**BRIEF REPORT**

**Ral function in muscle is required for flight maintenance in Drosophila**

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**ABSTRACT**

Ral is a small GTPase of the Ras superfamily that is important for a number of cellular functions. Recently, we found that expression of Ral is regulated by store-operated calcium entry (SOCE) in *Drosophila* neurons. In this study, through genetic and behavioural experiments, we show that Ral function is required in differentiated muscles for flight. Reducing Ral function in muscles, specifically reduced duration of flight bouts but not other motor functions, like climbing. Interestingly, unlike in the nervous system, Ral expression in the muscle is not regulated by SOCE. Moreover, either knockdown or genetic inhibition of SOCE in muscles does not affect flight. These findings demonstrate that a multiplicity of signalling mechanisms very likely regulate Ral expression in different tissues.

**Introduction**

Ral GTPases, members of the Ras superfamily regulate a variety of cellular processes including vesicle trafficking, cell polarity, and even oncogenesis. In mammals, two Ral GTPase genes are present — RalA and RalB and they interact with the exocyst complex. Ral function in various cell types has been studied, neuronal RalA and RalB regulate neurotransmitter release and neurite branching. RalA is critical for insulin secretion from pancreatic cells. Besides secretion, Ral proteins are also important in membrane targeting of proteins, like the Glut transporter in adipocytes.

In the genetic model system *Drosophila*, there exists a single homolog for Ral. Like the mammalian Ral proteins, *Drosophila* Ral also mediates its functions through the exocyst complex. It has several functions in development, including membrane transport and receptor trafficking. In a recent study, we identified Ral, as a regulator of *Drosophila* flight. Reduction of either Ral levels or function, across all neurons led to significant flight defects. However, Ral mutant flies were almost flightless, suggesting that Ral function might be required in tissues other than the nervous system, to regulate flight. In *Drosophila*, like most insects, the duration of flight bouts is regulated primarily by neuronal activity and muscle function. *Ral* is expressed in and functions in multiple tissues besides the nervous system. Thus, in this study we have investigated whether Ral function in muscles is required for flight.

Store-operated Calcium Entry (SOCE) is a mode of calcium entry in the cell that is triggered by emptying of endoplasmic reticular calcium stores by extracellular stimuli, and is involved in a variety of cellular processes like regulating gene transcription, muscle contraction and cell migration. SOCE is mediated primarily by the endoplasmic reticular calcium sensor STIM and the plasma membrane calcium channel Orai. Knockdown of either of these components in *Drosophila* neurons results in flight defects. In the previous study, we identified Ral expression to be regulated by SOCE in neurons and thus we have tested for similar regulation in the muscle in this paper.

**Results**

A dominant negative form of Ral, which harbours a single point mutation Ral(S25N) in the GTP binding domain of Ral (henceforth referred to as Ral(DN)) reduces Ral function. Flies in which *UAS-Ral(DN)* expression was driven using a muscle-specific *Mef2-GAL4* were tested for their ability to fly using the single flight assay. Flies with reduced Ral function in muscles had significantly lower flight durations than the corresponding controls (Fig. 1A). Reduction in Ral levels in muscles using an RNAi against Ral(RP) also significantly reduced the flight duration as compared with controls (Fig. 1A). This reduction in flight was not due to altered wing morphology because wings of flies, with Ral

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perturbations in muscles, appeared normal (Fig. 1A). A muscle requirement for Ral in the context of flight was further tested with another muscle driver, Mhc-GAL4, which expresses exclusively in differentiated muscles as compared with Mef2-GAL4 which also expresses in myoblasts. Mhc-GAL4 control flies themselves had slightly impaired flight bout durations as compared to the Mef2-GAL4 control (Fig. 1A and 1B) indicating an
effect of the genetic background on flight. Still, flies with expression of \( \text{Ral}^{\text{DN}} \) using \( \text{Mhc-GAL4} \) had significantly reduced flight bout durations as compared to controls with no effect on wing morphology (Fig. 1B). Taken together, these data demonstrate that the small GTPase Ral is also required in differentiated muscles for regulating the duration of \textit{Drosophila} flight.

Both the muscle GAL4s used, also express in muscles other than flight muscles. Therefore, next we tested if Ral function is specific to flight muscles or required more generally. For this, we tested the ability to climb 8 cm in 12 seconds. Flies with \( \text{Mef2-GAL4} \) driven expression of either \( \text{Ral}^{\text{DN}} \) or \( \text{Ral}^{\text{R}} \) in muscles could climb at a rate that was statistically indistinguishable from controls (Fig. 1C) suggesting that Ral function is very likely specific to flight muscles. If Ral function is important in the development of the indirect flight muscles, this could reflect in their morphology in adults.29 Thus, we looked at the morphology of the indirect flight muscles in the thorax which consist of dorsal longitudinal muscles (DLMs) and dorsal ventral muscles (DVMs).30 Mutations in genes required for formation of these muscle fibres can lead to malformed muscle fibres.31 However, expression of \( \text{Ral}^{\text{DN}} \) with \( \text{Mef2-GAL4} \) in all muscles through development and in adults resulted in muscle fibres that look identical to control (Fig. 1D).

SOCE in neurons regulates flight.32 and expression of Ral.15 Calcium dynamics in the muscle, mediated by Sarco-Endoplasmic Reticulum Calcium ATPase (SERCA) are important for muscle function.33 SERCA interacts with SOCE components in \textit{Drosophila} neurons.32 Having identified a role for Ral in flight muscles, we therefore tested whether SOCE-regulates Ral expression in muscles. We knocked down levels of \( \text{dStim} \), a calcium sensor on the endoplasmic reticulum that initiates SOCE upon store-depletion 24 in muscles with an RNAi against \( \text{dStim} \) (\( \text{dStim}^{\text{IR}} \)) and \( \text{Mef2-GAL4} \). Flies with \( \text{dStim} \) knockdown in muscles had normal wings and flight durations, no different from controls (Fig. 2A) despite significant reduction in \( \text{dStim} \) levels (Fig. 3A). Next, we tested the role of another molecule that mediates SOCE, the calcium release-activated calcium (CRAC) channel, Orai.25 Expression of the dominant-negative form of dOrai, \( \text{dOrai}^{\text{E180A}} \) results in abrogation of SOCE.34 Flies with UAS-\( \text{dOrai}^{\text{E180A}} \) without any GAL4 by itself had lower flight durations than other controls (Fig. 2B compared to Fig. 2A and Fig. 1A). Expression of \( \text{dOrai}^{\text{E180A}} \) in the muscle using the \( \text{Mef2-GAL4} \) however, resulted in no difference in flight times compared to the corresponding control (Fig. 2B). These data indicate that flight muscles do not require SOCE through the STIM/Orai pathway, and suggest that Ral expression in flight muscles is independent of SOCE. This idea was tested next by measuring Ral mRNA levels by qRT-PCR from dissected thoraces, in which the predominant tissues are flight muscles. Ral expression was the same between RNA from thoraces with knockdown of \( \text{dStim} \) and controls (Fig 3A and 3B) confirming that Ral levels in muscles are not regulated by SOCE.

**Discussion**

In this study, we have identified a role for Ral in flight muscles of \textit{Drosophila} flight. We also find that in muscles, unlike the nervous system, Ral expression is not regulated by store-operated calcium entry (SOCE).

Perturbation of Ral function with both \( \text{Mef2-GAL4} \) and \( \text{Mhc-GAL4} \) resulted in flight defects. However, \( \text{Mef2-GAL4} \) is expressed predominantly in developing muscles 27,35 whereas \( \text{Mhc-GAL4} \) is expressed mostly in differentiated muscles.28 Therefore, Ral function maybe required in developing as well as differentiated flight muscles. Based on the similar flight deficits observed in \( \text{Mef2}^{>\text{Ral}^{\text{DN}}} \) and \( \text{Mhc}^{>\text{Ral}^{\text{DN}}} \) (Figure 1 A and B) it is more likely that Ral function is primarily in differentiated flight muscles. Moreover, although \( \text{Mef2-GAL4} \) expresses in myoblasts, the gross anatomy of the adult thoracic flight muscles was not affected upon expression of \( \text{Ral}^{\text{DN}} \) supporting the idea that Ral function is not required for development of the flight muscles. However, our data does not rule out the possibility that loss of Ral function might affect the ultrastructure of these muscles. The requirement of Ral in differentiated muscles supports a role for Ral in muscle physiology. Further experiments are required to identify the temporal requirement of Ral in flight muscles which might help understand its function in this cell type better. Ral function in regulating the addition of post-synaptic membranes in differentiated muscles has been demonstrated earlier 13, and may be the underlying cause for the observed flight deficits. This previous study had also demonstrated a role for Ca\textsuperscript{2+} in activation of Ral but because perturbing SOCE in muscles did not lead to observed flight deficits, calcium through SOCE is unlikely to activate Ral.

Ral expression in neurons is down-regulated by knockdown of \( \text{dSTIM} \).15 However, in muscles, Ral levels were unaffected upon \( \text{dStim} \) knockdown. Concurrently, perturbations of SOCE in muscles did not alter flight durations. SOCE mediated by STIM and Orai has been documented in mammalian muscles 36 but so far has not been shown in \textit{Drosophila} muscles. Expression of \( \text{dStim} \) in \textit{Drosophila} thoracic muscles (Fig. 3), indicates the presence of SOCE. Thus, Ral expression is differentially regulated in the two tissues — neurons and muscles. The functional significance of this differential regulation needs further investigation.
Materials and methods

Fly rearing and stocks

Flies were reared on media containing cornmeal supplemented with yeast extract. Fly crosses were set up and allowed to lay eggs at 25°C, vials containing larvae were moved to 29°C and were maintained at the elevated temperature until testing. Fly strains used are as follows: Mef2-GAL4 (BL27390), Mhc-GAL4 (BL55133), UAS-RalDN (BL32094), UAS-RalIR (BL29580) and UAS-mcd8RFP (BL32219) from Bloomington Drosophila Stock Centre; dStimIR (v47073) from Vienna Drosophila Resource Centre and UAS-dOraiE180A.34

Fly images

Fly images were acquired using a Pro-Series camera attached to a stereo-microscope and images were obtained at the same zoom and image acquisition settings for all flies.

Single flight assay

In order to measure the ability of the flies to initiate and sustain flight, single flight assay was performed as described in.15 Briefly, 2—5 days old flies of either sex were anaesthetized on ice for about a minute and then tethered on to a thin metal wire between the head and thorax. After allowing a short duration of recovery, a gentle, mouth blow air-puff was given as a stimulus to initiate flight, and the duration of time from initiation to cessation of flight was noted at flight duration. Flight duration observation was capped at fifteen minutes.

Climbing assay

Flies were tested for their climbing ability using an assay described in.34 Briefly, a batch of ten 2—5 day old flies of either sex were dropped in a graduated glass cylinder, and tapped to collect them at the bottom. The number of flies that crossed the 8cm mark after this, in 12 seconds were noted as climbers. At least 3 independent batches of flies were used for this assay per genotype.

Preparation of thoracic flight muscles and microscopy

DVMs were dissected from adults of corresponding genotypes in ice cold 1x phosphate buffered saline (PBS) and fixed with 4% Paraformaldehyde (PFA) for an hour on ice. For visualizing DLMs, the thoraces were fixed with 4% PFA on ice for an hour and then cut longitudinally using a sharp blade. Both were mounted in 60% glycerol. The samples were imaged using a Leica SP5 confocal microscope using a 20x objective under similar image acquisition settings. Complete z-projects were obtained using Fiji37 and no post-acquisition processing was performed.

RNA isolation and qRT-PCR

RNA was isolated from thoraces of adult female flies. Four thoraces were used per sample to isolate RNA using

Figure 2. SOCE function in muscle is not required for flight. Representative images of flies from the indicated genotypes showing normal wing posture (top) and a box plot of flight durations of flies measured by single flight assay (bottom) from flies with dStim knockdown in the muscle (A) or dOrai dominant negative expression in the muscle (B). Box plot symbols are as described for Figure 1. All flies tested were from the same cross. Pairwise comparisons were performed by unpaired, two-tailed Student’s t-test and the exact p-values are indicated.

Figure 3. SOCE does not regulate Ral expression in the muscle. Change in the levels of dStim (A) and Ral (B) in the indicated genotypes, normalized to tubulin as measured by qRT-PCR. Flies used were from the same cross as that for Fig. 2. Bars represent means and error bars, standard errors of mean of the fold change. Pairwise comparisons were performed by unpaired, two-tailed Student’s t-test and the exact p-values are indicated.
TRIzol (Invitrogen), following manufacturer’s protocol. Approximately 500ng of total RNA was treated with DNaseI (Invitrogen) and reverse transcribed to cDNA using M-MLV (Invitrogen) as described in. For quantitative PCR, Kapa SYBR Fast qPCR kit (KAPA Biosystems) was used in a 10μl reaction on an ABI QuantStudio3 system. Three biological replicates from independently isolated RNA samples were used for each experiment. A melt curve was performed to ensure a single product. Fold changes were calculated using the ΔΔCt method. β-Tubulin was used as an internal control. Primer sequences used are as follows (5'-3'):

β-Tub(F) — CCAAGGGTCAATTACAGAGG, β-Tub (R)—ATCAGCAGGGTTCCCATACC

dStim(F) — GAAGCAATGGATGTGGTTCTG, dStim (R) — CCGAGTTCGATGAACTGAG

Ral(F) — GACTACGAGCCCACCAAG, Ral(R) — CCGCATAATCCTCTCGGC

Data representation and statistics

Flight data is represented as box plots generated using BoxPlotR. Otherwise, data is represented as bar graphs representing means and error bars, standard errors of mean. For analyses with more than two test conditions, One-way Analysis of Variance (ANOVA), followed by pairwise Tukey’s test was used. Statistical significance post ANOVA is denoted with small ‘s’ test was used and the exact p-values are indicated within the figures.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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References

1. Feig LA. Ral-GTPases: Approaching their 15 minutes of fame. Trends in Cell Biology. 2003;13:419-425. https://doi.org/10.1016/S0962-8924(03)00152-1. PMID:12888294

2. Shirakawa R, Horiuchi H. Ral GT-Pases: Crucial mediators of exocytosis and tumourigenesis. J. Biochem. 2015;157:285-299. https://doi.org/10.1093/jb/mv029. PMID:25796063

3. Chardin P, Tavitian A. Coding sequences of human ralA and ralB cDNAs. Nucleic Acids Res. 1989;17:4380. https://doi.org/10.1093/nar/17.11.4380. PMID:2662142

4. Moskalenko S, Tong C, Rosse C, Mirey G, Formstecker E, Daviet L, Camonis J, White MA. Ral GT-Pases Regulate Exocyst Assembly through Dual Subunit Interactions. J. Biol. Chem. 2003;278:51743-51748. https://doi.org/10.1074/jbc.M308702200. PMID:14525976

5. Li G, Han L, Chou T, Fujita Y, Arunachalam L, Xu A, Wong A, Chiew SK, Wan Q, Wang L, et al. RalA and RalB function as the critical GTP sensors for GTP-dependent exocytosis. J. Neurosci. 2007;27:190-202. https://doi.org/10.1523/JNEUROSCI.2537-06.2007. PMID:17202486

6. Lalli G, Hall A. Ral GT-Pases regulate neurite branching through GAP-43 and the exocyst complex. J. Cell Biol. 2005;171:857-869. https://doi.org/10.1083/jcb.200507061. PMID:16330713

7. Lopez JA, Kwan EP, Xie L, He Y, James D, Gaisano HY The RalA GT-Pase is a central regulator of insulin exocytosis from pancreatic islet beta cells. J. Biol. Chem. 2008;283:17939-17945. https://doi.org/10.1074/jbc.M800321200. PMID:18426794

8. Chen XW, Leto D, Chiang SH, Wang Q, Salitri AR. Activation of RalA Is Required for Insulin-Stimulated Glut4 Trafficking to the Plasma Membrane via the Exocyst and the Motor Protein Myo1c. Dev. Cell. 2007;13:391-404. https://doi.org/10.1016/j.devcel.2007.07.007. PMID:17765682

9. Sawamoto K, Winge P, Koyama S, Hirota Y, Yamada C, Miyao S, Yoshikawa S, Jin MH, Kikuchi A, Okano H. The Drosophila Ral GT-Pase regulates developmental cell shape changes through the Jun NH(2)-terminal kinase pathway. J. Cell Biol. 1999;146:361-72. https://doi.org/10.1083/jcb.146.2.361. PMID:10427090

10. Moskalenko S, et al. The exocyst is a Ral effector complex. Nat. Cell Biol. 2002;4:66-72. https://doi.org/10.1038/ncb728. PMID:11740492

11. Holly RM, Mavor LM, Zuo Z, Blankenship JT. A rapid, membrane-dependent pathway directs furrow formation through RalA in the early Drosophila embryo. Development. 2015;142:2316-2328. https://doi.org/10.1242/dev.120998. PMID:26092850

12. Cho B, Fischer JA. Ral GT-Pase promotes asymmetric Notch activation in the Drosophila eye in response to Frizzled/PCP signaling by repressing ligand-independent receptor activation. Development. 2011;138:1349-1359. https://doi.org/10.1242/dev.056002. PMID:21350007

13. Teodoro RO, Pekkurnaz G, Nasser A, Higashi-Kovtun ME, Balakireva M, McLachlan IG, Camonis J, Schwarz TL. Ral mediates activity-dependent growth of postsynaptic membranes via recruitment of the exocyst. EMBO J. 2013;32:2039-55. https://doi.org/10.1038/embj.2013.147. PMID:23812009
Small GTPases

14. Klose M, Duvall L, Li W, Liang X, Ren C, Steinbach JH, Taghert PH. Functional PDF Signaling in the Drosophila Circadian Neural Circuit Is Gated by Ral A-Dependent Modulation. Neuron. 2016;90:1-14. https://doi.org/10.1016/j.neuron.2016.04.002. PMID:27054611

15. Richhariya S, Jayakumar S, Abruzzi K, Rosbash M, Hasan G. A pupal transcriptomic screen identifies Ral as a target of store-operated calcium entry in Drosophila neurons. Sci. Rep. 2017;7:42586. https://doi.org/10.1038/srep42586. PMID:28195208

16. Dickinson MH, Tu MS. The function of dipteran flight muscle. Comparative Biochemistry and Physiology — A Physiology. 1997;116:223-238. https://doi.org/10.1016/S0012-1606(03)00162-4

17. Fry SN, Sayaman R, Dickinson MH. The aerodynamics of free-flight maneuvers in Drosophila. Science. 2003;300:495-8. https://doi.org/10.1126/science.1081944. PMID:12702878

18. Sadaf S, Reddy OV, Sane SP, Hasan G. Neural control of wing coordination in flies. Curr. Biol. 2015;25:80-86. https://doi.org/10.1016/j.cub.2014.10.069. PMID:25496964

19. Ghiglione C, Devergne O, Cerezo D, Noselli S. Drosophila RalA is essential for the maintenance of Jak/Stat signalling in ovarian follicles. EMBO Rep. 2008;9:676-82. https://doi.org/10.1038/embr.2008.79. PMID:18552769

20. Prakriya M, Lewis RS. Store-Operated Calcium Channels. Physiol. Rev. 2015;95:1383-1436. https://doi.org/10.1152/physrev.00020.2014. PMID:26400989

21. Feske S Calcium signalling in lymphocyte activation and disease. Nat. Rev. Immunol. 2007;7:690-702. https://doi.org/10.1038/nri2152. PMID:17703229

22. Stiber J, Hawkins A, Zhang ZS, Wang S, Burch J, Graham V, Ward CC, Seth M, Finch E, Malouf N, et al. STIM1 signalling controls store-operated calcium entry required for development and contractile function in skeletal muscle. Nat. Cell Biol. 2008;10:688-697. https://doi.org/10.1038/ncb1731. PMID:18488020

23. Yang S, Zhang JJ, Huang XY. Orai and STIM1 Are Critical for Breast Tumor Cell Migration and Metastasis. Cancer Cell. 2009;15:124-134. https://doi.org/10.1016/j.ccr.2008.12.019. PMID:19185847

24. Liu J, Kim ML, Heo WD, Jones JT, Myers JW, Ferrell JE JR, Meyer T. STIM Is a Ca2+-Sensor Essential for Ca2+-Store-Depletion-Triggered Ca2+ Influx. Curr. Biol. 2005;15:1235-1241. https://doi.org/10.1016/j.cub.2005.05.055. PMID:16005298

25. Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel SH, Tanasa B, Hogan PG, Lewis RS, Daly M, Rao A. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. Nature. 2006;441:179-185. https://doi.org/10.1038/nature04702. PMID:16582901

26. Venkiteswaran G, Hasan G. Intracellular Ca2+ signaling and store-operated Ca2+ entry are required in Drosophila neurons for flight. Proc. Natl. Acad. Sci. 2009;106:10326-10331. https://doi.org/10.1073/pnas.0902982106

27. Ranganayakulu G, Schulz RA, Olson EN. Wingless Signaling Induces nautilus Expression in the Ventral Mesoderm of the Drosophila Embryo. Dev. Biol. 1996;176:143-148. https://doi.org/10.1006/dbio.1996.9987. PMID:8654890

28. Klein P, Müller-Rischart AK, Motori E, Schönauer C, Schnorrer F, Winklhofer KF, Klein R. Ret rescues mitochondrial morphology and muscle degeneration of Drosophila Pink1 mutants. EMBO J. 2014;33:341-355. https://doi.org/10.1002/embj.201284290. PMID:24473149

29. Bernard F, Lalouette A, Gullaud M, Jeantet AY, Cossard R, Zider A, Ferveur JF, Silber J. Control of apertuous by vesicular drives indirect flight muscle development in Drosophila. Dev. Biol. 2003;260:391-403. https://doi.org/10.1016/S0012-1606(03)00255-0. PMID:12921740

30. Swank DM. Mechanical analysis of Drosophila indirect flight and jump muscles. Methods. 2013;56:69-77. https://doi.org/10.1016/j.jmeth.2011.10.015

31. Mukherjee P, Gildor B, Shilo B, Vijayraghavan K, Schetjer ED. The actin nucleator WASp is required for myoblast fusion during adult Drosophila myogenesis. Development. 2011;2357:2347-2357.

32. Venkiteswaran G, Hasan G. Intracellular Ca2+ signaling and store-operated Ca2+ entry are required in Drosophila neurons for flight. Proc. Natl. Acad. Sci. U. S. A. 2009;106:10326-10331. https://doi.org/10.1073/pnas.0902982106. PMID:19515818

33. Sanyal S, Consoulas C, Kurumi H, Basole A, Mukai L, Kidokoro Y, Krishnan KS, Ramaswami M. Analysis of conditional paralytic mutants in Drosophila sarcoplasmic reticulum calcium ATPase reveals novel mechanisms for regulating membrane excitability. Genetics. 2005;169:737-750. https://doi.org/10.1534/ genetics.104.031930. PMID:15520268

34. Pathak T, Agrawal T, Richhariya S, Sadaf S, Hasan G. Store-Operated Calcium Entry through Orai Is Required for Transcriptional Maturation of the Flight Circuit in Drosophila. J. Neurosci. 2015;35:13784-13799. https://doi.org/10.1523/JNEUROSCI.1680-15.2015. PMID:26446229

35. Bryantsev AL, Baker PW, Lovato TL, Jaramillo MS, Cripps RM. Differential requirements for Myocyte Enhancer Factor-2 during adult myogenesis in Drosophila. Proc. Natl. Acad. Sci. U. S. A. 2009;106:10326-10331. https://doi.org/10.1073/pnas.0902982106. PMID:19515818

36. Pan Z, Brotno M, Ma J. Store-operated Ca2+ entry in muscle physiology and diseases. BMB reports. 2014;47:69-79. https://doi.org/10.5483/BMBRep.2014.47.2.015. PMID:241466

37. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, et al. Fiji: an open source platform for biological image analysis. Nat. Methods. 2012;9:676-682. https://doi.org/10.1038/nmeth.1929. PMID:22743772

38. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^-DeltaCT Method. Methods. 2001;25:402-408. https://doi.org/10.1016/S0892-6822(00)00422-4. PMID:11846609

39. Spitzer M, Wildenhain J, Rappsilber J, Tyers M. BoxPlotR: a web tool for generation of box plots. Nat. Methods. 2014;11:121-2. https://doi.org/10.1038/nmeth.2811. PMID:24481215