Systematic Screen for Drosophila Transcriptional Regulators Phosphorylated in Response to Insulin/mTOR Pathway

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ABSTRACT

Insulin/insulin-like growth factor signaling (IIS) is a conserved mechanism to regulate animal physiology in response to nutrition. IIS activity controls gene expression, but only a subset of transcriptional regulators (TRs) targeted by the IIS pathway is currently known. Here we report the results of an unbiased screen for Drosophila TRs phosphorylated in an IIS-dependent manner. To conduct the screen, we built a library of 857 V5/Strep-tagged TRs under the control of Copper-inducible metallothionein promoter (pMt). The insulin-induced phosphorylation changes were detected by using Phos-tag SDS-PAGE and Western blotting. Eight proteins were found to display increased phosphorylation after acute insulin treatment. In each case, the insulin-induced phosphorylation was abrogated by mTORC1 inhibitor rapamycin. The hits included two components of the NURF complex (NURF38 and NURF55), bHLHZip transcription factor Max, as well as the Drosophila ortholog of human proliferation-associated 2G4 (dPA2G4). Subsequent experiments revealed that the expression of the dPA2G4 gene was promoted by the mTOR pathway, likely through transcription factor Myc. Furthermore, NURF38 was found to be necessary for growth in larvae, consistent with the role of IIS/mTOR pathway in growth control.

KEYWORDS

phosphorylation insulin transcription growth mTOR

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Several aspects of animal physiology, such as tissue growth, metabolism, reproduction, stem cell function and longevity, are regulated in response to nutrients. The insulin/insulin-like growth factor signaling (IIS) pathway is a central regulator of these processes in multicellular animals (Hietakangas and Cohen 2009; Teleman 2009; Partridge et al. 2011). Deregulation of IIS is the underlying cause of human diseases, such as diabetes and many types of cancer (Renehan et al. 2006; Guo 2014). Due to the conserved nature of IIS, Drosophila melanogaster serves as an important genetically tractable model system for the discovery of novel pathway components and targets as well as their physiological roles (Hietakangas and Cohen 2009; Teleman 2009).

IIS regulates cellular functions at different levels, including gene expression. The best-established transcription factor target of Drosophila IIS is the Forkhead transcription factor ‘O’ (FoxO), which is phosphorylated by protein kinase AKT, leading to inhibition of FoxO function through cytoplasmic retention (Puig et al. 2003; Hietakangas and Cohen 2007). Upon low IIS, FoxO promotes the transcription of growth inhibitory genes, such as 4EBP and sestrin (Puig et al. 2003; Lee et al. 2010). IIS also regulates the phosphorylation of transcriptional cofactors. Phosphorylation of CREB coactivator TORC/CRTC is increased upon insulin treatment through salt-inducible kinase 2 (SIK2), leading to the inhibition of TORC/CRTC activity (Wang et al. 2008). This pathway controls starvation resistance and lipid metabolism in adult flies through the central nervous system. A paralog of SIK2, SIK3, phosphorylates histone deacetylase 4 (HDAC4) in response to insulin, thereby inhibiting its deacetylase activity on FoxO (Wang et al. 2011). Upon low insulin signaling, HDAC4-mediated deacetylation promotes FoxO activity increasing lipolysis through the Brummer lipase (Wang et al. 2011).

The mechanistic target of rapamycin (mTOR) pathway is another important regulator of nutrient-responsive cell physiology.
The mTOR pathway integrates several nutrient derived signals, including IIS activity, which promotes mTOR activity (Hietakangas and Cohen 2009). mTOR complex 1 (mTORC1) is an activator of anabolic pathways, such as ribosome biogenesis through all three RNA polymerases (Wullschleger et al. 2006). In Drosophila, mTORC1 is known to promote the expression of ribosome assembly genes through transcription factor Myc, albeit the molecular mechanism remains poorly understood (Guerin et al. 2006; Teleman et al. 2008). mTOR also promotes the activities of RNA Pol I and Pol III, which transcribe ribosomal RNAs (rRNAs) and other non-coding RNAs needed for gene expression (Grewal et al. 2007; Marshall et al. 2012), but the phosphorylation targets of mTOR in this context have remained insufficiently characterized. One such target is chromatin binding protein PWP1, which is phosphorylated in an mTORC1-dependent manner and is necessary for promoting the transcription of rRNAs by RNA Pol I and Pol III (Liu et al., 2018, 2017). TORC1 also phosphorylates transcription factor Reptor, leading to its cytoplasmic retention (Tiebe et al. 2015). Upon mTORC1 inhibition by rapamycin, Reptor and its heterodimerization partner Reptor-BP activate gene transcription, controlling the majority of rapamycin-activated genes in Drosophila S2 cells.

While several TRs have been identified as phosphorylation targets for IIS/mTOR signaling, comprehensive understanding of the mediators of IIS/mTOR-dependent transcriptional control has not been achieved. Quantitative phosphoproteomics is the state-of-the-art approach for unbiased identification of phosphorylated proteins. Such approach was used to identify 191 proteins, whose phosphorylation changed upon insulin treatment of Drosophila S2R+ cells (Vinayagam et al. 2016). However, only few transcriptional regulators, such as Jun-related antigen, Modulo and Myb, were among the identified proteins (Vinayagam et al. 2016). Therefore, complementary approaches to detect phosphorylation changes in TRs are necessary. Phos-tag SDS-PAGE has emerged as a robust method to separate phosphorylated forms of proteins based on their reduced electrophoretic mobility (Kinoshita et al. 2006, 2015). When combined with ectopic expression of proteins of interest, it is possible to overcome the technical limitations posed by low endogenous expression levels. Here we have generated a library of constructs that allow Copper-inducible ectopic expression of 857 Strep/V5-tagged Drosophila TRs. Subsequent Phos-tag SDS-PAGE screen was used to identify targets of IIS/mTORC1 signaling.

**MATERIALS AND METHODS**

**Plasmids, cell culture and transfection**

*Drosophila* TRs were cloned into modified pMt-V5-HisA (Invitrogen) vectors by ligase independent cloning (Aslanidis and de Jong 1990). A C-terminal strepIII tag was inserted to the vector backbone, together with a hygromycin resistance gene. The library cDNAs were amplified from the *Drosophila* Genomics Resource Center (DGRC) gold collection library when available. When not in the DGRC collection, TR cDNAs were amplified from 5’RACE (Invitrogen) cDNA library made from adult female flies. Cloning was verified by digestion, the constructs of hits were confirmed by sequencing. *Drosophila* S2 cells were cultured in M3 medium (Sigma) supplemented with 1X Insect Medium Supplement (Sigma), 2% fetal bovine serum (LifeTechnologies) and penicillin/streptomycin (LifeTechnologies). Transfections were performed with Effectene Transfection Reagent kit (Qiagen) according to the manufacturer’s instructions. To analyze phosphorylation, S2 cells were incubated 10 min with...
10 μg/ml insulin (Sigma, 16634) alone, or after inhibition for 2 hr with 1 μM Rapamycin (Sigma, 37094) (Liu et al. 2017).

**Phos-tag Western blotting**
Phos-tag SDS-PAGE and Western blotting were performed according to previous studies (Liu et al. 2017). In brief, Phos-tag SDS-PAGE gels were made adding 30 μM Phos-tag reagent according to the manufacturer’s protocol (Wako Chemical). Samples were resolved on SDS-PAGE with Phos-tag and detected via Western blotting using mouse anti-V5 (Life Technologies, R960-25) and IRDye 680 goat anti-mouse (LI-COR, 926-32220). Bands were quantified using Image studio lite software (LI-COR). All bands that migrate slower than the most intensive band were considered as putative phosphorylated forms. A small subset of these bands displayed insulin/rapamycin responsiveness.

**Drosophila genetics**
Flies were grown at 25°C, on medium containing agar 0.6% (w/v), malt 6.5% (w/v), semolina 3.2% (w/v), baker’s yeast 1.8% (w/v), nipagin 2.4%, propionic acid 0.7%. In starvation experiments, larvae were kept on medium contain agar 0.5% (w/v), nipagin 2.5%, and propionic acid 0.7% in PBS, supplemented with or without 5% sucrose (w/v). Fly stocks used in this study were: Tub-Gal4 (Lee et al. 1999), Ey-Gal4 (BDSC 5534), w1118 (BDSC 6326), NURF38 RNAi (BDSC 31341), UAS-Rheb (BDSC, 9688), mTOR delP mutant (BDSC 7014), myc dm2 mutant (Maines et al. 2004), UAS-Myc (Johnston et al. 1999), UAS-Myc RNAi (VDRC 2947).

**Quantitative RT-PCR**
RNA was extracted using a Nucleospin RNA kit (Macherey-Nagel). cDNA was synthesized using SensiFAST cDNA Synthesis kit.
For this purpose, we constructed an epitope tagged downstream of IIS through a cell-based phosphorylation screen. This study aimed to identify novel transcriptional regulators (TRs) transcriptional regulators

RESULTS

Available at Bioline according to the manufacturer’s protocol. qPCR was performed with a Light cycler 480 Real-Time PCR System (Roche) using SensiFAST SYBR No-ROX qPCR Master Mix (Bioline). The primers used in this research are listed below:

CDK7-F: GGGTCAAGTTTGCCGAGGTTT
CDK7-R: GATCCACTCCAGATCCTGT
NURF38-F: GGCGCCCGTGGAGCAAGCGG
NURF38-R: GTAGCCCTTGTGCGGGAAGCAG
NURF55-F: GACTCTCTTTGGAATCCCAACCTC
NURF55-R: GGACTCTCTTGGAATCCCAACCTC
CDK7-R: GATCACCTCCAGATCCGTG
CDK7-F: GGGTCAGTTTGCCACAGTTT

NURF55-R: GGCCGGTGAAGATGTTCTTGGC
NURF38-F: GTAGCCCTTGTGCGGGAAGCAG
NURF38-R: GTGCCACGTTGGACCAACGCG

Drosophila melanogaster wandering third instar larvae) displayed a significant enrichment of Myc chromatin binding close to dPA2G4 transcriptional start site. *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t-test. Error bars indicate SDs.

Quantification and statistical analysis

Student’s t-tests were performed for statistical analyses. n indicates the number of biological replicates and is detailed in the figure legends. Error bars indicate standard deviation.

Data availability

Plasmids are available upon request. Information of the plasmid library is listed in Supplemental table 1. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. Supplemental material available at figshare: https://doi.org/10.25387/g3.12498020.

RESULTS

Construction of the epitope tagged library of Drosophila transcriptional regulators

This study aimed to identify novel transcriptional regulators (TRs) downstream of IIS through a cell-based phosphorylation screen. For this purpose, we constructed an epitope tagged Drosophila TR library, based on the annotation by Harvard medical school Drosophila RNAi Screening Center (DRSC) ‘Transcription Factors and Related’ RNAi Sub-Library (TRXN) (https://fgg.hms.harvard.edu/drs-focused-sub-libraries), TRXN includes a set of 963 Drosophila genes which are annotated as transcription factor, nuclear protein, and DNA binding protein. The proteins in the library include DNA binding factors, DNA remodeling factors, and histone modifying enzymes along with their associated proteins. Ligase-independent cloning (LIC) was performed to clone these genes into a modified pMti-Strep-V5-HisA expression vector (Figure 1AB). This modified vector was made by inserting a C-terminal Strep-tag and a hygromycin resistance gene to the backbone of the pMt-V5-HisA vector (Figure 1AB). The pMt promoter allows Copper-inducible expression of these TRs in cells. Strep-tag is optimal for immobilization and pulldown and V5 is a commonly used epitope tag for antibody detection (e.g., Western blot and immunofluorescence). In total 857 Drosophila genes were successfully included in our library, which provides 89% coverage of the TRXN gene set (Figure 1C).

Screen for TRs downstream of IIS

Next, we wanted to identify novel TRs regulated by insulin-dependent phosphorylation. To detect changes in phosphorylation status, we utilized the mobility shift of phosphorylated proteins on Phos-tag SDS-PAGE (Kinoshita et al. 2006, 2015). Individual TRs were expressed in S2 cells, treated with insulin, or left untreated. Cell lysates resolved on Phos-tag SDS-PAGE were immunoblotted using anti-V5 antibodies (Figure 2A). We were able to detect the expression of 61% of the TRs (504/857) (Figure 2B). In total eight TRs were identified to display increased intensity of a slow migrating band upon insulin treatment, consistent with increased phosphorylation (Figure 2C, Table 1). FoxO was used as a positive control. One of the hits was PWP1, reported earlier (Liu et al., 2017). The Phos-tag
SDS-PAGE approach was validated by phosphatase treatment of PWP1, which fully eliminated the insulin-responsive slow migrating bands (Liu et al., 2017). mTOR pathway is a downstream effector of IIS (Hietakangas and Cohen 2009). Hence, we tested whether mTOR pathway is involved in regulating these TRs by using rapamycin, an inhibitor of mTORC1. Indeed, rapamycin treatment fully inhibited the insulin-induced slow migrating band in the case of all TRs (Figure 2D). Interestingly, several of the identified TRs have been reported to control growth in Drosophila (Table 1).

dPA2G4 expression is controlled by mTORC1 and Myc

One of the hits was CG10576, which encodes the Drosophila ortholog of human proliferation-associated 2G4 (PA2G4) and will be referred to as Drosophila proliferation-associated 2G4 (dPA2G4). Downstream targets of nutrient sensing pathways are often regulated transcriptionally in parallel to posttranslational regulation. For example, our previous data showed that PWP1 is regulated by nutrients and IIS/mTOR pathway through both phosphorylation and gene expression (Liu et al., 2017). Thus, we wanted to test, whether dPA2G4 gene expression is also nutrient regulated in vivo. Indeed, dPA2G4 mRNA levels were downregulated in fasting larvae, while being prominently elevated upon re-feeding with protein-rich yeast diet, but not similarly induced by sugar-only diet (Figure 3A). Next, we asked whether this expression regulation is dependent on the mTOR pathway. Indeed, the protein-induced expression of dPA2G4 was blunted in mTOR mutant larvae (Figure 3B). Moreover, activation of mTORC1 pathway by overexpressing Ras homolog enriched in brain (Rheb) promoted dPA2G4 expression (Figure 3C). These data suggest dPA2G4 expression is controlled through the mTORC1 pathway.

The transcription factor Myc is an established downstream effector of the IIS/mTORC1 pathway (Teleman et al. 2008). To address whether Myc is responsible for the transcriptional regulation of dPA2G4, we analyzed dPA2G4 expression levels in larvae following myc loss- and gain-of-function. dPA2G4 expression was significantly upregulated upon Myc overexpression and reduced upon depletion of Myc (Figure 3D). In addition, induction of dPA2G4 mRNA expression by protein-rich diet was partially prevented in myc mutant larvae (Figure 3E), implying that Myc contributes to nutrient-responsive expression of dPA2G4. Moreover, in silico analysis of modENCODE ChIP-seq data (ENCSR191VCQ) in wandering 3rd instar larvae revealed a significant enrichment of Myc chromatin binding close to the transcriptional start site of dPA2G4 (Figure 3F). In conclusion, our data indicates that transcription of dPA2G4 is promoted by mTORC1 pathway through transcription factor Myc in response to protein-rich diet.

NURF38 is necessary for animal growth

Our Phos-tag data revealed that two subunits of the ATP-dependent chromatin remodeling complex NURF, NURF38 and NURF55 (Cafl-55), are phosphorylated upon insulin treatment, in mTORC1-dependent manner (Figure 2CD). Similar to dPA2G4, mRNA levels of NURF38 were significantly increased in response to re-feeding with a protein rich diet, and this elevated expression was partially blunted in mTOR mutant larvae (Figure 4A). The expression of NURF55 was also elevated upon yeast re-feeding, larvae and pupae. F. Representative images of control (w1118) and NURF38 eye (Ey-Gal4)-depleted adult eye. *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t-test. Error bars indicate SDs.
but this induction was insensitive to loss of mTOR (Figure 4B). In contrast to dPA2G4, NURF38 and NURF55 expression levels were not affected in myc mutants (Figure 4 CD). Thus, the NURF38 expression is regulated in response to nutrients through mTOR pathway, but is independent of Myc.

A previous study has shown that Drosophila mutants of NURF55 display a strong undergrowth phenotype (Wen et al. 2012). To explore the possible role of NURF38 in growth regulation, we depleted NURF38 ubiquitously in Drosophila larvae by Tub-GAL4 > NURF38 RNAi. The NURF38 depleted larvae remained viable for several days, but failed to increase in size, consistent with impaired tissue growth (Figure 4E). Moreover, eye-specific depletion of NURF38 using Ey-GAL4 led to strongly reduced eye size, suggesting a tissue autonomous role for NURF38-dependent growth regulation (Figure 4F).

### DISCUSSION

Animals coordinate their growth rate and metabolism in a changing nutrient landscape, which requires dynamic regulation of gene expression. Therefore, it is important to understand the identity and roles of the transcriptional regulators (TRs) involved. Here, we identify several TRs with a putative role in nutrient-dependent gene regulation. Specifically, we (1) identify two components of the NURF complex, Max and dPA2G4 as phosphorylation targets of IIS/mTORC1 signaling in Drosophila S2 cells; (2) provide evidence that expression of dPA2G4 is regulated by IIS/mTORC1 signaling, likely through Myc; (3) identify NURF38 as a necessary gene for tissue growth in larvae; (4) construct a library of 857 V5/Strep-tagged TRs compatible for Copper-induced expression in Drosophila cells. Collectively, this study demonstrates the potential of Phos-tag SDS-PAGE for unbiased identification of new phosphorylation targets of signaling pathways. Thus, it can be used to complement other approaches, such as phosphoproteomics. Some technical limitations were also observed. >350 proteins remained undetected, including Reptor (a known target of mTORC1). This might be due to high protein turnover or cell lethality or caused by technical reasons, such as insufficient transfer of large proteins or poor transfection efficiency. It should be also noted that some of the genes included in the library might be misannotated as TRs, including some of the hits (e.g., CG33671/Mevalonate kinase).

The Drosophila nucleosome remodeling factor (NURF) complex is a conserved ATP-dependent chromatin remodeling complex with four subunits: NURF38, NURF55, NURF301 and ISWI (Becker and Hörm 2002; Corona and Tamkun 2004). The NURF complex was initially discovered as a regulator of heat shock gene expression, a key mechanism to maintain cellular proteostasis (Tsukiyama and Wu 1995). Moreover, NURF55, one of the discovered IIS/mTOR phosphorylation targets, has been shown to be necessary for larval growth (Wen et al. 2012) as well as for regulation of lipid droplet size in the larval fat body (Yao et al. 2018). We found that another component of the NURF complex, NURF38, is necessary for larval growth along with its IIS/mTOR-dependent phosphorylation. Notably, however, mutants of NURF301 do not display a strong undergrowth phenotype, but an ecdysone-dependent pupariation phenotype (Badenhorst et al. 2005). Thus, it remains to be determined whether growth regulation by NURF38 and NURF55 is mediated through their function in the NURF complex and how they contribute to the gene regulatory effects of IIS/mTOR signaling.

Ribosome biogenesis is a key process in regulating cellular growth capacity and is tightly coordinated by the IIS/mTOR pathway. Ribosome biogenesis involves all three RNA polymerases and is likely regulated through multiple effectors. Myc is known to control ribosome biogenesis downstream of mTOR signaling, as inhibition of mTORC1 leads to downregulation of Myc protein levels (Telemann et al. 2008). However, the mechanistic details of how mTORC1 signaling regulates Myc have remained unresolved. Interestingly, our screen identified the Myc heterodimerization partner Max as a phosphorylation target by mTORC1. Earlier studies in mammals have shown that Max is a phosphoprotein, constitutively phosphorylated by CKII on N-terminal serines S2 and S11 (Berberich and Cole 1992; Koskinen et al. 1994). Future studies will be needed to address whether Max phosphorylation contributes to the mTORC1-dependent regulation of Myc-Max target genes in Drosophila. Another downstream target of mTORC1 signaling is PWP1, which is phosphorylated in an mTORC1-dependent manner and necessary for RNA Pol I and III-dependent expression of ribosomal RNA (Liu et al. 2017, 2018). In addition to phosphorylation, PWP1 gene expression is promoted by IIS/mTORC1 signaling through Myc transcription factor (Liu et al. 2017). Interestingly, we demonstrated here that dPA2G4 is under similar dual regulation by phosphorylation and gene expression. In mammalian cells, PA2G4 has been found to localize to the nucleolus, bind to Pol I, and promote rRNA expression (Karlsson et al. 2016; Nguyen et al. 2016). It can be therefore hypothesized that PA2G4 is a candidate to cooperate with PWP1 in regulating nutrient-responsive ribosome biogenesis in vivo.

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