A genome-wide dsRNA library screen for *Drosophila* genes that regulate the GBP/phospholipase C signaling axis that links inflammation to aging

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**Abstract**

**Objective:** Invertebrates are productive models for understanding how inflammation, metabolism and aging are intertwined. We have deployed a dsRNA library screen to search for genes in *Drosophila melanogaster*—and hence identify human orthologs—that encode participants in a G-protein coupled, Ca\(^{2+}\)-signaling pathway that regulates inflammation, metabolism and lifespan.

**Results:** We analyzed receptor-dependent, phospholipase C/Ca\(^{2+}\) signaling responses to the growth-blocking peptide (GBP) cytokine in *Drosophila* S3 cells plated in 384-well plates containing dsRNAs that target approximately 14,000 *Drosophila* genes. We used Z-scores of < −3 or > +3 to define gene hits. Filtering of ‘housekeeping’ genes from these hits yielded a total of 82 and 61 *Drosophila* genes that either down-regulate or up-regulate Ca\(^{2+}\)-signaling, respectively; representatives from these two groups were validated. Human orthologs of our hits may be modulators of Ca\(^{2+}\) signaling in general, as well as being candidates for acting in molecular pathways that interconnect aging and inflammation.

**Keywords:** Cytokine, Inflammation, Metabolism, Calcium-signaling, G-proteins, Receptor

**Introduction**

A systems-level understanding of cytokine-mediated, inter-tissue signaling can help to generate fundamental insight into links between longevity, metabolism and inflammation [1]. Research with human subjects indicates that aging is affected by the balance between circulating pro- and anti-inflammatory factors [2] which are subject to various environmental influences, among which calorific restriction receives particular attention [3]. However, little is known concerning the precise nature of the molecular entities and pathways that intertwine these biological phenomena in humans; most work in this area uses animal models.

Invertebrates are productive, genetically-tractable models for understanding how inflammation and aging are inter-related in humans [1, 4]. Recent work has established that a *Drosophila* cytokine, growth-blocking peptide (GBP), interconnects longevity, inflammation and dietary influences, through activation of the phospholipase C (PLC)/Ca\(^{2+}\) signaling cascade [5, 6]. The spatiotemporal control of stimulus-activated Ca\(^{2+}\) dynamics by PLC is a multiplex cellular process that coordinates three fundamental activities: maintenance of basal Ca\(^{2+}\) pools, release of Ca\(^{2+}\) from intracellular stores, and Ca\(^{2+}\) fluxes across the plasma membrane [7]. A complete understanding of the integration of these processes requires characterization of the networking of a multitude of individual regulatory components; this degree of systems-biology insight has not yet been attained. Some important information has been obtained by exploiting the amenability of *Drosophila* to the application of...
dsRNA libraries [8, 9]. However, the latter studies were technically limited to identification of the subset of proteins that impact Ca\(^{2+}\) fluxes across the plasma membrane; in the current study, we have sought to widen the knowledge-base through identification of regulatory factors that may impact release of Ca\(^{2+}\) release from intracellular stores and Ca\(^{2+}\) fluxes into the cell, i.e., total Ca\(^{2+}\) mobilization ([Ca\(^{2+}\)]\(_T\)).

For this work we deployed a fluorophore-based assay for GBP-mediated Ca\(^{2+}\) mobilization in Drosophila S3 cells in a 384-well, high-throughput screening format [5]. In that latter study, we screened a relative small dsRNA sub-library that targets 1729 genes known (or computationally predicted) to encode transmembrane proteins. In that way, we identified several integral membrane proteins that contribute to GBP-mediated Ca\(^{2+}\) release [5]. Nevertheless, that particular dsRNA sub-library only covered 12% of all Drosophila genes. In the current study, we performed a genome-wide dsRNA screen (almost 14,000 genes) so as to identify a more complete set of regulatory factors. This approach could be particularly beneficial in the context of diseases where changes in Ca\(^{2+}\)-signaling are causative, since that can provide identify novel therapeutic targets [7].

**Main text**

**Methods**

**Screening of the dsRNA library**

We purchased a genome-wide dsRNA library (v2.0) from the DRSC/TRiP Functional Genomics Resources (https://fgr.hms.harvard.edu/). This library contains 66 × 384 well-plates that target almost 14,000 Drosophila genes with either one or two dsRNA constructs. The library is provided in duplicate (i.e., as a total of 132 plates). The biological readout during the screening was the total, GBP-induced change in fluorescence (equivalent to [Ca\(^{2+}\)]\(_T\)) emitted from a genetically encoded Ca\(^{2+}\) sensor in Drosophila S3 cells, in response to the addition of 500 nM GBP (added after 5-days pre-treatment with the dsRNAs [5]. A FLIPRTETRA (Molecular Devices) was used to simultaneously record [Ca\(^{2+}\)]\(_T\) in every well of a 384-well plate. We describe these procedures in more detail in our previous study with a small dsRNA sub-library that targets 1729 genes [5].

**Evaluation and deposition of the screening data**

Z-scores were calculated for every unique dsRNA. A ‘positive’ gene-hit for any single dsRNA was defined by a reduction in [Ca\(^{2+}\)]\(_T\) that yielded a Z-score of less than −3; the Z-score of a dsRNA = ([average [Ca\(^{2+}\)]\(_T\) of plate])/SD of plate [Ca\(^{2+}\)]\(_T\)). This strict disambiguation approach is designed to avoid false positives. Additionally, we describe some unusual, ‘negative’ gene-hits: dsRNAs that yielded an increase in [Ca\(^{2+}\)]\(_T\) (with a Z-score greater than 3).

We encountered more plate-to-plate variability than in our previous study with the transmembrane dsRNA sub-library [5]. In the current study, for a number of plates, the cells in individual wells exhibited values for [Ca\(^{2+}\)]\(_T\) that clustered atypically close to that of the control, raising the possibility that knock-down efficiencies were abnormally low. We did not establish the cause of this variability, although it was not specific to any particular plate barcodes. We resolved this problem in the following manner: the Itp-r83A gene was one of our initial, and statistically most significant hits (Table 1); we independently validated this hit using alternate dsRNA constructs (Fig. 1a). Thus, we decided to use this as an internal control. This led us to establish that an adequate range of [Ca\(^{2+}\)]\(_T\) signals within a plate was typically observed whenever our Itp-r83A dsRNA caused at least 60% inhibition of GBP-mediated Ca\(^{2+}\) signaling. Thus, all data were discarded from any plate in which the inhibition by the internal Itp-r83A dsRNA control did not reach the 60% cut-off. We were able to procure new plates from the vendor to replace most of those plates that we discarded, and eventually we performed enough assays to screen every dsRNA in the genome-wide library at least once. Previous studies that used similar plates to screen for gene knock-downs that target Ca\(^{2+}\) entry into the cell [8, 9] do not state if a similar problem was experienced. However, a different version of the dsRNA library (i.e., v1) was deployed in those earlier studies; we used v2.

The complete list of Z-scores is available at http://www.flyrnai.org/. Here, we tabulate two filtered hit-lists (Tables 1 and 2), from which we have removed all hits that could reasonably be predicted to non-specifically affect protein synthesis, by virtue of their having the following categorizations in the Gene Ontology Database (http://www.geneontology.org/): mRNA splicing (GO:0000398); structural constituent of ribosome (GO:0003735); translation initiation factor activity (GO:0003743); transcription factor activity, sequence-specific DNA binding (GO:0003700); RNA polymerase II transcription cofactor activity (GO:0001104); RNA polymerase II activity (GO:0001055); DNA-directed RNA polymerase activity (GO:0003899); transcription factor binding (GO:0008134); transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding (GO:0001077); translation (GO:0006412); translation elongation factor activity (GO:0003746).

**Independent validation and additional screening**

Follow-up GBP-mediated Ca\(^{2+}\) mobilization in S3 cells, using unique dsRNA constructs different from those used...
Table 1 Filtered list of gene knock-downs that reduced GBP-mediated Ca\textsuperscript{2+} mobilization

| Flybase ID | Gene  | Amplicon # | Mean Z-score |
|------------|-------|------------|--------------|
| F8gn003246 | ACC   | DRSC06059  | −3.15        |
| F8gn0025725| alphaCOP | DRSC08706  | −5.27        |
| F8gn003884 | alpha Tub48B | DRSC12622, DRSC25011 | −3.32, −3.09 |
| F8gn003749 | Art102F | DRSC17195  | −4.18        |
| F8gn0033062| Ar2   | DRSC04893  | −3.06        |
| F8gn0010217| ATPsynbeta | DRSC17194  | −3.48        |
| F8gn0014127| barr  | DRSC03488  | −3.52        |
| F8gn0025724| betaCOP | DRSC03492, DRSC26650 | −4.27, −5.03 |
| F8gn0259876| Cap-G | DRSC21805  | −4.04        |
| F8gn0022213| Cas   | DRSC26857  | −3.13        |
| F8gn0022942| Cbp80 | DRSC18450  | −3.12        |
| F8gn0012058| Cdc27 | DRSC11112  | −3.3         |
| F8gn0030510| CG12177| DRSC19437  | −7.01        |
| F8gn0033429| CG12929| DRSC06242  | −6.12        |
| F8gn0031023| CG14200| DRSC19555  | −3.02        |
| F8gn0266917| CG16941| DRSC15166  | −3.92        |
| F8gn0031498| CG17260| DRSC00497  | −8.46        |
| F8gn0083978| CG17672| DRSC09230  | −3.49        |
| F8gn0035205| CG2469 | DRSC08562  | −3.14        |
| F8gn0031266| CG2807 | DRSC00535  | −5.48        |
| F8gn0031493| CG3605 | DRSC00619  | −5.79        |
| F8gn0058198| CG40198| DRSC21068  | −3.12        |
| F8gn0035983| CG4080 | DRSC10398  | −6.77        |
| F8gn0086758| chimmo| DRSC25847  | −3.26        |
| F8gn0259993| CR42491| DRSC25025  | −4.52        |
| F8gn0028836| CSN7  | DRSC06807, DRSC06808 | −3.03, −3.07 |
| F8gn0025455| CycT  | DRSC11124  | −3.6         |
| F8gn0086687| Desat1 | DRSC23578  | −5.23        |
| F8gn0026035| Diap1 | DRSC11404  | −4.59        |
| F8gn0039183| Dist3 | DRSC16034  | −3.99        |
| F8gn0004638| dkr   | DRSC07760  | −3.52, −3.54 |
| F8gn0049759| enok  | DRSC04096  | −3.07        |
| F8gn0033859| fand  | DRSC39027  | −4.62        |
| F8gn0004656| fis(1)h| DRSC29017  | −3.39        |
| F8gn0004435| Galphaq| DRSC07432  | −3.36        |
| F8gn001105| Gbeta13F| DRSC20247  | −5.32        |
| F8gn0014189| Hel2SE| DRSC03342  | −3.76        |
| F8gn0053818| His3-CG33818| DRSC21267  | −4.05        |
| F8gn0015393| hoip  | DRSC03546  | −5.3         |
| F8gn001218| Hsc70-3| DRSC25105  | −3.05        |
| F8gn0026599| Hsc70-4| DRSC29729  | −3.32        |
| F8gn0010051| Itp-83A| DRSC12354  | −6.95        |
| F8gn0004378| Klpl6F | DRSC28179  | −3.31        |
| F8gn001491| (I)110Bb| DRSC20346  | −3.32        |
| F8gn001986| (I)235Df| DRSC03560  | −4.51        |
| F8gn0011640| lark  | DRSC11362, DRSC25108 | −4.28, −5.71 |
| F8gn0035889| mkg-p | DRSC10777  | −3.13        |
| F8gn0032921| Mpp6  | DRSC03169  | −3.77        |
| F8gn0035132| mthl10| DRSC39158  | −3.58        |

The genes listed are the ‘hits’ that reduced GBP-mediated Ca\textsuperscript{2+} mobilization (i.e., Z-score > 3), after filtering of housekeeping genes (see “Methods”).

in the high-throughput assays, were performed exactly as described previously [5], using 50 nM GBP, and dsRNAs constructed using the following primers (for cDNA fragment and T7-cDNA, respectively):

\textit{Act5C-f}, ATGTTGACGAAAGAGGTGCTG; TAA TACGACTCAGATAAGGGTGAGAAAGTTGCTG

\textit{Act5C-r}, AAGCCTCCATTTCCCAAGAACG; TAA TACGACTCAGATAAGGGTGAGAAAGTTGCTG

\textit{Itp-83A-f}, CCTCAAGGTTTTGCTCATCG; TAA TACGACTCAGATAAGGGTGAGAAAGTTGCTG

\textit{Itp-83A-r}, CCTCAAGGTTTTGCTCATCG; TAA TACGACTCAGATAAGGGTGAGAAAGTTGCTG
Fig. 1 Identification and validation of genes that regulate GBP/PLC-mediated Ca\textsuperscript{2+} signaling. a Representative Ca\textsuperscript{2+}-signaling responses from Drosophila S3 cells in plate 116888 that were pre-treated with either control dsRNAi (traces ‘a’ and ‘c’) or our dsRNAi construct against Itp-r83A (trace ‘b’) before addition of either vehicle (trace ‘a’) or 500 nM GBP (traces ‘b’ and ‘c’). b Reproducibility of dsRNA replicates obtained from 23 plates assayed in duplicate. c Z-scores from all amplicons. d Effects upon [Ca\textsuperscript{2+}]\textsubscript{T} after treatment of cells with our dsRNA constructs against the indicated genes; n = 4; *p < 0.001
Results and discussion

The GBP/PLC signaling axis, which acts through a G-protein coupled receptor (GPCR), stimulates Ca\(^{2+}\) mobilization by a biphasic process; first, Ca\(^{2+}\) is released from the endoplasmic reticulum, which secondarily stimulates Ca\(^{2+}\) entry into the cell [5, 7]. To screen novel genes that regulate the entire Ca\(^{2+}\)-signaling process, we deployed a genome-wide dsRNA library (the DRSC/TRiP Functional Genomics Resources; https://fgr.hms.harvard.edu/). These dsRNAs were tested using a strain of Drosophila S3 cells that encode a fluorescent Ca\(^{2+}\) sensor, the GCaMP3 gene [5]. We screened all 66 library plates at least once, and obtained data for dsRNA knockdown of every gene in the screen (see "Methods"). A scatter-plot of those data that had replicates (see "Methods") indicate that most amplicons show good reproducibility (Fig. 1b). From the entire data set (Fig. 1c), we found that total Ca\(^{2+}\) mobilization ([Ca\(^{2+}\)]\(_T\)) was inhibited by 103 amplicons (Z score < −3; Table 1). A separate group of 80 amplicons (Table 2) increased [Ca\(^{2+}\)]\(_T\) (Z score > 3). All of these data for each individual dsRNA are available at: http://www.flyrnai.org/summary. These numbers of amplicons in the two categories were reduced to 82 and 61, respectively (Tables 1 and 2), after we filtered out housekeeping genes (see "Methods").

Any high throughput screen is susceptible to false positive and false-negative hits. We were concerned that plc21C and NorpA may have been false-negatives; neither of these genes were hits in our screen, yet being that they are orthologs of mammalian PLC-\(\beta\), one or both of these gene products was expected to mediate GBP-dependent, GPCR-coupled Ca\(^{2+}\) mobilization. Thus, we performed

### Table 2 Filtered list of gene knock-downs that increased GBP-mediated Ca\(^{2+}\) mobilization

| Flybase ID  | Gene name | Amplicon # | Mean Z-score |
|-------------|-----------|------------|--------------|
| FBgn0027084 | Aats-lys  | DRSC25618  | 3.62         |
| FBgn0000043 | Act42A    | DRSC04835  | 4.46         |
| FBgn0000044 | Act57B    | DRSC04042  | 5.98         |
| FBgn0000042 | Act5C     | DRSC17723; DRSC25024 | 7.32; 8.13 |
| FBgn0000045 | Act79B    | DRSC11604  | 3.69         |
| FBgn0087035 | AGO2      | DRSC10847; DRSC40785 | 4.89; 3.95 |
| FBgn0041188 | Atx2      | DRSC40785  | 4.40         |
| FBgn00000212 | brm      | DRSC11330; DRSC26226 | 4.33; 9.95 |
| FBgn0029856 | CG11700   | DRSC23384  | 3.67         |
| FBgn0035307 | CG12909   | DRSC27706  | 3.07         |
| FBgn0039601 | CG1523    | DRSC15033; DRSC26315 | 3.49; 3.10 |
| FBgn0035569 | CG15876   | DRSC08484  | 3.71         |
| FBgn0067622 | Lsm-4     | DRSC02845  | 3.44         |
| FBgn0032240 | CG17768   | DRSC02845  | 3.44         |
| FBgn0034325 | CG18539   | DRSC06766  | 3.12         |
| FBgn0034326 | CG18540   | DRSC06767  | 3.10         |
| FBgn0011824 | CG4038    | DRSC25331  | 3.79         |
| FBgn0036691 | CG5872    | DRSC11785  | 3.64         |
| FBgn0035872 | CG7185    | DRSC10781  | 3.49         |
| FBgn0259236 | comm3     | DRSC09995  | 3.24         |
| FBgn0011726 | tsr       | DRSC04718; DRSC40832 | 6.64; 6.60 |
| FBgn00035124 | ttm2     | DRSC08268  | 3.84         |
| FBgn0039530 | Tusp      | DRSC15838  | 3.17         |
| FBgn0023143 | Uba1      | DRSC07567  | 4.15         |
| FBgn00263697 | Uba3     | DRSC06377  | 3.27         |
| FBgn0035853 | UbcE2M    | DRSC10828  | 6.22         |

The genes listed are the ‘hits’ that increased GBP-mediated Ca\(^{2+}\) mobilization (i.e., Z-score < −3), after filtering of housekeeping genes (see “Methods”).

### Table 2 (continued)

| Flybase ID  | Gene name | Amplicon # | Mean Z-score |
|-------------|-----------|------------|--------------|
| FBgn0003392 | shi       | DRSC20373; DRSC29498 | 5.20; 5.03 |
| FBgn0019890 | Smg5      | DRSC03124  | 3.63         |
| FBgn0264357 | SNF4Agamma | DRSC16847; DRSC40676 | 4.95; 4.12 |
| FBgn0011715 | Smr1      | DRSC12369  | 6.12         |
| FBgn0034175 | ste24b    | DRSC07315  | 3.24         |
| FBgn0033902 | Tango7    | DRSC07142; DRSC26908 | 3.95; 5.70 |
| FBgn0024921 | Trn       | DRSC25104  | 4.88         |
| FBgn0011726 | tsr       | DRSC04718; DRSC40832 | 6.64; 6.60 |
| FBgn0035124 | ttm2      | DRSC08268  | 3.84         |
| FBgn0039530 | Tusp      | DRSC15838  | 3.17         |
| FBgn0023143 | Uba1      | DRSC07567  | 4.15         |
| FBgn00263697 | Uba3     | DRSC06377  | 3.27         |
| FBgn0035853 | UbcE2M    | DRSC10828  | 6.22         |

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We screened all 66 library plates at least once, and obtained data for dsRNA knockdown of every gene in the screen (see “Methods”). A scatter-plot of those data that had replicates (see “Methods”) indicate that most amplicons show good reproducibility (Fig. 1b). From the entire data set (Fig. 1c), we found that total Ca\(^{2+}\) mobilization ([Ca\(^{2+}\)]\(_T\)) was inhibited by 103 amplicons (Z score < −3; Table 1). A separate group of 80 amplicons (Table 2) increased [Ca\(^{2+}\)]\(_T\) (Z score > 3). All of these data for each individual dsRNA are available at: http://www.flyrnai.org/screensummary. These numbers of amplicons in the two categories were reduced to 82 and 61, respectively (Tables 1 and 2), after we filtered out housekeeping genes (see “Methods”).

Any high throughput screen is susceptible to false positive and false-negative hits. We were concerned that plc21C and NorpA may have been false-negatives; neither of these genes were hits in our screen, yet being that they are orthologs of mammalian PLC-\(\beta\), one or both of these gene products was expected to mediate GBP-dependent, GPCR-coupled Ca\(^{2+}\) mobilization. Thus, we performed
additional validation assays using our own, unique dsRNA constructs. Both plc21C and NorpA were hits in these follow-up assays (Fig. 1d); knock-down of either significantly reduced Ca\(^{2+}\) mobilization. The simultaneous knockdown of both genes elicited an approximately additive effect (Fig. 1d). These data suggest partial functional redundancy of the two PLC-\(\beta\) genes, which can account for their being false negatives in a dsRNA screen. As mentioned above, we separated our hits into two groups, based on whether \([Ca^{2+}]_T\) was either decreased (Table 1) or increased (Table 2). We selected representatives from each group for validation. We used our unique dsRNAs at an early stage of this project to validate that knockdown of the \(Itp-r83A\) reduced \([Ca^{2+}]_T\) (see “Methods,” and Figs. 1a, d); we subsequently deployed \(Itp-r83A\) as a positive control to interrogate dsRNA plate integrity (see “Methods”). A second hit, \(Mthl10\) (Table 1), was also validated in secondary assays with our own, independent dsRNAs (Fig. 1d).

Among hits that elevate \([Ca^{2+}]_T\) (Table 2), we selected two—\(Tsr\) and \(Act5C\)—for further testing with our independent dsRNAs; in both cases, we confirmed that knockdown of either gene significantly increased \([Ca^{2+}]_T\). These data indicate that both of these genes normally constrain \([Ca^{2+}]_T\); it should be interesting to study further the biological significance of such a phenomenon.

Another aspect of our data that is of interest is the determination that GBP-dependent Ca\(^{2+}\) signaling is regulated by a family of genes that encode proteins that are components of the proteosome (PSMA2, PSMB6, PSMD6, Rpn11/PSMD14; Tables 1, 2). This multiprotein complex can regulate PLC/GPCR signaling by controlling the cell-surface levels of the receptor [10]; our data highlight the likely participation of the proteosome in regulating the activity of the GBP/Mthl10 signaling axis. Another hit, \(Gqo\) (Table 1), is a subunit of a heterotrimeric G-protein that couples GPCRs to the activation of PLC-\(\beta\). These are all data that underscore the value of a systems-level approach to fully understanding all aspects of the Ca\(^{2+}\)-signaling process.

We propose that human orthologs of our complete list of filtered gene hits (Tables 1 and 2) are not only candidates for acting in molecular pathways that interconnect aging and inflammation, but also potential new modulators of Ca\(^{2+}\) signaling in general. Thus, our data may drive several new, future research directions.

**Limitations**

A limitation in this study—as is the case for all high throughput screening exercises—is the possibility of false positives and false negatives. The possibility that gene redundancy may lead to false negatives is highlighted by Fig. 1d. In a genome-wide study such as this, it is not feasible to validate every hit, so false positives remain a possibility for future studies that pursue our data.

**Abbreviations**

[Ca\(^{2+}\)]; total calcium mobilization; GBP: growth-blocking peptide; GPCR: G-protein coupled receptor; PLC: phospholipase C.

**Authors’ contributions**

SBS designed the study, EJS performed experiments and analyzed data. EJS and SBS wrote the manuscript. Both authors read and approved the final manuscript.

**Acknowledgements**

We thank Drs. Gary Bird and Yoichi Hayakawa for helpful advice and discussions.

**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and materials**

The data supporting our findings are either included in the current study, or are publically accessible at the DRSC Functional Genomics Resources repository (http://www.flyrnai.org/screensummary); the unique identifier associated with that dataset is the Pubmed ID for the current study.

**Consent to publish**

Not applicable.

**Ethics approval and consent to participate**

Not applicable.

**Funding**

This research was supported by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences (ES080046-28). The DRSC Functional Genomics Resources (Harvard Medical School) is supported by NIH Funding NIGMS R01 GM067761. Neither of these funding bodies played any role in the design of the study, the collection, analysis and interpretation of the data, and the writing of the manuscript.

**Publisher’s Note**

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Received** 1 November 2018  **Accepted** 6 December 2018

**Published online** 13 December 2018

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