ReLo: a simple colocalization assay to identify and characterize physical protein-protein interactions

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ABSTRACT

Characterizing protein-protein interactions (PPIs) is fundamental for understanding biochemical processes. Many methods have been established to identify and study direct PPIs; however, screening and investigating PPIs involving large and poorly soluble proteins remain challenges. As a result, we developed ReLo, a simple cell culture-based method to detect and investigate interactions in a cellular context. ReLo allows both the identification of protein domains that mediate the formation of complexes and screening of interfering point mutations. ReLo is sensitive to drugs that mediate or interfere with a specific interaction. Furthermore, protein conformation- and protein arginine methylation-dependent interactions can be studied. Importantly, ReLo can be used for specifically detecting direct but not indirect PPIs and can be applied for describing the binding topology of subunits within multiprotein complexes. Because of these attributes, ReLo is a simple, quick and versatile tool for identifying and studying binary PPIs, as well as for characterizing multisubunit complexes.

INTRODUCTION

Identifying and characterizing protein-protein interactions (PPIs) is a routine laboratory practice and lays the foundation for understanding biological processes. PPIs can be identified through various established, mass spectrometry-coupled screening methods, including coimmunoprecipitation (co-IP), tandem affinity purification, and proximity-dependent labeling approaches, such as BioID or APEX (Bosch et al., 2021; Gingras et al., 2019; Masters, 2004; Ransone, 1995; Rigaut et al., 1999; Roux, 2013). Thus, a list of interacting protein candidates is obtained, and these candidates are usually ranked according to their abundance in eluate fractions. Determining which of these candidates are truly direct binding partners requires subsequent validation, which is often performed through *in vitro* methods, such as GST pull-down assays, which depend upon the availability of purified proteins. In situations where proteins are poorly soluble and not obtainable through recombinant protein expression or in cases where expertise in recombinant protein expression and purification methods is lacking, PPIs can be validated alternatively using cell-based assays.

Yeast two-hybrid (Y2H) and protein complementation assays (PCA) are well-established techniques, in which an interaction results in the reconstitution of a split reporter protein, such as a transcription factor, ubiquitin, an enzyme, or a fluorescent protein, which is subsequently detected (Blaszczak et al., 2021; Cui et al., 2019;

Lalonde et al., 2008; Remy and Michnick, 2007; Walport et al., 2021; Wang et al., 2020). Y2H and PCA are, however, not particularly suitable for analysis of large and/or unstable proteins, as these proteins may be barely expressed or rapidly degraded in a cell, causing unreliable, mostly false-negative results (Cui et al., 2019; Lalonde et al., 2008). Therefore, to be conclusive, negative results require additional assessment of protein expression levels, complicating the process, especially when probing many PPIs.

Cell-based PPI methods that are better suited for testing interactions involving large and/or potentially unstable proteins are based on fluorescent protein tagging and colocalization readouts and thus permit simultaneous monitoring of both PPI and protein expression levels via fluorescence microscopy. The readout of these colocalization assays is usually the translocation of one protein upon its association with a second distinctly localized protein (e.g., localization to a membrane, the nucleus, or granules). "Cytoskeleton-based assay for protein-protein interaction" (CAPPI), "membrane recruitment assay" (MeRA), and "knocksideways in plants" (KSP) are powerful translocation assays developed for use with plant cells (Grefen et al., 2008; Lv et al., 2017; Winkler et al., 2021). "Nuclear translocation assay" (NTA), "emerging circle of interactive proteins at specific endosomes" (ECLIPSE), and "protein interactions from imaging of complexes after translocation" (PICT) are assays, which require the addition of a compound (e.g., rapamycin) to monitor the translocation upon PPI (Dixon and Lim, 2010; Gallego et al., 2013; Lee et al., 2010). Other translocation assays are based on oligomerization/aggregation readouts (Martin et al., 2007; Taslimi et al., 2014; Watanabe et al., 2017) and thus may not be suitable for studying interactions with proteins that form granules on their own within a cell. Importantly, none of the described translocation assays has been assessed for its ability to distinguish direct from indirect interactions.

We developed a simple and fast translocation PPI assay named ReLo for use in animal cell culture. This assay is based on relocalization of a protein upon its interaction with a second membrane-anchored protein. We applied ReLo to many large proteins, of which most carry long disordered regions and are known to be insoluble upon recombinant protein expression experiments. Using this set of proteins, we demonstrate that ReLo can be used to identify and characterize PPIs. Importantly, using a structurally well-characterized multidomain protein complex, we provide evidence showing that ReLo can be used to detect only physical interactions, a

prerequisite for analyzing previously unknown protein complexes through *in vitro* and structural biology methods. Furthermore, ReLo is responsive to drug treatment, which enables the study of drug-induced interactions as well as the screening of small PPI inhibitors. In summary, ReLo is a simple, quick and versatile tool that enables the comprehensive, initial description of direct PPI networks.

RESULTS

ReLo: a simple and robust cell culture-based PPI assay

For being studied with ReLo, two proteins of interest were fused to a red (mCherry) and a green (EGFP, mEGFP) fluorescent protein, respectively. Importantly, one of the constructs carried an additional fusion to a "membrane anchoring" protein domain, which led to distinct subcellular membrane localization of the resulting fusion protein. Upon interaction, the second protein is expected to colocalize with the anchored protein to the membrane; that is, it would "relocalize" with respect to its original location (**Figure 1A**). Thus, we refer to the assay as "relocalization PPI assay", abbreviated "ReLo".

ReLo is based on a simple methodology in which cells are seeded onto 4-well chambered coverslips and cotransfected with the desired combination of plasmids. Protein localization is then analyzed after one or two days through live-cell confocal fluorescence microscopy (**Figure 1A**). We used S2R+ cells, which were derived from semiadherent Schneider's-line-2 (S2) cells, which in turn were established from late *Drosophila* embryos (Schneider, 1972; Yanagawa et al., 1999). Compared to S2 cells, S2R+ cells show greater adherence to dishes, which facilitates live-cell microscopy without the need for fixing cells onto a coated dish. All ReLo plasmids carry an in-frame blunt end restriction site, allowing the application of a simple, fast, and straightforward cloning procedure (see Methods section).

To anchor cytoplasmic proteins of interest to a membrane, we chose the pleckstrin homology (PH) domain of rat phospholipase C δ_1 (PLC δ_1), which is known to specifically recognize phosphatidylinositol 4,5-bisphosphate (Garcia et al., 1995; Lemmon et al., 1995) and to thus direct the fusion construct to the plasma membrane of a cell (**Figure 1A**). In our assay, the membrane localization was independent of whether the PH domain was fused to the N- or C-terminus of a protein (**Supplemental Figure 1A**). Unfortunately, nuclear proteins fused to the PH domain were only inefficiently retained within the cytoplasm and barely localized to the plasma

membrane (**Supplemental Figure 1C**). Hence, testing PPIs with these proteins may result in false-negative outcomes when the interaction partner resides in the cytoplasm. Therefore, we tested alternative membrane-anchoring domains to assess their ability to retain nuclear proteins within the cytoplasm. Hence, we selected the minimembrane protein subunit 4 of the yeast oligosaccharyltransferase complex (OST4), which has been previously shown to localize a fusion protein to the endoplasmic reticulum (ER) (Kim et al., 2003; Stagljar et al., 1998) (**Supplemental Figure 1D**). As the plasma membrane localization of the PH domain distinguishes more from a ubiquitous cytoplasmic localization than the ER localization of OST4, we prefer using the PH domain in ReLo whenever possible.

PPI mapping and mutational analysis

As proof of concept for PPI studies with ReLo, we tested a previously characterized protein complex consisting of the extended LOTUS (eLOTUS) domain of Oskar (Oskar 139-240) and the C-terminal RecA-like domain (CTD) of the ATP-dependent DEAD-box RNA helicase Vasa (Vasa-CTD, Vasa 463-661) (Jeske et al., 2017) (**Figure 1B**). Only the short isoform of Oskar (Short Oskar, aa 139-606) interacts with Vasa (Breitwieser et al., 1996), and therefore, only Short Oskar and the domains it contains were used in the following experiments (**Figure 1B**). In our setup, the eLOTUS domain of Oskar was fused to PH-mCherry and localized to the plasma membrane. Vasa-CTD was fused to EGFP and localized ubiquitously within the cytoplasm and nucleus. When coexpressed with PH-mCherry-eLOTUS, but not with PH-mCherry alone, EGFP-Vasa-CTD relocated to the plasma membrane (**Figure 1C**). The unstructured region of Vasa (Vasa 1-200) and the N-terminal RecA-like domain (Vasa 200-463) did not interact with the Oskar-eLOTUS domain, and similarly, neither the unstructured region of Oskar (Oskar 241-387) nor its OSK domain (Oskar 388-606) interacted with Vasa-CTD (**Figure 1C**). Surface point mutations that had been previously shown to interfere with the Vasa-Oskar interaction (Jeske et al., 2017) were also found to be inhibitory in the ReLo assay (**Figure 1D**). Together, these data confirmed the specific interaction between Vasa-CTD and Oskar-eLOTUS and demonstrated that ReLo can be applied to map PPIs and to screen for mutations that interfere with PPIs.

Oskar-eLOTUS and Vasa-CTD form a transient complex characterized by a dissociation constant (K_D) of $\sim 10 \mu\text{M}$, and although this complex has been crystallized, it is not sufficiently stable to be detected with size exclusion chromatography (Jeske et

al., 2015, 2017). Nevertheless, the relocalization upon the Oskar-eLOTUS and Vasa-CTD interaction was clearly detectable in the ReLo assay. In 38 of 40 cotransfected cells (i.e., 95%) carrying both red and green fluorescent signals, relocalization of Vasa-CTD toward the Oskar-eLOTUS location on the plasma membrane was observed (**Supplemental Figure 2**), indicating that relocalization is a highly frequent event and that the assay is well suited to study low-affinity complexes. Most other interactions tested revealed relocalization in 100% of the cells (see below; **Supplemental Figure 3**).

Conformation-dependent interactions

Previous data have suggested that the Oskar-Vasa interaction depends on the conformation of Vasa (Jeske et al., 2017). We aimed to test the interaction between Oskar and different Vasa conformations in a full-length protein context. However, in contrast to the eLOTUS domain of Oskar, full-length Short Oskar did not localize to the cytoplasm but exclusively localized to the nucleus in S2R+ cells (**Supplemental Figure 1B**) (Jeske et al., 2017). Therefore, we made use of the OST4-mCherry-Oskar construct, which localized to membranous structures within the cytoplasm (**Supplemental Figure 1D and Figure 1E**). Upon coexpression, wild-type Vasa relocalized and colocalized with OST4-Oskar at the ER (**Figure 1E**), confirming the Vasa-Oskar interaction with the ReLo assay.

The cores of Vasa and other ATP-dependent DEAD-box RNA helicases are composed of two RecA-like domains, which display different orientations relative to each other, depending on whether ATP and RNA are bound (Linder and Jankowsky, 2011). In a substrate-unbound form, the helicase core adopts an open conformation, and closes upon substrate binding (**Figure 2A**). To assess the conformation-dependent Vasa interaction using ReLo, we used Vasa variants with well-characterized point mutations that stabilize either the open conformation (K282N; Vasa-open) or the closed conformation (E400Q; Vasa-closed) (Gorbalenya et al., 1988; Walker et al., 1982; Xiol et al., 2014). When testing the interaction of OST4-anchored Oskar with the Vasa mutants through ReLo assays, we observed an interaction with Vasa-open but not with Vasa-closed (**Figure 2B**), revealing that Oskar prefers to bind to the open conformation of Vasa. These outcomes were consistent with our previous observations (Jeske et al., 2017) and demonstrated that the ReLo

assay allows the study of PPIs that are dependent on a specific conformation of an interaction partner.

sDMA-dependent interactions

Protein interactions may depend on posttranslational modifications, such as arginine methylation. The symmetric dimethylated arginine (sDMA) modification is catalyzed by a subset of protein arginine methyltransferases (PRMTs) (Stopa et al., 2015), and sDMA methylation activity has been previously reported in S2 cells (Gonsalvez et al., 2006). To test whether ReLo is suitable to investigate interactions involving sDMA modifications in S2R+ cells, we tested the previously characterized strictly sDMA-dependent interaction between the PIWI protein Aubergine (Aub) and Tudor (Kirino et al., 2010; Liu et al., 2010) (**Figure 2C**). Using ReLo, we indeed observed an Aub-Tudor interaction (**Figure 2D**). In contrast, the Aub-Tudor interaction was not observed in Y2H tests (**data not shown**). Aub carries four sDMAs within its RG-rich N-terminus (R11, R13, R15, and R17), which are specifically recognized and bound by extended Tudor (eTud) domains of Tudor (Kirino et al., 2009, 2010; Liu et al., 2010; Nishida et al., 2009). Substituting these four arginine residues with lysine residues (Aub R->K), rendered Aub unmodifiable by PRMT5 (Kirino et al., 2009) and abolished the Aub-Tudor interaction (**Figure 2D**). Together, these data demonstrated that the S2R+ cells contained sufficient sDMA activity to effectively modify proteins expressed after transient transfection of the cells. We conclude that ReLo is suitable to study PPIs that depend on PRMT5-catalyzed sDMA modification.

Effect of small molecules on PPIs

Next, we tested whether the ReLo assay can be used to study PPIs that are induced by the addition of small molecules to the cell culture medium. To this end, we tested the previously characterized rapamycin-dependent interaction between human FK506-binding protein 12 (FKBP12) and the FKBP12-rapamycin-binding domain (FRB) of human mTOR (Choi et al., 1996). In the absence of rapamycin, using the dimethyl sulfoxide (DMSO) control medium, FKBP12 and FRB did not interact in the ReLo assay but did interact in the presence of 100 nM rapamycin in the cell culture medium (**Figure 2E**).

Furthermore, we tested whether ReLo can be used to inhibit PPIs through drug treatment. We chose to interfere with the interaction between human p53 and the

human ortholog of mouse double minute 2 (MDM2) by the known peptidomimetic inhibitor nutlin-3 (Vassilev et al., 2004). In the ReLo assay, we used only the N-terminal domains of p53 and MDM2, which were sufficient to mediate the p53-MDM2 interaction (Kussie et al., 1996). Indeed, while the interaction between p53 1-50 and MDM2 1-118 was detected in the DMSO control experiment, it was not observed in the presence of 5 μ M nutlin-3 in the cell culture medium (**Figure 2F**).

Together, these data suggest that the ReLo assay can be used to study interactions involving non-*Drosophila* proteins as well as to screen drugs for their ability to either enable or inhibit a specific PPI.

ReLo specifically reveals direct but not indirect interactions

Thus far in our study, we had tested known direct interactions between proteins that are not endogenously expressed in S2R+ cells. PPIs observed with ReLo may not necessarily involve physical contact. Instead, PPIs may also result from indirect contacts caused by incorporation of the coexpressed pair of proteins into cell-endogenous protein complexes and subsequent indirect bridging of this pair through one or more common interaction partners within such a complex. To understand the extent to which direct or indirect associations underlie a relocalization event observed in ReLo, we assessed interactions between individual subunits of the CCR4-NOT complex, which is endogenous to S2R+ cells. The CCR4-NOT complex is an essential eukaryotic deadenylase, comprising six subunits that form the core, which has a well-characterized architecture at the molecular and structural levels (Collart and Panasenko, 2017; Temme et al., 2014). In this complex, NOT1 is the scaffolding subunit for the assembly of all the other subunits. Specifically, the CAF1-CCR4 subcomplex and CAF40 bind to the central region of NOT1, and the NOT2-NOT3 subcomplex associates with the C-terminal region of NOT1 (**Figure 3A**). Using ReLo, we performed a systematic pairwise screen in which each subunit was tested against all other subunits of the *Drosophila* CCR4-NOT core complex (**Figure 3B and Supplemental Figure 4A**). In S2R+ cells, NOT1 and NOT3 localized exclusively to the cytoplasm, while NOT2, CAF1, CAF40 and CCR4 localize to both the cyto- and the nucleoplasm (**Supplemental Figure 4B**). Remarkably, we observed interactions only between proteins that had been previously shown to associate directly but not between indirectly linked combinations. In our assays, NOT1 specifically bound to CAF1, CAF40, NOT2, and NOT3, CAF1 bound to CCR4, and NOT2 bound to NOT3 (**Figure**

3B, C, Supplemental Figure 4A, B). We also tested CAF1 interactions using the OST4 membrane anchor and observed results similar to those obtained using the PH anchor (**Supplemental Figure 4C**). Together, these data reveal that due to the overexpression situation present in ReLo the degree of protein incorporation into endogenous complexes is too low to be detected, and thus, physical but not indirect interactions are observed by ReLo. Despite the overexpression of proteins with ReLo, we did not detect false-positive interactions.

Topological description of multisubunit complexes

As has been described for S2 cells (Yang and Reth, 2012), we usually observe a very high cotransfection efficiency of the S2R+ cells in ReLo experiments (**data not shown**). Therefore, we tested whether bridging of CCR4-NOT subunits that do not directly interact is observable when one or two common binding partners are added to the mixture. Specifically, S2R+ cells were cotransfected with three or four plasmids, two plasmids expressing the PH-mCherry or mEGFP fusion construct each, and one or two plasmids expressing nonfluorescent bridging factors (**Figure 3D**). Indeed, NOT3 failed to interact with CAF1 or CAF40 in the presence of the control plasmid but interacted when exogenous NOT1 was added (**Figure 3E, F**). In addition, we tested bridging of the NOT3-CCR4 interaction, which requires not only NOT1 but also the CAF1 subunit. The NOT3-CCR4 interaction was not observed in the presence of NOT1 alone but was observed when both NOT1 and CAF1 were simultaneously coexpressed (**Figure 3G**). Similar results were obtained when testing indirect interactions with CAF40 (**Supplemental Figure 4D**). Together, these data show that bridging experiments with ReLo can be used to reconstitute the binding topology of multisubunit complexes such as the CCR4-NOT complex.

Assessing PPIs between the CCR4-NOT complex and mRNA repressor proteins

The CCR4-NOT complex can be recruited specifically to mRNAs through adapter RNA-binding proteins, leading to accelerated deadenylation and eventually degradation and/or translation repression of the targeted mRNA. Next, we assessed PPIs between some of these repressor proteins and the six core subunits of the CCR4-NOT complex using ReLo (**Figure 4A**). Co-IP experiments combined with structural analysis had previously revealed the specific subunit(s) of the CCR4-NOT complex through which adapter proteins target the complex to a specific mRNA. For example,

Drosophila Bag-of-marbles (Bam) has been shown to bind specifically to CAF40 (Sgromo et al., 2018). We confirmed this finding with ReLo; that is Bam bound to CAF40 but not to any other CCR4-NOT complex subunit (**Figure 4B and Supplemental Figure 5A**). A point mutation in Bam (M24E), which is known to interfere with CAF40 binding (Sgromo et al., 2018), also abolished CAF40 binding in the ReLo assay (**Figure 4B**). *Drosophila* Nanos has been shown to bind the NOT1, NOT2, and NOT3 subunits of the CCR4-NOT complex (Raisch et al., 2016). With ReLo, we detected the interaction of Nanos with NOT1 but not with NOT2 or NOT3, suggesting that the NOT1 interaction might be predominant (**Figure 4C, Supplemental Figure 5B**). In addition, we detected Nanos binding to the CAF40 subunit (**Figure 4C**), an interaction not previously reported. *Drosophila* Roquin has been demonstrated to bind CAF1, CAF40, NOT1, NOT2, and NOT3 (Sgromo et al., 2017). Of these proteins, we detected clear binding of Roquin to CAF40, NOT1, and NOT3 using ReLo (**Figure 4D and Supplemental Figure 5C**). In addition, we detected a previously unknown interaction between Roquin and CCR4 (**Figure 4D**).

Encouraged by these validating data, we tested the interaction of the CCR4-NOT complex with less well-characterized potential adaptor proteins. *Drosophila* meiosis regulator and mRNA stability factor 1 (MARF1) is an oocyte-specific protein that recruits the CCR4-NOT complex to target mRNAs and thereby controls meiosis (Zhu et al., 2018). Whether MARF1 directly associates with the CCR4-NOT complex was unknown. Using ReLo, we found that MARF1 interacted with the NOT1 subunit, suggesting that MARF1 is indeed a direct recruiter of the CCR4-NOT complex (**Figure 4E and Supplemental Figure 5D**).

In co-IP experiments, Cup interacted with the CAF1, CCR4, NOT1, NOT2, and NOT3 subunits of the CCR4-NOT complex (Igreja and Izaurralde, 2011). We did not detect Cup binding to an individual subunit of the CCR4-NOT core complex using ReLo (**Supplemental Figure 5E**), suggesting that if Cup directly recruits the CCR4-NOT complex, then it binds through weak multivalent interactions. Cup has also been reported to bind the repressors Nanos and Bruno (Kim et al., 2015; Nakamura et al., 2004; Verrotti and Wharton, 2000), and through ReLo, Cup interactions with both of these repressors were confirmed (**Figure 4F**). Consistent with a recent report (Bansal et al., 2020), we observed that Bruno binds to Nanos in the absence of Cup (**Figure 4G**). We then wondered whether Bruno and Nanos bind to Cup as a complex. However, in the presence of Cup, the interaction between Bruno and Nanos was not

detectable (**Figure 4G**), suggesting that Bruno and Nanos compete for binding to the same region in Cup. Bruno has thus far not been shown to establish a direct contact to the CCR4-NOT complex, and we did not detect Bruno binding to any of the core CCR4-NOT complex subunits (**Supplemental Figure 5F**).

In summary, our assessment of PPIs using ReLo, involving a selection of repressor proteins, confirmed many previously described interactions, while new interactions were also discovered. Therefore, these data indicate that ReLo is a powerful method for identifying PPIs that is complementary to other methods such as co-IP experiments.

DISCUSSION

Detecting and characterizing PPIs are crucial for uncovering regulatory mechanisms that underlie cellular processes. Here we described ReLo, a quick procedure to identify and investigate pairwise as well as multisubunit PPIs. We provided strong evidence showing that PPIs identified with ReLo are based on physical contacts. Thus, newly identified PPI partners using ReLo are highly promising candidates for use in subsequent studies involving *in vitro* and experimental structural biology methods. Alternatively, PPI mapping data obtained from ReLo experiments may be used to guide subsequent modeling experiments using AlphaFold2 or AlphaFold-Multimer to obtain structural information on protein-protein complexes (Evans et al., 2021; Jumper et al., 2021). A thus identified protein-protein interface may then quickly be validated through mutational analysis using ReLo, and those mutations that specifically interfere with the PPI may finally be tested *in vivo* without the need for purifying a protein or experimentally determining a protein (complex) structure.

We refrained from quantifying the relocalization event observed with ReLo through image processing tools to assess or compare PPIs. Although only qualitative, the results obtained were typically clear. For example, in control experiments, we observed no relocalization; in experiments in which proteins interacted, most or all cells showed the relocalization (**Supplemental Figures 2 and 3**). Furthermore, implementing a relocalization score as a quantified readout of an interaction may have easily led to the false assumption that the score represents a measure of binding affinity between proteins. Such false assumptions are also an issue with Y2H dot assays, which appear to provide quantitative data but do not. The degree of relocalization identified with a ReLo assay or yeast cell growth in a Y2H assay does

not only reflect protein complex affinity but also relies on protein expression levels, protein stability, subcellular localization, and other factors. With this thought in mind, we prefer to consider ReLo a qualitative PPI method, the strength of which lies in its speed and versatility.

As is, ReLo is easy to perform in every laboratory equipped with cell culture technology and with access to a confocal fluorescence microscope. In contrast to Y2H, the expression levels of the proteins tested in ReLo assays are directly monitored during microscopy, facilitating data interpretation. The *Drosophila* S2R⁺ cell line we used for our ReLo testing required very simple handling. Similar to S2 cells, S2R⁺ cells grow at ambient temperature, do not need an incubator with CO₂, and passaging neither requires coated dishes nor scraping or trypsinization of cells (Yang and Reth, 2012). Using S2R⁺ cells, we successfully investigated not only *Drosophila* PPIs but also human PPIs. However, when a cell line derived from an alternative organism is required for a ReLo assay, only the PH or OST4 membrane anchors need to be inserted into the expression vectors compatible with the desired cell line.

As an advantage over Y2H, ReLo detection of PPIs appears to be successful regardless of the length of the protein of interest, as we successfully investigated PPIs with NOT1 and Tudor, two large proteins comprising 2505 and 2515 amino acid residues, respectively, with ReLo assays. Similar to any other cell-based PPI assay, attention is required when working with toxic proteins. To reduce toxicity, we recommend testing the splitting of the toxic protein into its domains or, when possible, testing protein variants with mutated active sites.

PPIs are highly relevant as putative therapeutic targets for the development of new treatments (Buchwald, 2010; Ivanov et al., 2013). In ReLo, complex formation is reversible, and we demonstrated that ReLo is as a tool for testing the effect of small molecules on PPIs. Due to its simple setup, ReLo can be easily adjusted to a high-throughput approach using automated imaging, thereby allowing for large drug screening experiments. In this setup, where a single specific PPI is subjected to a drug screening experiment, it might be advantageous to express the two protein partners from one plasmid, as equimolar protein expression within a cell may facilitate data interpretation.

Taken together, our data show that investigations with ReLo are quick, simple, and reliable. We recommend using ReLo as an initial tool to screen and characterize PPIs, especially when Y2H or more complicated approaches fail. Subsequently, ReLo

can be complemented with biochemical, structural, or genetic approaches to further characterize or ultimately validate the biological relevance of a given PPI.

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METHODS

Plasmid backbone construction

pAc5.1-EGFP (T5-MJ) and pAc5.1-mCherry (T7-MJ) were described previously (Jeske et al., 2017). pAc5.1-mEGFP (T6-MJ) encodes the monomeric A206K EGFP variant and was generated by site-directed mutagenesis of pAc5.1-EGFP. The pAc5.1- λ N-HA vector (T8-MJ) (Behm-Ansmant et al., 2006) was used to create nonfluorescent constructs. The *Saccharomyces cerevisiae* OST4 sequence was amplified from a pDHB1 vector (Stagljar et al., 1998) and inserted into the KpnI site of pAc5.1-mCherry to yield pAc5.1-OST4-mCherry. For cloning into the pAc5.1-EGFP, pAc5.1-mEGFP, pAc5.1-mCherry, pAc5.1- λ N-HA, and pAc5.1-OST4-mCherry vectors, sequences of interest were inserted into the blunt-end EcoRV site. The rat PLC δ ₁-PH sequence was amplified from the pETM11-His6-PH-Sumo3-sfGFP vector (Vonkova et al., 2015) and inserted into the KpnI site of pAc5.1-mCherry and pAc5.1-mEGFP to obtain pAc5.1-PH-mCherry (HK49) and pAc5.1-PH-mEGFP (JM50), respectively. Alternatively, the PH sequence was inserted into the EcoRV site of pAc5.1-mCherry to obtain the pAc5.1-mCherry-PH vector (EB3). For all PH-containing vectors, a new unique in-frame FspAI blunt end site was introduced 3' or 5' with respect to the fluorescent protein sequences, and it was used to insert the sequences of interest.

Cloning

Ligation reactions were assembled in a 10- μ l reaction containing T4 DNA ligase (Thermo Scientific), a 50 ng of vector DNA, and a 5- to 20-fold molar excess of DNA inserts and were incubated for 1 to 2 h at room temperature. DNA inserts were generated by PCR amplification. To prevent religation, the reaction was supplemented with 0.25 μ l of a blunt end restriction enzyme that was also used for linearization of the respective vector, except when this site was present in the insert sequence. Positive clones were screened with colony PCR using a primer that binds to the vector and one that binds to the insert. All constructs were verified by sequencing. Detailed information on all DNA constructs used in this study is listed in **Supplemental Table 1**.

Cell culture

S2R⁺ cell culture and imaging were previously described (Jeske et al., 2017). Schneider's *Drosophila* medium + (L)-glutamine (Thermo Scientific) was supplemented with 10% fetal bovine serum (Sigma) and 1 x Gibco™ Antibiotic-Antimycotic (Thermo Scientific). Cells were seeded in six-well glass-bottom plates (Cellvis) or four-well polymer μ -slides (Ibidi) and cotransfected using jetOPTIMUS (Polyplus transfection) according to the instruction manual. After one or two days of incubation at 25°C, images of live cells were taken with a 100x oil objective and a Nikon TE2000 laser scanning or Nikon Ti-E spinning disc confocal fluorescence microscope and processed with Fiji software (Schindelin et al., 2012).

Drug treatment

Rapamycin and nutlin-3 (both from MedChemExpress) were dissolved in DMSO to stock concentrations of 10.9 mM and 10 mM, respectively, and they were diluted with SF-4 Baculo express medium (BioConcept) to generate working concentrations of 100 nM and 5 μ M, respectively. S2R⁺ cells were grown in SF-4 medium and cotransfected with the desired plasmids using FuGENE® HD transfection reagent (Promega) according to the instruction manual. Twenty-four hours post-transfection, the cells were treated with the drug by replacing the medium with drug-containing medium. Cells were imaged after 24 h of incubation with the drug or DMSO control medium at 25°C.

AUTHOR CONTRIBUTIONS

H.K.S. and J.M. acquired, analyzed, and interpreted the data. M.J. conceived and supervised the project and wrote the manuscript.

COMPETING INTEREST

The authors declare no competing interests.

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FIGURES AND LEGENDS

Figure 1. The ReLo assay and its use in PPI mapping and mutational analysis.

(A) Plasmids encoding fluorescently tagged proteins 1 and 2 were cotransfected into S2R+ cells. Protein 1 carried an additional fusion to a membrane anchoring (MA) domain, i.e., PH or OST4. After two days, protein localization was analyzed by confocal fluorescence microscopy. When protein 2 interacts with protein 1, protein 2 is relocalized to the plasma membrane (PH domain) or ER (OST4). (B) Domain organization of *Drosophila* Oskar and Vasa proteins. Oskar is expressed as two isoforms, with Short Oskar lacking amino acids (aa) 1-138. Previously, the eLOTUS domain of Oskar was shown to interact with the C-terminal domain (CTD) of Vasa, and a crystal structure of the complex has been resolved (Jeske et al., 2015, 2017) (yellow stripe). (C) Vasa 463-661, but not Vasa 1-200 or 200-463, interacted with the Oskar eLOTUS domain. Vasa 463-661 did not interact with Oskar 241-396 or Oskar 398-606. (D) Oskar A162E/L228E and Vasa F504E point mutations (MUT) interfered with the Oskar-Vasa interaction. (E) OST4-mCherry Oskar localized to the ER (top panel), and Vasa fully relocalized with Oskar to the ER (bottom panel). The scale bar is 10 μ m.

Figure 2. ReLo PPI studies with respect to conformation, posttranslational modification and drug sensitivity.

(A) Pictorial representation of Vasa N- and C-terminal RecA-like domains (NTD and CTD, respectively) in the open and closed conformations. Vasa-open and Vasa-closed carry the K282N or E400Q mutations, respectively. (B) EGFP-Vasa-open, but not EGFP-Vasa-closed, interacted with OST4-mCherry-Oskar. White dotted lines indicate cell boundaries. (C) Symmetric dimethyl arginine (sDMA) modifications can be catalyzed by PRMT5 and recognized by eTudor domains. (D) Wild-type Aub interacted with Tudor, while Aub carrying nonmethylatable 4R→K point mutations (R11K/R13K/R15K/R17K) did not. (E) Rapamycin induced the interaction between human FKBP12 and FRB. The control contained 0.0009% dimethyl sulfoxide (DMSO). (F) The interaction between p53 1-50 and MDM2 1-118 was inhibited upon nutlin-3 treatment. The control contained 0.05% DMSO. The scale bar is 10 μm.

Figure 3. ReLo identifies direct PPIs.

(A) Subunit organization of the *Drosophila* CCR4-NOT core complex. (B) NOT1-mCherry-PH recruited CAF1, CAF40, NOT2, and NOT3 to the plasma membrane but not CCR4. See **Supplemental Figure 3A** for the PPI analysis of the other CCR4-NOT complex subunits. (C) Results summary of the pairwise screen of CCR4-NOT complex core subunits using ReLo. Blue, interaction, and gray, no interaction. (D) Schematic showing ReLo assay using coexpression of four different protein constructs. (E) NOT3 interacted with CAF1 in the presence of NOT1. (F) NOT3 interacted with CAF40 in the presence of NOT1. (G) NOT3 recruited CCR4 to the plasma membrane only in the presence of both NOT1 and CAF1. The scale bar is 10 μ m.

Figure 4. PPIs between the CCR4-NOT complex and repressor proteins.

Results summary (A) of the pairwise screen of CCR4-NOT complex core subunits and Bam (B), Nanos (C), Roquin (D), MARF1 (E), and other repressor proteins. Blue, interaction, and gray, no interaction. (F) Cup interacted with Bruno and Nanos. (G) Bruno interacted with Nanos in the absence of Cup but not in its presence. The scale bar is 10 μ m.