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Definition of a RACK1 Interaction Network in *Drosophila melanogaster* Using SWATH-MS

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ABSTRACT Receptor for Activated protein C kinase 1 (RACK1) is a scaffold protein that has been found in association with several signaling complexes, and with the 40S subunit of the ribosome. Using the model organism *Drosophila melanogaster*, we recently showed that RACK1 is required at the ribosome for internal ribosome entry site (IRES)-mediated translation of viruses. Here, we report a proteomic characterization of the interactome of RACK1 in *Drosophila* S2 cells. We carried out Label-Free quantitation using both Data-Dependent and Data-Independent Acquisition (DDA and DIA, respectively) and observed a significant advantage for the Sequential Window Acquisition of all Theoretical fragment-ion spectra (SWATH) method, both in terms of identification of interactants and quantification of low abundance proteins. These data represent the first SWATH spectral library available for *Drosophila* and will be a useful resource for the community. A total of 52 interacting proteins were identified, including several molecules involved in translation such as structural components of the ribosome, factors regulating translation initiation or elongation, and RNA binding proteins. Among these 52 proteins, 15 were identified as partners by the SWATH strategy only. Interestingly, these 15 proteins are significantly enriched for the functions translation and nucleic acid binding. This enrichment reflects the engagement of RACK1 at the ribosome and highlights the added value of SWATH analysis. A functional screen did not reveal any protein sharing the interesting properties of RACK1, which is required for IRES-dependent translation and not essential for cell viability. Intriguingly however, 10 of the RACK1 partners identified restrict replication of Cricket paralysis virus (CrPV), an IRES-containing virus.

KEYWORDS

Drosophila melanogaster
mass spectrometry
translation
RACK1
ribosome
virus
IRES
Lark
AGO2

Infectious diseases represent a major cause of death for animals, including humans. Among them, viral infections are particularly hard to

treat because viruses replicate inside host cells. Many cellular proteins are hijacked by viruses to complete their replication cycle and represent putative targets for host-targeted antiviral drugs. Using the model organism *Drosophila melanogaster*, we recently showed that RACK1 is an essential host factor for the replication of fly and human viruses (Majzoub *et al.* 2014). More specifically, we demonstrated that RACK1, a component of the 40S subunit of the ribosome, is required for translation driven by the 5' IRES element of two members of the Dicistroviridae family in flies, *Drosophila C virus* (DCV) and CrPV. Related to Picornaviridae, these viruses are used as models to decipher the genetic basis of host–virus interactions in flies. Importantly, RACK1 is also essential for translation driven by the IRES of human hepatitis C virus in human hepatocytes. By contrast, RACK1 is not required for general 5' cap-dependent translation, indicating that this factor regulates selective translation at the level of the ribosome (Majzoub *et al.* 2014). Thus, RACK1 could be used as target for the development of new host-targeted antiviral drugs (Martins *et al.* 2016). The ribosomal proteins RpS25 (Landry *et al.* 2009), RpL40 (Lee *et al.* 2013), and RpL38 (Kondrashov *et al.* 2011) are also required for selective translation, bringing support for the

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existence of a ribosomal code (Mauro and Edelman 2002; Topisirovic and Sonenberg 2011; Barna 2015).

RACK1 is a 36 kDa protein containing seven WD40 β -propeller domains, evolutionarily conserved throughout eukaryotes (Wang *et al.* 2003; Kadrmas *et al.* 2007). RACK1 was also identified as an interacting partner of many proteins, including kinases, phosphatases, and adhesion molecules, suggesting that it functions as a scaffold protein (Gibson 2012; Long *et al.* 2014; Li and Xie 2015). Of note, we identified RACK1 as a factor pulled down with Argonaute (AGO) 2, a key component of the *Drosophila* antiviral RNA interference (RNAi) pathway, in virus-infected cells (Majzoub *et al.* 2014). Independent studies confirmed that RACK1 can interact with components of the RISC complex and impacts microRNA (miRNA) function (Jannot *et al.* 2011; Speth *et al.* 2013). In summary, RACK1 appears to be the central node of a molecular hub at the interface of the ribosome and signaling complexes. Hence, a comprehensive characterization of the RACK1 interactome is of central importance to gain insight into the function of this molecule.

Affinity purification followed by mass spectrometry (AP-MS) is a popular strategy for identifying interactions between an affinity-purified bait and its copurifying partners (Rinner *et al.* 2007; Gingras *et al.* 2007; Wepf *et al.* 2009; Collins *et al.* 2013; Lambert *et al.* 2013). This approach is particularly appreciated because experiments can be performed under near physiological conditions and because dynamic changes can be assessed by quantitative techniques operated under DDAs, with or without labeling strategies (Gavin *et al.* 2006, 2011; Krogan *et al.* 2006; Kühner *et al.* 2009). In the past few years, targeted proteomics as well as techniques derived from DIAs, such as sequential windowed acquisition termed MS/MS^{ALL} with SWATH acquisition (Gillet *et al.* 2012), have emerged as a complement to these more widely used discovery proteomic methods. DIA results in comprehensive high resolution data with qualitative confirmation and no tedious method development (Bisson *et al.* 2011; Chang *et al.* 2012; Picotti and Aebersold 2012; Picotti *et al.* 2013; Selevsek *et al.* 2015). Moreover, one can acquire useful information for all analytes in a single run, thus enabling retrospective *in silico* interrogation to explore unexpected biological pathways for example (Gillet *et al.* 2012). Here, we applied these techniques to define the RACK1 interactome in tissue culture *Drosophila* S2 cells infected or not by the dicistrovirus CrPV.

MATERIALS AND METHODS

Cell culture and immunoaffinity purification

Drosophila S2 cells were grown in Schneider medium complemented with 10% fetal bovine serum, 1% glutamax, and 1% Penicillin/Streptomycin. RACK1 immunoprecipitation (IP) was performed after the transient transfection (Effectene, QIAGEN) of RACK1 tagged with the 3xHA or 3xFLAG versions in 30 million cells in triplicate. Cells were either mock-infected or infected with DCV or CrPV at multiplicity of infection (MOI) 1 for 16 hr. Protein purification and identification was performed as previously described (Fukuyama *et al.* 2013). Next, 1 ml of TNT lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% Glycerol, 1% Triton X-100, 100 mM NaF, 5 μ M ZnCl₂, 1 mM Na₃VO₄, 10 mM EGTA pH 8.0, and Complete Protease Inhibitor Cocktail containing EDTA from Roche) was used and kept on ice for 30 min before centrifugation at 13,000 rpm for 30 min at 4°. Supernatants were mixed with 150 μ l of either prewashed anti-DYKDDDDK (Clontech #635686) or anti-HA (Sigma #A2095) beads and incubated for 1 hr at 4°. Beads were washed three times with 1 ml wash buffer I (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% Glycerol, 0.1% Triton X-100, 100 mM NaF, 5 μ M ZnCl₂, 1 mM Na₃VO₄, and 10 mM EGTA pH 8.0), one time

with 1 ml wash buffer II (wash buffer I without Triton X-100), and suspended in 1 ml wash buffer II plus Complete Protease Inhibitor Cocktail containing EDTA. The elution was performed with Laemmli 1 \times buffer. Eluates from RACK1 and control cell lines were separated by SDS-PAGE: a precast gradient 4–12% acrylamide gel was used followed by Coomassie Blue staining. Each gel lane was cut into 48 consecutive bands, with the exception of the two bands containing the light and heavy chains of immunoglobulins, and submitted to proteomic analysis.

Label-free quantification using DDAs and DIAs

A Spectral Counting (SpC) strategy was carried out using the Mascot identification results and Proteinscape 3.1 package. A total number of MS/MS spectra (including modified and shared peptides) was attributed to each protein in each of the 18 conditions. The partner quality was positively assessed if Ratio(RACK-Cter/Control) > 2 and/or Ratio(RACK-Nter/Control) > 2. The MS1 label-free strategy was carried out using the PeakView v1.2 and MarkerView v1.2 software from Sciex. Resulting tables were then submitted to a Student's *t*-test: peptides and proteins validated with a *P*-value < 0.05 were considered statistically significant. The SWATH strategy was carried out using an AB Sciex informatics package to extract the quantitative information from the files acquired in Data-Independent mode (MS/MS^{ALL} with SWATH acquisition). The Paragon results file (group) was imported into PeakView v1.2 to create an experimental in-house *Drosophila* spectral library. Data were further evaluated in MarkerView using a Principal Component Analysis (PCA) (Pareto) and a Student's *t*-test. The same significance criteria were applied to the ion, peptide, and protein tables. More detailed presentation of the mass spectrometry data analysis can be found in the supplemental information (File S1).

Functional classification and network analysis of RACK1 identified partners

Gene Ontology (GO) annotations were retrieved from the PANTHER classification system (v10.0 Released 2015-05-15) with the following parameters: (i) Enter IDs: UniProtKB accession numbers; (ii) Organism: *D. melanogaster*; and (iii) Analysis: functional classification viewed in pie chart. GO enrichment analysis was performed using the same classification system with the following parameters: (i) Enter IDs: UniProtKB accession numbers; (ii) Organism: *D. melanogaster*; and (iii) Analysis: statistical overrepresentation test release 20160302. The network of RACK1-interacting proteins was further constructed by STRING (<http://string-db.org/v10.0>) while considering the following active interaction sources: "Coexpression," "Databases," "Experiments," and "Textmining."

RNAi screen and RT-qPCR

Target genes were amplified by PCR with specific primers containing the T7 RNA polymerase binding site in their 5'-end. After PCR product purification by GE Illustra GFX PCR DNA purification kit and verification on agarose gels for correct sizes, 1 μ g of DNA template was used to generate dsRNA with the MEGAscript T7 Ambion kit. After overnight incubation, dsRNA was precipitated with 0.3 M NaAc and absolute ethanol and resuspended in nuclease-free water. Then, 3 μ g of dsRNA was mixed with 2 \times 10⁴ S2 cells in serum-free medium for 2–3 hr in 96-well plates, allowing the penetration of dsRNA into the cells. Four replicates of the same dsRNA were tested. Afterward, complete medium was added. After 1 wk incubation, cells were infected for 1 d with DCV (MOI 1) and CrPV (MOI 0.1). Cell lysis, retrotranscription, and qPCR against the target virus genome were performed using

the Cell-To-Ct Ambion kit. Cells were lysed in 50 μ l lysis buffer for 5 min. Reverse transcription was performed on 10 μ l lysate in SYBR RT buffer and enzyme mix in a final volume of 50 μ l. Quantitative PCR on 4 μ l cDNA samples was done in 20 μ l final volumes with 10 μ l SYBR Green power master mix and 0.5 mM of each primer. An unpaired two-tailed *t*-test was then performed, comparing control dsRNA against GFP with all tested dsRNA. At least three independent biological replicates were performed for each experiment. All primers used are presented in the supplemental information.

Cell viability upon dsRNA treatment was tested with CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) reagent (Promega) or assessed on the genome RNAi database (<http://www.genomernai.org>).

Luciferase assay

Drosophila S2 cells (Invitrogen) were soaked with dsRNA. Four days later, reporter plasmids (CrPV5' IRES-Renilla and Cap-Firefly) were transfected using an Effectene kit (QIAGEN). After 48 hr, cells were lysed and luciferase activity was measured with the Promega dual-luciferase assay, using a Berthold Luminometer.

Data availability

Datasets have been deposited to the ProteomeXchange Consortium with identifiers PXD002965 (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository.

RESULTS

Identification of 37 RACK1-interacting proteins using data-dependent acquisition

In order to define the RACK1 interactome in *D. melanogaster*, N- or C-terminal FLAG-tagged RACK1 was transiently expressed in *Drosophila* S2 cells, in mock- or virus-infected conditions (Figure 1). A vector expressing RACK1 with a hemagglutinin (HA) tag was used as control, which is not recognized by the anti-FLAG antibody, so that cells expressing similar levels of RACK1 were compared. Biological triplicates were analyzed for each of the six samples. We first optimized the AP-MS protocol at three critical steps to improve specificity (type of tag, incubation time and salt concentration in the washing buffer, and type of virus, see Supplemental Material, in Figure S1 in File S1). We also ran a quality control sample in triplicate (500 ng of a trypsin-digested HeLa lysate) to ascertain the technical reproducibility of the MS instrument. As expected, the variability of the affinity purification replicates is higher than that of the technical replicates of injection (Table S1 in File S2). Purified complexes were eluted from the beads with Laemmli buffer and separated by SDS-PAGE. Proteins bands were in-gel digested with trypsin before being submitted to liquid chromatography MS analysis. DDA was used in the first instance to estimate relative changes between all conditions via SpC (Table S2 in File S2). After normalization, we calculated the ratio RACK1/control for the N- and C-terminally tagged protein in mock- and virus-infected cells, to assess the quality of the partners. A protein was considered as a RACK1 partner if it was enriched in the condition where RACK1 was overexpressed and pulled-down, using the following criterion: ratio (IP/Ctrl) > 2 and *P*-value < 0.05 (*t*-test). The *P*-values were not corrected by multiple testing in this initial step, in which the goal was to identify a list of putative partners for RACK1. This criterion identified 34 potential interacting proteins (Figure 2A), having either an “on/off” behavior or being enriched by a factor of ≥ 2 when RACK1 was pulled-down. The same DDA data were then submitted to an MS1 label-free analysis, using the vendor’s processing package and composed from PeakView

v1.2 and MarkerView v1.2 software (Sciex). This identified 19 RACK1 partners in either mock- or virus-infected samples (Figure 2, A and B and Table S2 in File S2). Of note, the average coefficient of variation (CV) of the 18 samples is 25% higher than the average CV of the nine noninfected samples. Altogether, close to 75% of the partners were identified with both tagged versions of RACK1. This highlights the overall good reproducibility and attests to the reliability of the approach, even if the position of the tag appears to influence the recovery of some partners, possibly reflecting their interaction with the extremities of RACK1.

SWATH-MS quantification reveals an additional 15 RACK1-interacting proteins

We next used the MS/MS spectra obtained with the DDA mode to build a spectral library to be used for 18 consecutive DIA injections. Up to 10 peptides per protein and 5 transitions per peptide were considered for SWATH-MS quantification leading a total of 3368 transitions. A careful adaptation of the retention time window reduced the sensitivity of peak picking interferences, as reflected by the very low chromatographic shift observed all along the separation (1.48 min). Each protein detected as being a RACK1 partner was manually inspected and validated or corrected (Figure S2 in File S1). As in the MS1 label-free quantification, the CV of the SWATH data decreases by 21% when only the nine noninfected samples are taken into account. The PCA analysis revealed a clear-cut difference between the control and the co-IP samples (Figure 2C). A total of 48 RACK1 partners were identified, which included 17 out of the 19 partners identified using the MS1 quantification method. This indicates that SWATH quantification is as reliable as the standard MS1 label-free approach, yet more sensitive (Figure 2A and Table S2 in File S2). The IP bait, RACK1, identified both by MS1 and SWATH, was enriched by an average factor of 27.7 with SWATH, which is significantly higher than with the MS1 quantification (average fold change of 8.4, Figure S3 in File S2). Most of the partners identified by SWATH (53.5%) were validated with both C- and N-terminally tagged constructions.

The selective requirement for RACK1 in IRES-dependent translation suggests that infection by an IRES-containing virus, such as CrPV, may involve an association with specific cofactors. However, our approach did not reveal specific factors recruited to RACK1 in the context of CrPV infections. As the infection can affect the post-translational status of RACK1 and its partners (*e.g.*, Valerius *et al.* 2007), an extended Mascot search was performed using an “Error Tolerant Search” strategy. This did not lead to the identification of novel interactants. Despite the fact that RACK1 is a phosphoprotein itself and that ubiquitination has been demonstrated for the orthologs in yeast and human cells (Starita *et al.* 2012; Yang *et al.* 2017), the only modifications we detected were: (i) the acetylation of the second residue (S2) with the loss of the initiation methionine, and (ii) deamidation on N24 and N52. Regarding the involvement of RACK1 in cell signaling, we did identify some signaling proteins, such as the serine/threonine kinase Polo, but we did not isolate the kinases previously reported to interact with RACK1, such as protein kinase C β (PKC β) or Src. We note that these proteins were also not detected in the RACK1 interactome in *Aedes albopictus* cells, in which the endogenous protein was pulled down (González-Calixto *et al.* 2015).

Characteristics of the RACK1 interactome in *Drosophila* S2 cells

The PANTHER classification system (<http://www.pantherdb.org>) was used to assess the GO annotations of the 52 different proteins retrieved

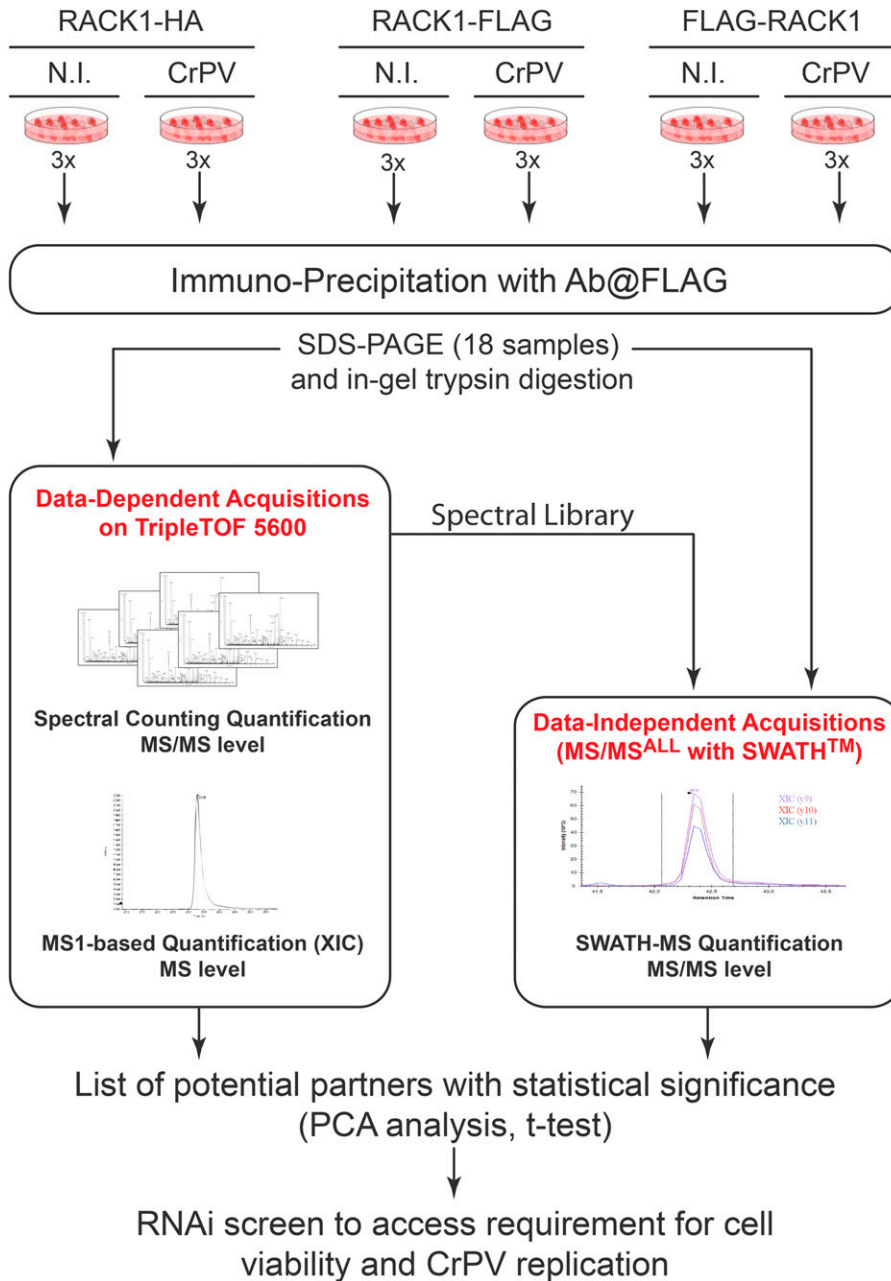


Figure 1 Immunoprecipitation and proteomic workflows used to identify RACK1 partners. Thirty million *D. melanogaster* S2 cells were transiently transfected with RACK1, tagged either at the N- or C-terminus with the indicated peptide epitopes. Cells were then left uninfected or challenged with CrPV MOI 0.1 for 24 hr and co-IP experiments were performed using an anti-FLAG antibody. Following SDS-PAGE, in-gel trypsin digestion was performed, before nanoLC-MS/MS analyses. Quantification was made under either Data-Dependent Acquisition mode, thus enabling Spectral Counting and MS1 label-free methods, or Data-Independent Acquisition mode, dedicated to MS/MS^{ALL} with SWATH-MS quantification method. The biological relevance of potential RACK1 partners, identified and statistically validated by these three quantification methods, was finally tested using RNAi. co-IP, co-immunoprecipitation; CrPV, Cricket paralysis virus; MOI, multiplicity of infection; MS, mass spectrometry; N.I., noninfected; RACK1, Receptor for Activated protein C kinase 1; RNAi, RNA interference; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SWATH, Sequential Window Acquisition of all Theoretical fragment-ion spectra.

(Figure 3 and Table S3 in File S2). The PANTHER overrepresentation test used a reference list of 13624 *D. melanogaster* accessions, as well as adjusted *P*-values (correction for multiple testing using the Benjamini-Hochberg method). Of note, the PANTHER Protein Classes “Nucleic Acid Binding proteins” and “Chaperones” were well represented, and 37.5% of the proteins were annotated as “Macromolecular complexes.” Interestingly, when considering each of the three quantitative methods independently, the SWATH approach identified more proteins involved in nucleic acid binding ($n = 13$) than the SpC or MS1 approaches ($n = 5$ for each). It also recognized eight proteins involved in RNA interaction or translation regulation, including several ribosomal proteins (Table S3 in File S2). Thus, the SWATH analysis appears to best reflect the known cellular functions of RACK1 in regulation of mRNA translation.

The whole set of 52 RACK1-interacting partners was further submitted to a PANTHER overrepresentation test, which was subsequently run with the 37 RACK1 partners identified by the SpC and MS1 methods only (Table S4 in File S2). Figure 4A displays the fold enrichment returned by PANTHER with or without the SWATH-specific RACK1 partners for each of the three GO terms, as well as the significance of the fold enrichment (P -value < 0.05). Nine GO annotations exhibit increased fold enrichment when the 16 additional SWATH-specific interactors are included. Eight of them are related to translation, RNA helicase activity, and nucleic acid binding. Moreover, the fold enrichment systematically becomes significant for the nine GO terms when including the SWATH dataset. To further elucidate the relationships between the set of 52 RACK1-interacting proteins and to identify functional complexes, the STRING interaction database was used to map

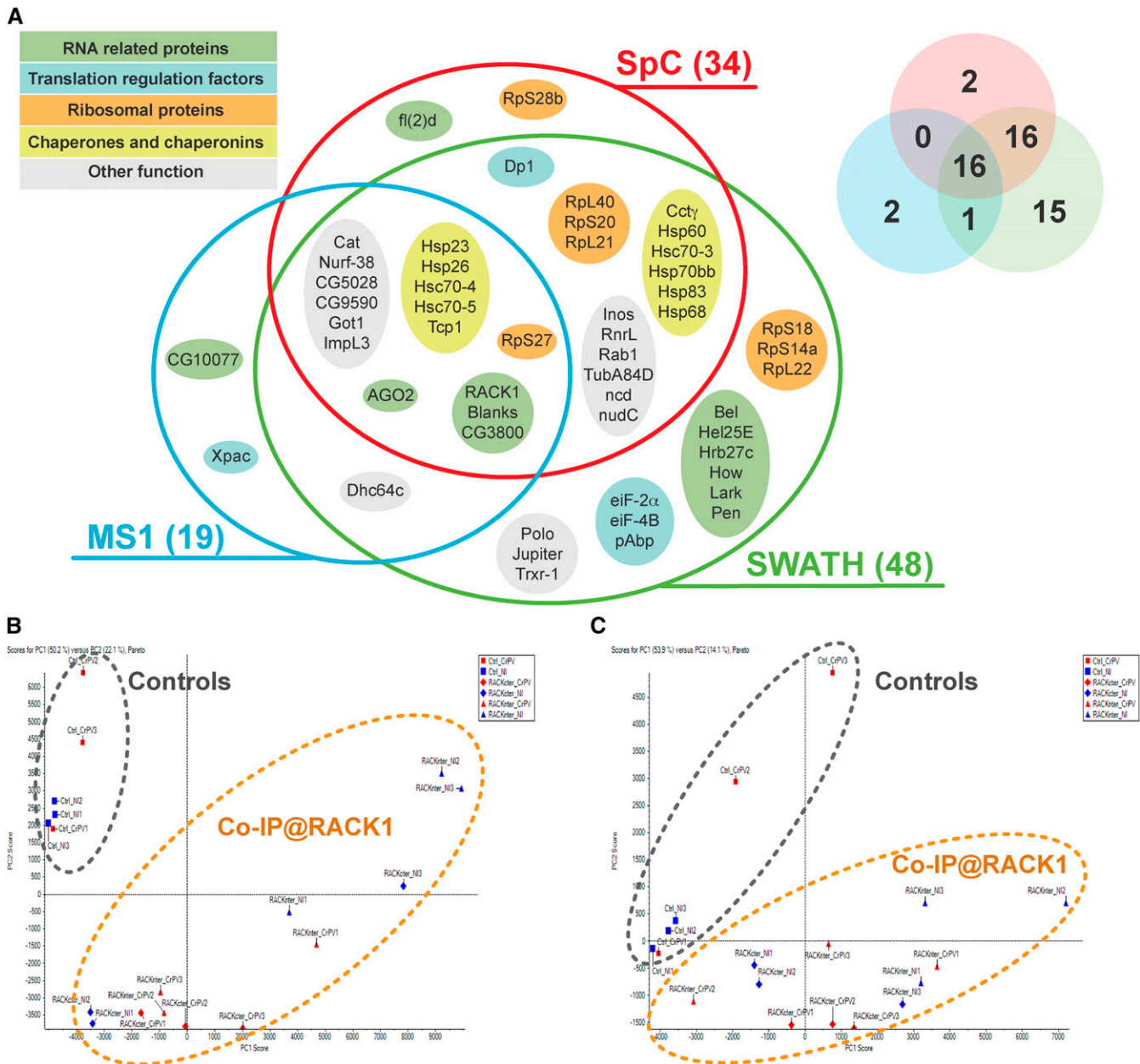


Figure 2 RACK1 partners identified by either the DDA (Spectral Counting = SpC, MS1 label-free = MS1) or DIA approach (MS/MS^{ALL} with SWATH = SWATH). (A) Proteins identified as RACK1 partners by the three types of quantitative methods. The Venn diagram shows the global overlap between the three strategies. Five functional categories are represented. (B) Principle Component Analysis for the MS1 label-free dataset: 231 proteins were identified by Paragon algorithm and further quantified after the automatic reconstruction of peptide features at the MS level (XIC). (C) Principle Component Analysis for the SWATH dataset: proteins were quantified by interrogation of a home-made spectral library at the MS/MS level. co-IP, co-immunoprecipitation; DDA, Data-Dependent Acquisition; DIA, Data-Independent Acquisition; MS, mass spectrometry; RACK1, Receptor for Activated protein C kinase 1; SWATH, Sequential Window Acquisition of all THEoretical fragment-ion spectra.

the RACK1 network (Figure 4B). This analysis reveals that the vast majority of the protein nodes are connected together. It also shows a high connectivity with a total of 21 protein nodes between the group of ribosomal proteins, to which RACK1 belongs, and three other groups: (i) RNA-related proteins; (ii) chaperones and chaperonins; and (iii) translation regulation factors.

One family of molecules reported to interact with RACK1 and possessing interesting properties in the context of the regulation of translation and the control of viral infections are members of the AGO

family. Indeed, RACK1 is involved in miRNA function in the plant *Arabidopsis thaliana* (Speth *et al.* 2013), the nematode *Caenorhabditis elegans* (Jannot *et al.* 2011), and humans (Otsuka *et al.* 2011). In *Drosophila* as well, we previously reported that RACK1 participates in silencing triggered by miRNAs, although its impact was stronger for some miRNAs than others (Majzoub *et al.* 2014). In *Drosophila*, most miRNAs are loaded onto AGO1, with only a small subset loaded onto AGO2. Interestingly, we recovered AGO2, but not AGO1, in the RACK1 interactome (Figure 2A). The functional significance of the

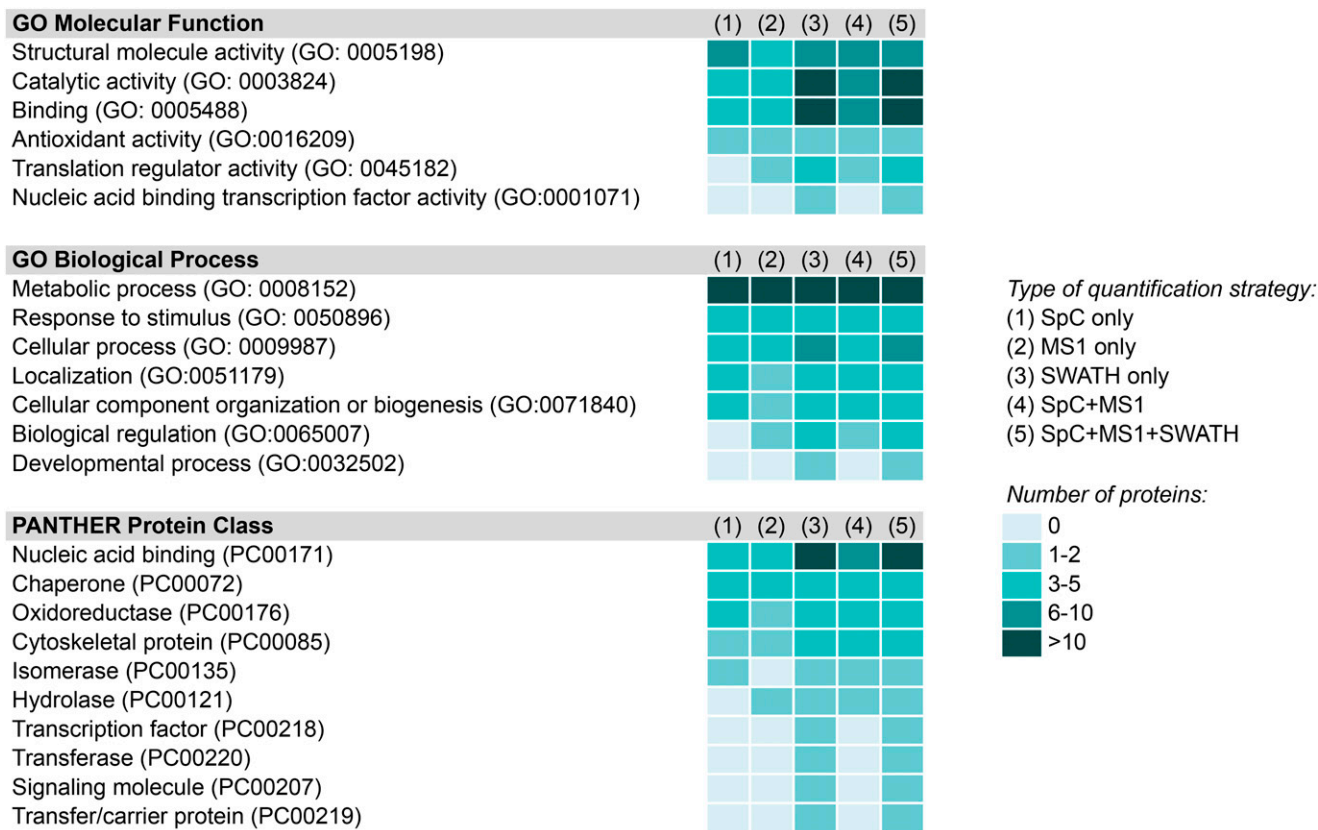


Figure 3 Heat map displaying the Gene Ontology (GO) annotations of the Molecular Process of the identified proteins. The classification system made by PANTHER (<http://www.pantherdb.org>). Proteins included in heat map were identified by either one or several of the three quantification methods [Spectral Counting (SpC), MS1 Label-Free (MS1), and MS/MS^{ALL} with SWATH (SWATH, Sequential Window Acquisition of all Theoretical fragment-ion spectra)].

interaction of RACK1, which promotes translation driven by viral IRES elements, and AGO2, a major effector of antiviral immunity in flies, deserves further investigation.

Functional characterization of the RACK1 interactome

To assess the biological significance of the interactions identified in the context of viral infection, we used RNAi in S2 cells to silence expression of the RACK1-interacting proteins (Figure 5A). Silencing of 17 of the 52 identified proteins affected cell viability or proliferation, preventing further characterization. As expected, these included the majority of the ribosomal proteins, with the notable exception of RpS20 and RACK1. We next tested the impact on CrPV replication of the remaining 35 genes. Genes were silenced for 4 d prior to CrPV infection and accumulation of viral RNA was monitored by RT-qPCR 16 hr later. Twenty-three genes (66%) did not significantly impact CrPV replication. Interestingly, 10 genes (28%) led to increased CrPV RNA in infected cells when their expression was knocked-down, suggesting that they encode factors restricting viral infection. Indeed, these include AGO2, a central component of the antiviral siRNA pathway (van Rij *et al.* 2006; Mueller *et al.* 2010) (Figure 5A). The others were not previously associated with the control of viral infections. Besides RACK1, only one other gene, *Lark*, led to decreased CrPV replication when it was silenced (Figure 5A). To rule out off-target effects, we synthesized two dsRNA targeting different regions of the *Lark* gene. Both dsRNAs efficiently silenced *Lark* expression (Figure 5B) and suppressed CrPV replication, although not as efficiently as silencing of RACK1 (Figure

5C). This suggests that *Lark*, an RNA-binding protein, might participate in selective mRNA translation together with RACK1. Because RACK1 is also required for translation of the related virus DCV, we next tested replication of this virus in *Lark*-silenced cells. However, silencing *Lark* had no significant impact on DCV (not shown). Finally, we tested directly whether *Lark* had an effect on viral translation, using a CrPV-5' IRES luciferase reporter (Majzoub *et al.* 2014). As expected, silencing RACK1 had a strong impact on the expression of the reporter. By contrast, silencing of *Lark* did not affect its activity (Figure 5D). We conclude that *Lark* and RACK1 promote CrPV replication by different mechanisms.

DISCUSSION

The present study represents a first description in the model organism *Drosophila* of the interactome of RACK1, an intriguing cytoplasmic protein at the interface of the ribosome and cell signaling pathways. In spite of its limitations (transient overexpression of the bait; analysis of a single cell line; interactions not confirmed by alternative techniques; and only one time point analyzed for viral infection), the study confirms the power of SWATH for the establishment of the RACK1 interaction network under the biological conditions described in this study, and reveals some interesting findings. Indeed, 48 out of the 52 RACK1 interactants were identified using SWATH, and 9 of the 15 partners identified only by this method are RNA-related proteins (Bel, Hel25E, How, *Lark*, Pen, and Hrb27c) or translation regulation factors (eIF-2 α , eIF-4B, and pAbp). Overall, our data are consistent

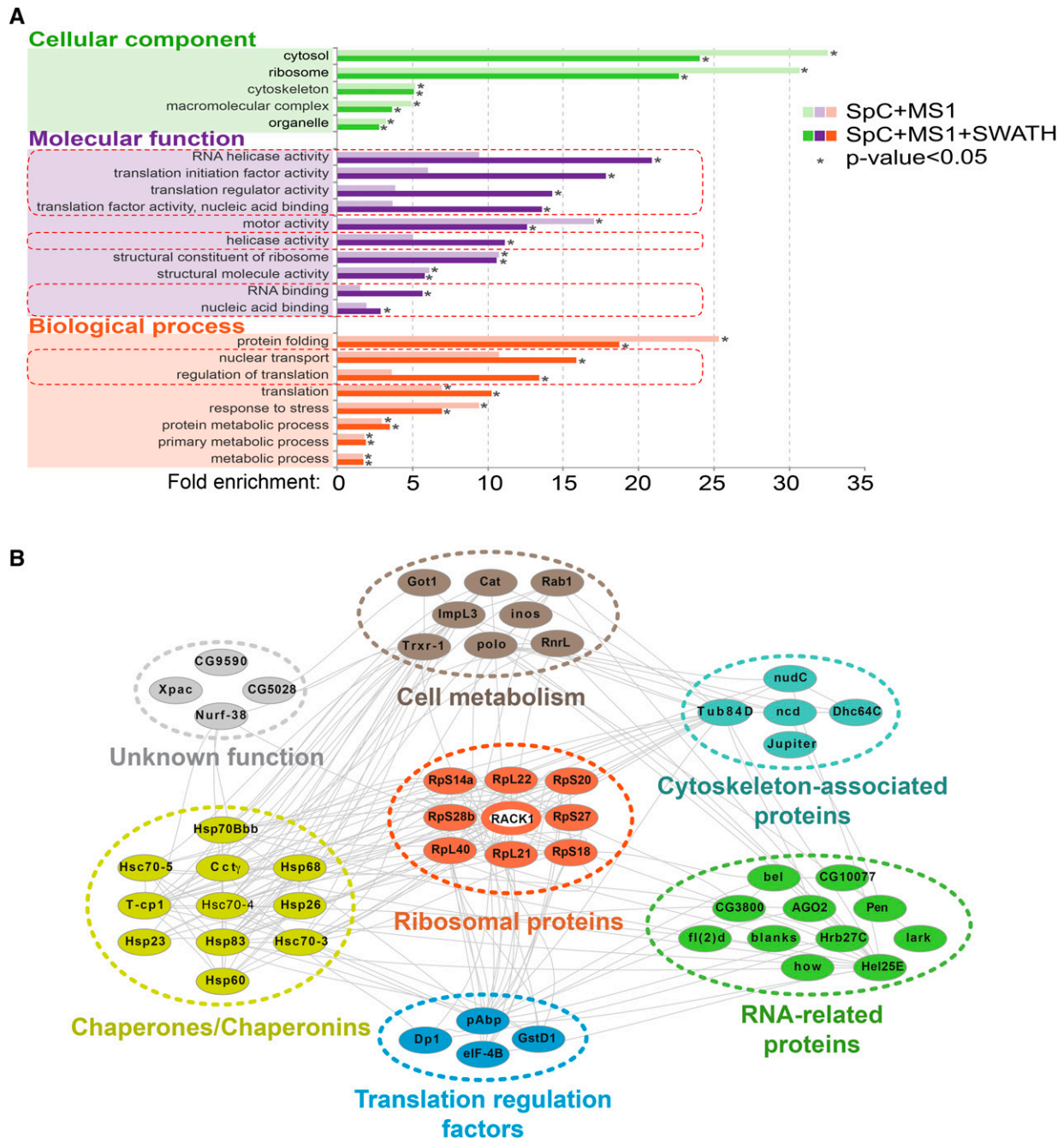


Figure 4 Functional Classification and Enrichment analysis of the RACK1-interacting proteins identified by the three quantification methods. (A) STRING network prediction of the 52 proteins identified as partners by SpC, MS1, and SWATH approaches. (B) Gene Ontology terms overrepresentation analysis by PANTHER: GO terms with an increased fold enrichment when considering SWATH data and for which P -value becomes significant (< 0.05) are highlighted by a red box. GO, Gene Ontology; MS1, MS1 Label-Free; RACK1, Receptor for Activated protein C kinase 1; SpC, Spectral Count; SWATH, Sequential Window Acquisition of all THEoretical fragment-ion spectra.

with RACK1 playing a major role at the level of the ribosome on translational control. RACK1 has been proposed to interact with an array of signaling molecules and to act as a scaffold protein (Adams *et al.* 2011; Li and Xie 2015). Indeed, RACK1 was identified as a partner of several kinases (*e.g.*, PKC β (Ron *et al.* 1994; Sharma *et al.* 2013), Src (Chang *et al.* 1998), p38 MAPK (Belozarov *et al.* 2014), a phosphatase (PP2A, Long *et al.* 2014), and membrane receptors [*e.g.*, Flt1 (Wang *et al.* 2011) and integrins (Liliental and Chang 1998)]. It is intriguing that we only identified a few signaling proteins (*e.g.*, polo kinase, Rab1,

and a myo-inositol 1-phosphatase). Interestingly, the interactome of RACK1 in a mosquito cell line also revealed that 25% of the RACK1 partners were annotated as involved in ribosomal structure and/or translation (González-Calixto *et al.* 2015). This study also detected a few signaling proteins, which differ from the ones reported here. Our failure to identify signaling proteins associated with RACK1 could reflect the experimental settings used (*e.g.*, use of cell line, and high detergent and salt concentration in the washing steps to minimize nonspecific interactions, at the risk of elimination of weak interactors).

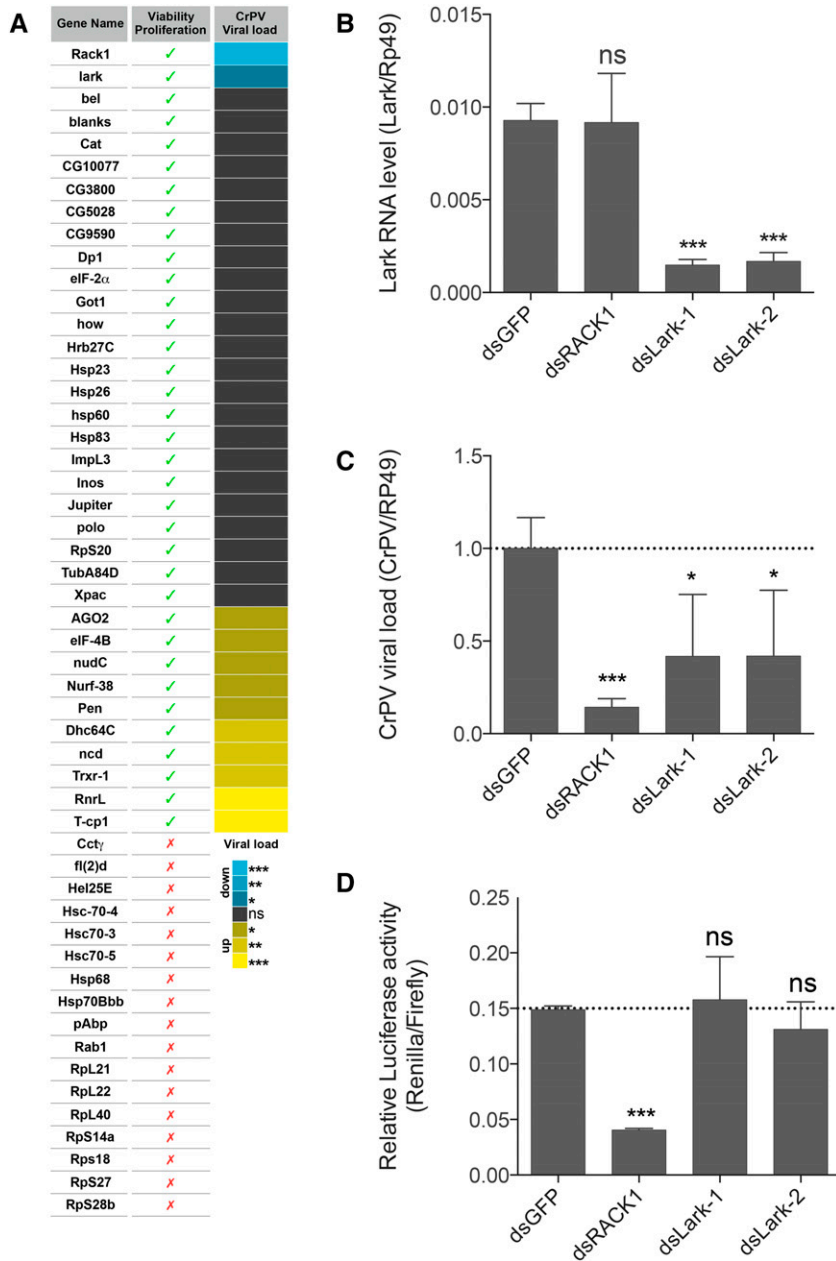


Figure 5 Functional characterization of the 52 RACK1 interactors identified (A). Impact of the silencing of the 52 genes on cell number and CrPV replication. Cell viability/proliferation was monitored by counting nuclei following DAPI staining. Viral load was monitored only on cells not impacted by silencing of the candidate genes (B). Incubation of S2 cells with two dsRNAs targeting different regions of the gene result in efficient *Lark* silencing. (C) Silencing of *Lark* affects CrPV replication in S2 cells. (D) Silencing of *Lark* does not affect translation driven by 5' IRES from CrPV, unlike silencing RACK1. Statistical analysis with t-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and ns, not significant. CrPV, Cricket paralysis virus; DAPI, 4',6-diamidino-2-phenylindole; dsRNA, double-stranded RNA; GFP, green fluorescent protein; IRES, internal ribosome entry site; RACK1, Receptor for Activated protein C kinase 1.

It could also reflect a transient, signal-dependent nature of the interaction. This hypothesis could also account for the lack of interaction induced by CrPV infection. Although RACK1 is known to be subject to post-translation modification, we did not detect any (Adams *et al.* 2011; Schmitt *et al.* 2017; Yang *et al.* 2017). Additional experiments in conditions stabilizing these modifications (*e.g.*, in the presence of phosphatase inhibitors) could clarify this issue and confirm that RACK1 acts as a scaffold protein (Adams *et al.* 2011). However, we cannot rule out the possibility that all functions so far attributed to RACK1 indirectly result from its presence at the ribosome (Schmitt *et al.* 2017). This hypothesis is consistent with the fact that RACK1 appears to be exclusively associated with ribosomes and polysomes in *Drosophila* cells (E. Einhorn, F. Martin, C. Meignin and J. Imler, unpublished data).

Our aim was to identify proteins functioning together with RACK1 in IRES-dependent translation. However, none of the 52 interacting proteins identified behaved like RACK1 in our functional assays. In-

terestingly however, one of them, *Lark*, appears to be required for CrPV replication, although it is not required for translation driven by the 5' IRES of the virus. *Lark* encodes a protein composed of an N-terminal Zinc knuckle domain, followed by two RRM motifs, initially characterized for its role in mRNA splicing and regulation of the circadian rhythm (Huang *et al.* 2007). Interestingly, *Lark* is evolutionarily conserved, and both *Lark* and its mammalian homolog RBM4 participate in miRNA-dependent inhibition of translation by AGO proteins (Höck *et al.* 2007; Lin and Tarn 2009). Thus, the functional significance of the interaction between RACK1 and *Lark*/RBM4 deserves to be tested in other settings (Otsuka *et al.* 2011; Jannot *et al.* 2011; Speth *et al.* 2013). Of note, our functional analysis is limited to the genes not affecting cell viability or proliferation, which could explain our lack of success in identifying functional partners of RACK1.

One unexpected finding of our study was that 20% of the identified interacting proteins (10 out of 52) restrict CrPV replication. This may at

first sight seem surprising in light of the opposite effect of RACK1 on this virus. However, translation control is a critical step in the viral replication cycle, where the viral RNAs are exposed to host cell molecules, including restriction factors. Thus, it is possible that RACK1, a critical molecule for viral IRES-dependent translation, is used as a surveillance platform for proteins participating to cellular intrinsic antiviral responses. Although we cannot rule out that the antiviral effect of some of these genes is indirect at this stage, AGO2 has antiviral functions that have been well characterized *in vitro* and *in vivo* (Wang *et al.* 2006; van Rij *et al.* 2006; Nayak *et al.* 2010; van Mierlo *et al.* 2012). Therefore, this protein represents a prime candidate to elucidate the biological significance of the interaction between factors restricting viral replication and RACK1.

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