged at 21,000 x g for 10 min. The RNAlater supernatant fraction was removed and replaced with 240 µl of lysis binding buffer from the mirVana miRNA Isolation Kit (ThermoFisher, AM1560). RNA was purified according to the manufacturer's guidelines. Purified RNA was reverse transcribed using Transcriptor Reverse Transcriptase (Roche, 03531295001) and random primer (Primer, random p(dN)6, Roche, 11034731001). Quantitative PCR was performed with SYBR Green Supermix (Bio-Rad, 1708882) on a iQ5 Thermal Cycler (Bio-Rad). Respectively, the 5' and 3' PCR primers used were: Dusp2: TTCTTGCGAGGCGGTTTCAA and AGTTGCTATTTTCGGCCCCA; Dusp4: GAGAGTCT-CCGGGAGGACAG and

GTCCTTTACTGCGTCGATGT; and Dusp5: TCGCCTACAGAC-CAGCCTAT and GTAGTGTAGGTGGGTGGTGC.

Immunoblotting

Immunoblotting protocol is customized from our earlier studies[15]. Whole cell cardiomyocyte lysates were generated by re-suspending cardiomyocytes in 250 µl of RIPA buffer (Cell Signaling, 9806S) supplemented with phosphatase inhibitor cocktail 2 and 3 (Sigma-Aldrich, P5726-1ML and P0044-1ML), 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, 93482-50ML-F) and protease inhibitor cocktail (Roche, 11697498001); cardiomyocytes were lysed by sonication and then pelleted by centrifuged at 21,000 x g for 30 min. The resulting supernatant fractions were aliquoted into fresh Eppendorf tubes and then snap-frozen in liquid nitrogen. Aliquots, stored at –80 °C, were allowed to thaw on ice immediately before use. Initially, a 5 to 10 µl aliquot (~20 µg protein) was mixed with an equal volume of 2x Laemmli sample buffer (Bio-Rad, 1610737), heated for 5 min at 95–99 °C and then immediately cooled on ice for 5 min. The samples were then centrifuged briefly before loading onto a SDS–polyacrylamide gel (12–18%) for electrophoresis (SDS-PAGE), which was performed at 200 volts for 5 min, and then at 150 volts for 30 min to 2 h, and then transferred to a PVDF membrane by electroblotting (Turbo Transfer; Bio-Rad). Depending upon the molecular weight of the proteins or protein complexes, the transfer time on Turbo Transfer was varied for high (11 min), average (7 min) and low (5 min) molecular weight proteins. After transfer, all blots were pre-blocked for 30–60 min in Superblock (Thermo Scientific, 37536). Initially, the samples were probed with GAPDH antibody. Based on GAPDH, the loading for each sample was adjusted to run gels containing all samples with an equal amount of GAPDH. Membranes were probed with the target protein-specific primary antibody. For quantitative analysis, the membrane was then stripped and re-probed with GAPDH to ensure

that loading was normalize for each sample. For stripping, the membrane was washed twice with 1x Tris-buffered saline (TBS, Thermo Scientific, BP2471-1) 5 min each and then incubated with Restore™ Western Blot Stripping Buffer (Thermo Scientific, 21059) for 5–15 min and then washed again twice with 1x TBS and pre-blocked with Superblock (Thermo Scientific, 37536) for 1 h before incubating with GAPDH antibody. Primary antibodies (see below) were also diluted in Superblock and incubated for 2 h at 22 °C, or overnight at 4 °C, followed by horseradish peroxidase (HRP)-labeled secondary antibody (1:10,000) for 45 min at 22 °C. The signals were detected using Super Signal West Dura Detection Reagent (Thermo Scientific, 34075) and images captured on a Bio-Rad GelDoc system equipped with CCD camera and ImageLab program (Bio-Rad). Quantification was performed by densitometry using the ImageLab program.

For studies involving nuclear protein quantification, nuclear and cytoplasmic fractions were separated from cardiomyocytes using NE-PER Nuclear Cytoplasmic Kit (Thermo Scientific, 78833) as per the manufacturer's protocol. Nuclear and cytoplasmic fractions were then similarly resuspended in RIPA buffer and stored and analyzed as described above.

For studies involving developmental quantification of cardiac specific proteins, we generated tissue lysates from ventricular apex, mid or base at each postnatal age specified. The husbandry of these mice is detailed above. We harvested hearts at each postnatal day between P1 to P17 and snap-froze them in liquid nitrogen. The ventricular apex, mid or base was then separated from these hearts and 200 µl of RIPA buffer (Cell Signaling, 9806S) supplemented with phosphatase inhibitor cocktail 2 and 3 (Sigma, P5726-1ML and P0044-1ML), 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF, Sigma, 93482-50ML-F) and protease inhibitor cocktail (Roche, 11697498001) was added, and the samples then homogenized using Polytron® PT 1200E handheld homogenizer (Kinematica). The resulting lysate was centrifuged at 21,000 xg for 30 min,

and the supernatant fraction harvested and aliquoted into fresh Eppendorf tubes and then snap-frozen in liquid nitrogen. These samples were subsequently fractionated by SDS-PAGE and analyzed as detailed above for cardiomyocyte lysates.

For studies involving different cardiac regions, the heart was divided into atria, RV and LV. In some studies, the LV was further subdivided into apex, mid and base region using a sharp scalpel to cut the hearts into pieces of equal width from the apex. On average the fraction of LV myocardium in LV apex, mid and base was approximately 18%, 38% and 44%, respectively. These regions were then immediately snap frozen in liquid nitrogen and subsequently used to prepare lysates, as above.

Antibodies used for immunoblots are detailed in Supplementary Table S1. Most of these antibodies are profiled in 1DegreeBio and were additionally validated using siRNA knockdown.

Supplementary Table S1. Information about the Antibodies Used in this Study

Antibody	Vendor	Catalogue number
c-Jun	Cell Signaling	9165
cTnT	Miltenyi Biotec	130-119-674
Cyclin A2	Abcam	ab181591
Cyclin B1	Abcam	ab32053
Cyclin D1	Abcam	ab134175
DUSP5	Abcam	ab200708
ERK1/2	Cell Signaling	4695
GAPDH	Cell Signaling	2118
IGF-1	Abcam	Ab9572
IGF-1R	Cell Signaling	9750
MEK1/2	Cell Signaling	9126
Phospho-ERK1/2 (T202/Y204)	Cell Signaling	4370
Phospho-histone H3 (S10)	Cell Signaling	8481
Phospho-MEK (S217/221)	Cell Signaling	9121

Most of these antibodies are profiled in 1DegreeBio and were also validated using siRNA.