A GID E3 ligase assembly ubiquitinates an Rsp5 E3 adaptor and regulates plasma membrane transporters

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Abstract

Cells rapidly remodel their proteomes to align their cellular metabolism to environmental conditions. Ubiquitin E3 ligases enable this response, by facilitating rapid and reversible changes to protein stability, localization, or interaction partners. In Saccharomyces cerevisiae, the GID E3 ligase regulates the switch from gluconeogenic to glycolytic conditions through induction and incorporation of the substrate receptor subunit Gid4, which promotes the degradation of gluconeogenic enzymes. Here, we show an alternative substrate receptor, Gid10, which is induced in response to changes in temperature, osmolarity, and nutrient availability, regulates the ART-Rsp5 ubiquitin ligase pathway, a component of plasma membrane quality control. Proteomic studies reveal that the levels of the adaptor protein Art2 are elevated upon GID10 deletion. A crystal structure shows the basis for Gid10-Art2 interactions, and we demonstrate that Gid10 directs a GID E3 ligase complex to ubiquitinate Art2. Our data suggest that the GID E3 ligase affects Art2-dependent amino acid transport. This study reveals GID as a system of E3 ligases with metabolic regulatory functions outside of glycolysis and gluconeogenesis, controlled by distinct stress-specific substrate receptors.

Keywords GID; metabolism; nutrient signaling; Rsp5; ubiquitin E3 ligase

Introduction

The ubiquitin system is an integral part of cellular responses to environmental changes. The post-translational modification of proteins with ubiquitin modulates virtually every cellular pathway and all aspects of protein fate, including gene expression, and protein activity, stability, localization, and binding partners (Varshavsky, 2012). Because of their wide-reaching effects, substrate selection by E3 ubiquitin ligases must be strictly controlled to maintain cellular homeostasis upon environmental perturbations (Sui et al., 2020). Indeed, the cell simultaneously employs several control mechanisms to ensure faithful selection of E3 ligase substrates, but how these mechanisms are coordinated across cellular pathways remains poorly understood.

The transfer of one or more ubiquitins to substrate proteins requires a hierarchical pathway, in which ubiquitin is first activated by an E1 ubiquitin activating enzyme, and then transferred to an E2 ubiquitin conjugating enzyme, which together with an E3 ubiquitin ligase covalently attaches ubiquitin to the substrate (Cappadocia & Lima, 2018). The two largest E3 ligase families are the HECT (Homologous to the E6AP Carboxyl Terminus)-type E3 ligases, which first pass the ubiquitin from the E2 to the E3 before substrate attachment, and the RING (Really Interesting New Gene) E3 ligases, which facilitate the direct transfer of ubiquitin from the E2 to a remotely bound substrate (Deshaies & Joazeiro, 2009; Rotin & Kumar, 2009).

E3 ligases can encompass substrate binding and a catalytic HECT or RING domain all within a single subunit, or substrates can be recruited through receptor subunits. Although many HECT E3 ligases are thought to be single subunit enzymes, some members of the Nedd4 family employ adaptor proteins for substrate selection (Duprè et al., 2004; Foot et al., 2008; Lin et al., 2008; Mund & Pelham, 2009; Trimpert et al., 2017). Although constellations of WW domains in Nedd4 family E3 ligases directly recognize PYx(Y/F) motifs in some substrates, in some cases, an intervening PYx(Y/F)-containing adaptor bridges the E3 ligase and substrate (Huibregtse et al., 1993; Lu et al., 1999; Ogunjimi et al., 2005; Polo & Di Fiore, 2008; Persaud et al., 2014). Nedd4 family adaptor proteins can also modulate the E3’s sub-cellular localization, which can promote ubiquitination of specific substrates while sequestering the ligase away from others (Plant et al., 2000; Hettema et al., 2004; Shearwin-Whyatt et al., 2006). In the budding yeast Saccharomyces cerevisiae, Rsp5 is the sole Nedd4 family E3 ubiquitin ligase, and its PYx(Y/F)-containing
adaptor proteins, termed ARTs (Arrestin-Related Trafficking adaptors), are best recognized for regulating endocytosis of plasma membrane nutrient transporters to serve cellular metabolic needs (Lin et al., 2008; Lauwers et al., 2010; Zhao et al., 2013). The ART family consists of 14 such adaptor proteins, in a complex network involving activation in response to specific environmental stimuli (Becuwe et al., 2012; Ivashov et al., 2020; Kahlhofer et al., 2020).

Nutrient signaling originating at the plasma membrane also simultaneously regulates cellular synthesis of metabolites. For example, in the absence of glucose, glucose transporters are rapidly endocytosed, and the cell additionally initiates transcriptional and translational programs to promote gluconeogenesis. When glucose becomes available again, cells rapidly restore glucose transporters to the plasma membrane, terminate gluconeogenesis, and resume translational programs to promote gluconeogenesis. When glucose is the more energetically favorable glycolysis (Gancedo, 1998; Barnett et al., 2012), nutrient signaling originating at the plasma membrane also transiently induces the expression of the SRs Gid4 and Gid10. Each SR is transiently induced under distinct environmental conditions, turned-over in a manner which depends on itself, and can influence binding of the other SR. Furthermore, using a mass spectrometry (MS)-based method (Karayel et al., 2020), we identify the ART-Rsp5 network as a novel regulatory target of GIDSR10, demonstrating cross-talk between the two E3 ligase pathways.

**Results**

**Gid10 has hallmark features of a GID E3 substrate receptor in vivo**

Previous studies implicated Gid10 as a SR of the GID E3 ligase. For example, it has been shown that both Gid4 and Gid10 bind the GIDAnt scaffolding subunit Gid5 (Menssen et al., 2012; Melnykov et al., 2019; Qiao et al., 2020). In addition, a high resolution cryo-EM structure of GIDSR4 showed Gid4 binding a concave surface of Gid5, through key interactions mediated by its C-terminal tail, and a low resolution structure demonstrated that Gid10 forms a homologous complex (Qiao et al., 2020). Consistent with this, a yeast two-hybrid analysis confirmed that both Gid10 and Gid4 bind directly to Gid5 (Fig 1A). To investigate if the same intermolecular interactions are required for Gid4 and Gid10 binding, we probed the effect of structure-based mutants in the Gid5-SR binding interface. While Gid4 and Gid10 were able to bind WT GIDAnt to a similar extent, binding was significantly abrogated to GIDAnt containing Gid5 point mutations (Gid5W606A, Y613A, Q649A) on the concave binding surface (Fig 1B), which also disrupts ubiquitination by GIDSR4 (Qiao et al., 2020). Furthermore, deletion of the C-terminal residues in Gid4 or Gid10 also significantly reduced the binding of each SR to GIDAnt (Fig 1B), indicating that Gid4 and Gid10 bind to the same surface on Gid5 through homologous residues on each SR.

Gid4 and Gid10 share many sequence and structural elements (Appendix Fig S1) and might carry out redundant functions in the cell. Indeed, GIDSR10 is capable of ubiquitinating Mdh2 in vitro, albeit to a lesser extent than GIDSR4 (Qiao et al., 2020). However, Gid10 is unable to substitute for loss of Gid4 in vivo to promote Mdh2 or Fbp1 degradation, even when placed under the control of the Gid4 promoter (Melnykov et al., 2019). Because ubiquitination of Mdh2 in vitro by GIDSR10 is less efficient than by GIDSR4, higher levels of Gid10 might be required to compensate for lack of Gid4 in vivo. To test this, we employed the promoter reference technique, which allows the fate of existing proteins to be monitored without the use of global transcription or translation inhibitors (Chen et al., 2017; Oh et al., 2017). During the switch between gluconeogenic and glycolytic conditions, the GIDSR4 substrates Mdh2 and Fbp1 are significantly stabilized in a Gid4 deletion strain, but not in a Gid10 deletion strain (Figs 1C and EV1A), in agreement with previously published results (Santt et al., 2008; Melnykov et al., 2019). In addition, constitutive overexpression of Gid10 from the Tdh3 promoter alone did not significantly alter the rate of Mdh2 degradation, and could not compensate for loss of Gid4 (Fig 1C). Thus, even overexpressed Gid10 is not competent to promote recognition or degradation of Gid4 substrates during carbon recovery in vivo.
Figure 1. Gid4 and Gid10 expression is regulated by the GID E3 ligase.

A Yeast two-hybrid interactions between SR-Gal4 activation domain (AD) and Gid5-DNA binding domain (DBD). Growth on -His-Ade+Aureobasidin A (AbA) is indicative of an interaction between the two test proteins. Spots represent 1.5 serial dilutions.

B Streptactin pull-down of GIDAnt (strept-tagged at Gid8 C-terminus) probing binding of Gid10\(^{57,202}\) and Gid4\(^{177,362}\) to the complex visualized with Coomassie-stained SDS-PAGE. The experiment was performed with WT and C-terminal deletion (AC) of the substrate receptors (Δ289–292 (FEIA) and Δ359–362 (FEIA) for Gid10 and Gid4, respectively) and WT and mutant (mut, Gid4\(^{PGK}\)) strains overexpressing (OE) Gid10 and Gid4 strains respectively and WT and mutant (mut, Gid10ΔC) strains. Growth on -His-Ade and Gid10ΔC strains expressing endogenously tagged -C-terminal deletion (ΔC) and Gid8-2xS, Gid9, Gid8-2xS, Gid2, Gid4/Gid10, Gid4/Gid10-α-FLAG, 3xFLAG-Gid4, 3xFLAG-Gid10 and 3xFLAG-Gid10-α-FLAG. Growth on -His-Ade and 3xFLAG-Gid10-α-FLAG and 3xFLAG-Gid4 or 3xFLAG-Gid10 that were grown in SD complete at 30°C (no stress), SD complete for 30°C (no stress), SD complete supplemented with 0.5 M NaCl for 1 h (HS), SD complete supplemented with 0.5 M NaCl for 1 h (OS), or SD-N for 1 h (-N) were run on an SDS-PAGE gel and immunoblotted with α-FLAG (two exposures from the same gel are shown) and α-PGK.

C Tetracycline reference-based chase performed during transition from ethanol to glucose media with wildtype, ΔGid4 and ΔGid10 strains, and in wildtype and ΔGid4 strains overexpressing (OE) Gid10. Points represent mean, error bars represent standard deviation (n \(>\) 3 biological replicates).

D Lysates from wildtype and ΔGid2 yeast strains expressing endogenously tagged 3xFLAG-Gid4 or 3xFLAG-Gid10 that were grown at 30°C for the indicated timepoints were run on an SDS-PAGE gel and immunoblotted with α-FLAG and α-PGK.

E Lysates from wildtype and ΔGid2 yeast strains expressing endogenously tagged 3xFLAG-Gid4 or 3xFLAG-Gid10 that were grown at 30°C for the indicated timepoints were run on an SDS-PAGE gel and immunoblotted with α-FLAG and α-PGK.

F Lysates from wildtype and ΔGid2 yeast strains expressing endogenously tagged 3xFLAG-Gid10 that were grown at 30°C for 1 h, and then returned to 30°C for the indicated timepoints were run on an SDS-PAGE gel and immunoblotted with α-FLAG and α-PGK.

G Lysates from wildtype, Gid4\(^{V666A/Y661A/Q664A}\) (Gid4\(^{mut}\)), and Gid10\(^{3289–292}\) (Gid10\(^{AC}\)) strains expressing endogenously tagged 3xFLAG-Gid10 that were grown at 42°C for 1 h, and then returned to 30°C for the indicated timepoints were run on an SDS-PAGE gel and immunoblotted with α-FLAG and α-PGK.
While Gid10 protein levels are not induced during carbon starvation or recovery, they are transiently induced in response to a variety of other stress conditions, including heat shock, osmotic shock, and amino acid and nitrogen starvation (Figs 1D and EV1B–G) (Gasch et al., 2000; Melnykov et al., 2019; Qiao et al., 2020). Interestingly, while Gid10 is induced during heat shock, Gid4 is transiently induced during recovery from heat shock (Fig 1E), suggesting complementary roles of the two SRs during stress and recovery. To gain a better understanding of how the transient expression of SRs is regulated, we first examined the requirements of the GID complex for SR turnover. All of the subunits of GIDAnt were previously shown to be required for GID complex assembly, we first examined the requirements of the GID complex for SR turnover. All of the subunits of GIDAnt were previously shown to be required for GID complex assembly, and therefore better able to compete for potential substrates. Following a 1h heat shock at 42°C, only 0.01 and fold change > 4, Fig 3A) (Lu et al., 1996; Lussier et al., 1997). Importantly, regulation of both proteins was Gid10-specific as their abundance did not change in a Gid4 deletion strain (Fig 3B). To determine whether Art2 or Nhp10 might also be regulated under other growth conditions where the GID E3 ligase is known to be active, we reanalyzed our previously published data set characterizing GID-dependent protein regulation during recovery from ethanol starvation (Karayel et al., 2020). We selected for proteins that contain a proline in position 2 or 3, and are significantly upregulated during growth in ethanol, compared with glucose, and downregulated during recovery compared to ethanol. Interestingly, during recovery from ethanol starvation, Art2 also appears to be regulated by Gid2 (although this did not reach statistical significance), but not by Gid4 (Fig EV2A). In addition, constitutive overexpression of Gid10 during recovery from carbon starvation results in decreased levels of Art2 (Fig EV2B).

GIDSR4 is an N-degron E3 ligase that recognizes substrates with an N-terminal proline, although peptides with other N-terminal residues have been shown to bind Gid4 or Gid10 with lower affinity (Hämmerle et al., 1998; Chen et al., 2017; Dong et al., 2018, 2020; Melnykov et al., 2019). On this basis, we probed the potential for Gid10 interaction to bind the Art2 or Nhp10 N-terminal sequences by yeast two-hybrid. Gid10 efficiently bound the Art2, but not the Nhp10, N-terminus (Figs 4A and EV3A). Moreover, the Gid10-Art2 interaction is Gid10-specific as we did not observe an interaction between Gid4 and the Art2 N-terminus (Fig 4A), and dependent on the N-terminal proline of Art2 (Fig 4B). In contrast, Gid4, but not Gid10, interacted with the N-terminus of the classic Gid4 substrate Mdh2 (Fig 4A), suggesting that Gid10 and Gid4 indeed prefer discrete regulatory targets.

To further confirm Gid10 binding to the Art2 N-terminus, we quantified the interaction using isothermal titration calorimetry. The putative Gid10 substrate-binding domain (Gid101-56) bound the first 9 amino acids of Art2 (PFITSRPVA) synthesized with a C-terminal tryptophan to enable concentration determination based on extinction coefficient at 280 nm, with a K_D of 1.03 μM (Fig 4C). Notably, this is 2-fold higher affinity than any published peptide examined for interaction with Gid4 (Dong et al., 2018, 2020).

To determine the molecular mechanism of this interaction, we determined a crystal structure showing degron recognition by Gid10. A co-crystal structure of Gid101-56 bound to a peptide corresponding to the first seven residues of Art2 (PFITSRPVA) synthesized with a C-terminal tryptophan to enable concentration determination based on extinction coefficient at 280 nm, with a K_D of 1.03 μM (Fig 4C). Notably, this is 2-fold higher affinity than any published peptide examined for interaction with Gid4 (Dong et al., 2018, 2020). The structures of the two SRs are nearly identical, with an RMSD of 0.87 Å (Fig EV3B and C). The structures of the two SRs are nearly identical, with an RMSD of 0.87 Å (Fig EV3B and C). The structures of the two SRs are nearly identical, with an RMSD of 0.87 Å (Fig EV3B and C). The structures of the two SRs are nearly identical, with an RMSD of 0.87 Å (Fig EV3B and C). The structures of the two SRs are nearly identical, with an RMSD of 0.87 Å (Fig EV3B and C). The structures of the two SRs are nearly identical, with an RMSD of 0.87 Å (Fig EV3B and C). The structures of the two SRs are nearly identical, with an RMSD of 0.87 Å (Fig EV3B and C).

To further characterize Art2 as a GIDSR10 substrate, we asked if GidSR10 was capable of ubiquitinating Art2. Towards this end, we performed ubiquitination assays using Art2-3xFLAG immunoprecipitated from yeast lysates, and reconstitantly expressed GIDAnt, GIDSR10, or GIDSR4. In this system, GIDSR10, but not GIDSR4 or GIDAnt, was able to efficiently poly-ubiquitinate Art2. In contrast,
only GIDSR4 was capable of ubiquitinating Mdh2 (Fig 5A and B). Moreover, GIDSR10-mediated ubiquitination of Art2 is dependent on Gid10 binding to GidSR10, and the ability of Art2 to bind Gid10 via its N-terminal proline (Fig 5C). Of all ubiquitin’s lysines, only K48 was able to support robust poly-ubiquitination of Mdh2 by GIDSR4 (Qiao et al., 2020). To test if the molecular mechanism of GIDSR10 poly-ubiquitination is similar, we tested the effect of ubiquitin mutants of K48 and non-K48 ubiquitin chains. To further confirm the ubiquitination activity of GIDSR10 toward Art2, we used a peptide substrate consisting of residues 2–29 of Art2, which includes an endogenously ubiquitinated lysine at position 26 (Swaney et al., 2013). In the presence of GIDSR10, we observed poly-ubiquitination of this peptide substrate, which was dependent on the N-terminal proline. GIDSR4 also mediated low-level ubiquitination of the peptide substrate (Fig 5D), which suggests the potential to interact in the context of a fully-assembled E3. Taken together, our results indicate that Art2 is a substrate of GIDSR10.

**A GID phenotype for amino acid uptake**

Art2 is one of a suite of ART adaptors that guides the Nedd4 family E3 ubiquitin ligase Rsp5 to plasma membrane transporters, and directs their selective endocytosis (Lauwers et al., 2010; Babst, 2020). Intriguingly, Rsp5, the Rsp5-associated deubiquitinase Ubp2, and the σ-arrestin Art3 also contain a proline at position 2, and therefore may parallel other GID E3 substrates and be processed by mithionine aminopeptidase to exist in cells with an N-terminal proline. Thus, we tested if Gid10 and/or Gid4 bind the N-terminal sequences of Rsp5, Ubp2 or members of the σ-arrestin family. However, our yeast two-hybrid assay revealed only the Gid10-Art2 interaction, further highlighting its specificity (Fig EV4B–E).

Compared with other ARTs, the functions of Art2 are relatively poorly characterized, in part because of redundancy with other more functionally dominant family members, and in part because its relatively large size and protein properties make biochemical...
and an Art2P2S mutant also showed delayed growth on thialysine stabilized at the plasma membrane. Importantly, both Art2 deletion rationale that cells will be hypersensitive to thialysine when Lyp1 is plays a role in Lyp1 regulation by examining phenotypes on the 6 al starvation, nitrogen starvation, and cycloheximide treatment (Lin et al., 2008; Zhao et al., 2013; Baile et al., 2019), suggesting that GID-dependent regulation of Art2 may not be the main mechanism to promote Lyp1 import during heat stress. Indeed, we observe that thialysine toxicity is increased and Lyp1 import and degradation is reduced in an ART1 deletion, compared with wildtype, and a double deletion of ART1 and ART2 is further impaired (Fig EV5B and C). These data suggest that Art1 is the main regulator of Lyp1 during heat stress, but Art2 also contributes to this regulation. Notably, as observed for ART1ART2 double deletion, in the absence of Art1, deletion of a core subunit or Gid10 resulted in increased toxicity during growth on thialysine (Fig 6D and E).

Deletion of the GID core subunits Gid2 or Gid5 results in impaired Lyp1 import and degradation in the ΔArt1 background (Fig EV5D). Interestingly, when compared with single deletions, deletion of both Art2 and a GID core subunit resulted in a slightly stronger phenotype on thialysine (Fig EV5E), and slightly greater impairment in Lyp1 import and degradation (Fig EV5F and G), raising the possibility that the GID E3 ligase plays both Art2-dependent and Art2-independent roles in plasma membrane nutrient transporter regulation. Taken together, these data indicate that GID$^{SR10}$, and to a lesser extent GID$^{SR4}$, modulate Art2 function to affect the flux of plasma membrane nutrient transporters; however, future experiments will be required to identify the exact mechanism.

**Genetic interaction between the GID E3 ligase and an Art2-Rsp5 pathway**

Art2 recruits Rsp5 to plasma membrane nutrient transporters and thereby promotes their endocytosis. Thus, to better understand the molecular mechanism although which the GID ligase regulates plasma membrane nutrient transporters, we probed the genetic interaction between the GID ligase, Art2, and Rsp5. Notably, ubiquitination of other Rsp5 adaptor proteins promotes their interaction with Rsp5 in a manner depending on Rsp5’s ubiquitin-binding exosite (MacDonald et al., 2020). Thus, one intriguing possibility is that ubiquitinated Art2 may also employ this exosite in binding to Rsp5. Ubiquitin binding to such HECT E3 exosites has pleiotropic mechanistic roles, including allosterically activating ubiquitin transferase activity, contributing to processivity of the analyses challenging. Nonetheless, Art2 has been associated with endocytosis and vacuolar degradation of the lysine permease, Lyp1, during some environmental perturbations, including amino acid starvation, nitrogen starvation, and cycloheximide treatment (Lin et al., 2008; Ivashov et al., 2020). Thus, we tested if the GID complex plays a role in Lyp1 regulation by examining phenotypes on the toxic lysine analog, thialysine (S-Aminoethyl-l-cysteine), with the rationale that cells will be hypersensitive to thialysine when Lyp1 is stabilized at the plasma membrane. Importantly, both Art2 deletion and an Art2$^{P2S}$ mutant also showed delayed growth on thialysine (Fig 6A), suggesting that the Art2 deletion effect can at least partly be attributed to regulation by the GID E3 ligase. Furthermore, individual deletions of all GID core subunits, with the exception of Gid7, which is dispensable for some substrates (Negoro et al., 2020; Kong et al., 2021; Sherpa et al., 2021), as well as a double deletion of GID4 and GID10 resulted in cellular toxicity during growth on thialysine, even in the absence of an additional stress condition (Fig 6B and C). The requirement for both SRs (Fig 6C) suggests that there may be some overlap in SR function in vivo, consistent with the low level of GID$^{SR4}$ activity observed in the in vitro ubiquitination assay (Fig 4D). The similar phenotypes for all core subunits suggest a role in regulation of Lyp1 receptor localization or activity.

Thus, we used a GFP protection assay to determine whether the GID E3 ligase impacts Lyp1 import and degradation. This assay takes advantage of the fact that GFP is resistant to vacuolar degradation, but proteins fused to it typically are not. Thus, vacuolar degradation of GFP-tagged proteins can be monitored by Western blot, where undegraded protein will appear as a full-length-GFP band, and the appearance of a free GFP band reflects delivery of the GFP-tagged protein to the vacuole (Cheong & Klionsky, 2008). Surprisingly, performing this assay with Lyp1-GFP showed that Lyp1 import and degradation during heat shock remained unchanged in the GID mutant strains (Fig EV5A), despite the effect of these mutations on yeast growth on thialysine. In addition to Art2 regulation of Lyp1, another ART protein, Art1, has also been shown to regulate Lyp1 import and degradation in response to lysine excess, thialysine treatment, and heat stress (Lin et al., 2008; Zhao et al., 2013; Baile et al., 2019), suggesting that GID-dependent regulation of Art2 may not be the main mechanism to promote Lyp1 import during heat stress. Indeed, we observe that thialysine toxicity is increased and Lyp1 import and degradation is reduced in an ART1 deletion, compared with wildtype, and a double deletion of ART1 and ART2 is further impaired (Fig EV5B and C). These data suggest that Art1 is the main regulator of Lyp1 during heat stress, but Art2 also contributes to this regulation. Notably, as observed for ART1ART2 double deletion, in the absence of Art1, deletion of a core subunit or Gid10 resulted in increased toxicity during growth on thialysine (Fig 6D and E).
poly-ubiquitination reaction, and recruiting ubiquitinated partner proteins including adaptor proteins. Nonetheless, an Rsp5 point mutant (F618A) that impairs ubiquitin binding is useful for probing genetic interactions (French et al., 2009; Kim et al., 2011; Maspero et al., 2011; Kathman et al., 2015; Zhang et al., 2016).

While the Rsp5F618A mutation alone showed a strong growth defect on thialysine, the combination of a deletion of a GID core subunit and Rsp5F618A resulted in stronger toxicity on thialysine than either mutation alone (Fig6F), suggesting that GID-dependent ubiquitination of Art2 is acting independently from the ubiquitin binding function of Rsp5. Next, we tested if GID-dependent Art2 ubiquitination might affect its ability to interact with Rsp5 though the canonical WW-PPx(Y/F) interaction motifs. While Rsp5 and Art2 contain multiple WW and PPx(Y/F) domains, respectively, the Rsp5-Art2 interaction is primarily mediated by the third WW domain of Rsp5 and the second PPx(Y/F) domain of Art2 (Ivashov et al., 2020). The combination of a core GID subunit deletion and either a mutation in the second Art2 PPx(Y/F) motif or the third WW Rsp5 domain did not result in stronger toxicity on thialysine than either mutation alone (Fig 6G and H), suggesting that the effect of the GID E3 ligase is dependent on the canonical WW-PPx(Y/F) interaction. Taken together, our data suggest that the GID E3 ligase ubiquitinates Art2 and thereby affects its ability to interact with Rsp5.

Discussion

Here, we demonstrate that the GID E3 ligase is a multifunctional metabolic regulator that incorporates different SRs in response to distinct stresses. We show that Gid10 is a bona fide substrate receptor by identifying Art2 as a protein that binds Gid10 through specific contacts directed by its N-terminal proline, and is ubiquitinated by GIDSR10. Furthermore, we identify for the first time a
Through ubiquitination of Art2, the GID E3 could affect the activity of another E3, Rsp5. Indeed, Art2 is a modulator of Rsp5, and we found that GID influences the import and degradation of the Rsp5 target Lyp1. This regulation depends on the interaction between the Art2 PPx(Y/F) domain and the Rsp5 WW domain, although the exact mechanism remains elusive. In addition, reversing the activity of Gid2 has been shown to disrupt growth on low-tryptophan media (MacDonald et al., 2017), implicating GID in the regulation of even more plasma membrane transporters. Both plasma membrane protein trafficking and Rsp5 interactions are intricately regulated by ubiquitin. Thus, Art2 ubiquitination could impact the ART-Rsp5 network in several ways. First, ubiquitination of Art2 by GIDSR10 may affect its protein-protein interactions or activities. Ubiquitinated Art2 may also selectively affect its ability to interact with its plasma membrane targets, leading to a higher affinity for some targets, but a lower affinity for others. Future studies will be required to identify precisely how post-translational modifications modulate the ART-Rsp5 network and the molecular details underlying the relationship between the GID E3 ligase and plasma membrane nutrient transporters.

Figure 5. GidSR10 ubiquitates Art2.
A Art2-3xFLAG was immunocaptured from yeast cells grown in YPD and incubated with GIDSR10, GIDSR4, GIDAnt, or Gid10 for the indicated timepoints. Progress of the reaction was followed by aFLAG immunoblot.
B Mdh2-3xFLAG was immunocaptured from yeast cells following growth in YPE for 19 h and incubated with GIDSR10, GIDSR4, GIDAnt, or Gid4 for the indicated timepoints. Progress of the reaction was followed by aFLAG immunoblot.
C Art2-3xFLAG or Art2PPS-3xFLAG was immunocaptured from yeast cells grown in YPD and incubated with GIDSR10, or GIDSR10ΔC for the indicated timepoints. Progress of the reaction was followed by aFLAG immunoblot.
D In vitro ubiquitination assay probing the ability of Gid10 and Gid4 to promote ubiquitination of Art2-29 N-terminus and its P25 mutant. Progress of the reaction was monitored by fluorescent scan of the gel visualizing the Art2 peptide with FAM appended to its C-terminus (pep*).
Interestingly, previous studies have also linked the GID E3 ligase to regulation of plasma membrane proteins. First, some GID complex subunits have been shown to play a role in the Vacuolar Import and Degradation (VID) pathway, which brings proteins to the vacuole for degradation following their endocytosis from the plasma membrane (Brown et al., 2010; Giardina et al., 2013). Second, it has been shown that the signals which lead to the degradation of Fbp1 and the hexose transporter Gal2 likely originate from the same biochemical pathway (Horak et al., 2002). Third, Gid11 was recently identified as an additional SR of the GID complex. The Gid11 protein also regulates metabolic enzymes involved in amino acid and nucleotide biosynthesis (Kong et al., 2021), and deletion of GID11 leads to defects in plasma membrane electron transport (Herst et al., 2008), suggesting an additional role for Gid11 in regulation of plasma...
membrane proteins. Importantly, expression of each SR is only induced during a distinct subset of environmental perturbations. Because each environmental change leads to vast, but distinct, remodeling of cellular metabolism, we propose that the GID E3 ligase may have evolved as a common node to regulate nutrient import across the plasma membrane and subsequent cellular synthesis of the necessary metabolites.

Although the GID E3 ligase has long been characterized as functioning during glucose-induced glycolysis, we show that the GID E3 ligase additionally regulates amino acid transporters, and also that there is a GID phenotype linked to amino acid metabolism, similar to effects observed when deleting ART proteins. What advantages might arise from a singular core E3 complex with distinct inputs from and outputs to multiple metabolic pathways? Because environmental changes are often abrupt, the activation of the complex through incorporation of a single protein allows yeast cells to respond rapidly. In addition, the transient expression of SRs, which we show depends on both GID complex activity and SR-GID\textsuperscript{SR} binding, ensures that substrate selection by GID\textsuperscript{SR} is limited to a pulse following a switch in environmental conditions. Thus, the GID E3 ligase employs rapid “on” and “off” switches which allow it to rapidly and specifically respond to environmental perturbations.

We speculate that the GID E3 may be poised like other post-translational modifying enzymes that serve as metabolic nodes. For example, abundance or paucity of particular metabolites regulate kinase activities of mTOR, which like the GID E3 assembles into different complexes to regulate specific sets of biosynthetic and catabolic processes. Although the GID E3 is not essential in yeast under normal growth conditions, our work suggests there could be distinct requirements under particular environments. Moreover, the GID complex in higher eukaryotes, (termed CTLH—for C-Terminal to LiS) regulates important physiology and is essential for viability (Salemi et al., 2017; Lampert et al., 2018). Intriguingly, the CTLH complex serves as a regulator of autophagic flux and mTOR signaling, key pathways that integrate cellular responses to environmental changes (Liu et al., 2020). While more studies are needed to characterize additional GID regulatory targets, it is clear that the GID E3 ligase is implicated in diverse cellular pathways throughout eukaryotes and serves to enable rapid and robust cellular responses to environmental perturbations.

Materials and Methods

All plasmids and yeast strains used in this study are listed in Tables 1 and 2, respectively.

Yeast strains and growth conditions

All yeast strains were constructed using standard techniques (Knop et al., 1999; Storici & Resnick, 2003; Janke et al., 2004). Yeast were grown inYPD (1% yeast extract, 2% peptone, 2% glucose) or SD complete (0.67% yeast nitrogen base without amino acids, 2% glucose, containing 87.5 mg/l alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, leucine, lysine, methionine, myo-inositol, isoleucine, phenylalanine, proline, serine, threonine, tyrosine and valine, 43.7 mg/l histidine, tryptophan and uracil.

### Table 1. Plasmids used in this study.

| Plasmid   | Description                                      | Reference      |
|-----------|--------------------------------------------------|----------------|
| pCSJ95    | pRS313-PDsH(Mouse-Gid)-Fbp1-3XFLAG-CYC-P(DHFR)   | Chen et al (2017) |
| pCSJ125   | pRS313-PDsH(Mouse-Gid)-Mdh2-3XFLAG-CYC-P(DHFR)   | Chen et al (2017) |
| pGADCg    | Y2H expression vector. Contains the P\textsubscript{Adh1} promoter, used to produce Gal4-AD-HA fusions. | Addgene (Cat#20161) |
| pGBKcG    | Y2H expression vector. Contains the P\textsubscript{Adh1} promoter, used to produce Gal4-DBD-Myc fusions. | Addgene (Cat#20162) |
| pCSJ182   | pGADCg-NLS-Gid4-3xFLAG-Gal4-AD                  | Chen et al (2017) |
| pCSJ392   | pGADCg-NLS-Gid10-3xFLAG-Gal4-AD                 | Melnykov et al (2019) |
| CRPL81    | pGBKcG-Gids-Gal4-DBD                             | This study      |
| CRPL83    | pGADCg-DHFR-Gal4-AD                              | This study      |
| VBP43     | pGBKcG-Rsp1\textsuperscript{1-10}-DHFR-Gal4-DBD | This study      |
| VBP44     | pGBKcG-Nhp1\textsuperscript{1-10}-DHFR-Gal4-DBD | This study      |
| VBP45     | pGBKcG-Art1\textsuperscript{1-10}-DHFR-Gal4-DBD | This study      |
| VBP55     | pGBKcG-Mdh2\textsuperscript{1-10}-DHFR-Gal4-DBD | This study      |
| VBP57     | pGBKcG-Fbp1\textsuperscript{1-10}-DHFR-Gal4-DBD | This study      |
| VBP58     | pGBKcG-DHFR-Gal4-DBD                             | This study      |
| VBP60     | pGBKcG-Art2\textsuperscript{1-10}-PDT-DHFR-Gal4-DBD | This study |
| VBP69     | pGBKcG-Art1\textsuperscript{1-10}-DHFR-Gal4-DBD | This study      |
| VBP70     | pGBKcG-Art3\textsuperscript{1-10}-DHFR-Gal4-DBD | This study      |
| VBP71     | pGBKcG-Art4\textsuperscript{1-10}-DHFR-Gal4-DBD | This study      |
| VBP72     | pGBKcG-Art5\textsuperscript{1-10}-DHFR-Gal4-DBD | This study      |
| VBP73     | pGBKcG-Art6\textsuperscript{1-10}-DHFR-Gal4-DBD | This study      |
| VBP74     | pGBKcG-Art7\textsuperscript{1-10}-DHFR-Gal4-DBD | This study      |
| VBP75     | pGBKcG-Art8\textsuperscript{1-10}-DHFR-Gal4-DBD | This study      |
| VBP76     | pGBKcG-Art9\textsuperscript{1-10}-DHFR-Gal4-DBD | This study      |
| VBP77     | pGBKcG-Art10\textsuperscript{1-10}-DHFR-Gal4-DBD | This study      |
| VBP78     | pGBKcG-Bul\textsuperscript{1-10}-DHFR-Gal4-DBD  | This study      |
| VBP79     | pGBKcG-Bul\textsuperscript{2-10}-DHFR-Gal4-DBD  | This study      |
| VBP80     | pGBKcG-Ubp\textsuperscript{2-10}-DHFR-Gal4-DBD  | This study      |
| JCD501    | pLIB-GidS                                       | Qiao et al (2020)   |
| JCD502    | pLIB-GidS-W606A/Y613A/Q649A                     | Qiao et al (2020)   |
| JCD503    | pBIG2-Gid1.Gid8-TEV-2xS.Gid8.Gid2.Gid9         | Qiao et al (2020)   |
| JCD504    | pBIG2-Gid1.Gid8-TEV-2xS.Gid2.Gid9              | Qiao et al (2020)   |
| JCD505    | pGEX-GST-TEV-Gid4 (117-362)                    | Qiao et al (2020)   |
| JCD506    | pGEX-GST-TEV-Gid4 (117-358)                    | Qiao et al (2020)   |
| JCD507    | pGEX-GST-TEV-Gid10 (57-292)                    | Qiao et al (2020)   |
Table 1 (continued)

| Plasmid | Description | Reference |
|---------|-------------|-----------|
| JD508   | pGEX-GST-TEV-Gid10 (57-288) | Qiao et al (2020) |
| JD509   | pGEX-GST-TEV-Gid10 (65-284) | This study |
| JD510   | pRSF-Ubc8-6xHis | Qiao et al (2020) |
| JD511   | PET3b-Ub | Qiao et al (2020) |
| JD512   | pUB-2x5-3c-Gid4 | This study |
| JD513   | pBIG2-Gid1-Gid8.Gid5.2x5-3c-Gid10.Gid2.Gid9 | This study |

22.5 mg/l adenine, and 8.7 mg/l para-aminobenzoic acid) media. Where plasmids are used, the appropriate amino acids were omitted from SD complete media. YPE and SE growth media indicate replacement of the glucose in YPD or SD complete, respectively, with 2% ethanol. For nutrient starvation, yeast cultures were grown to OD600 = 1.0 in SD complete, washed once with pre-warmed SD-AA (0.67% yeast nitrogen base without amino acids, 2% glucose, and 20 mg/l uracil) or SD-N (0.17% yeast nitrogen base without amino acids or ammonium sulfate, 2% glucose), resuspended in pre-warmed SD-AA or SD-N to an OD600 = 1, and grown for the indicated time-points. Unless otherwise specified, yeast cultures were grown at 30°C.

Yeast growth assays

For yeast two-hybrid experiments, pGADG and pGBKCG-based plasmids containing the indicated protein fusions were transformed into the yeast strain Y2HOLD (Takara Bio), and double transformants were selected by growth on SD media lacking leucine and tryptophan. Cells were then grown in SD media lacking leucine and tryptophan and supplemented with 0.1 mg/ml adenine to an OD600 of 1.0–2.0. A 240 µl dilution containing 0.096 ODs of cells was transferred to the first column of a sterile 96-well microtiter plate. Serial dilutions were made, at the dilutions specified, and yeast cells were spotted using a 48 Pin Multi-Blot Replicator (V&P Scientific VP480) on SD media lacking leucine and tryptophan, supplemented with 0.1 mg/ml adenine, and SD media lacking leucine, tryptophan, histidine and adenine (and, where indicated, supplemented with Aureobasidin A). Plates were grown at 30°C.

For growth assays on thialysine, yeast cells were grown in SD complete media to an OD of 1.0–2.0. Yeast cultures were diluted as described above and spotted on SD lacking lysine, or SD lacking lysine supplemented with thialysine at the indicated concentrations.

Yeast cell lysis and western blotting

Protein degradation assays using the promoter reference technique were done as previously described (Oh et al, 2017). Cells were transformed with a plasmid expressing a test substrate and DHFR from identical promoters containing tetracycline-repressible RNA-binding elements. Yeast cells were grown in SD media lacking histidine to an OD600 of 1.0–1.5, pelleted by centrifugation at 3,000 rpm for 3 min, washed once with pre-warmed SE media lacking histidine, resuspended to an OD600 of 1.0 in pre-warmed SE media lacking histidine, and grown for 19 h. Cells were then pelleted by centrifugation at 3,000 rpm for 3 min, resuspended to an OD600 of

Table 2. Yeast strains used in this study.

| Strain   | Genotype | Reference         |
|----------|----------|-------------------|
| BY4741   | MATα His3Δ11 leu2-3,112 met15Δ10 ura3Δ10 | Euroscarf (Cat#Y00000) |
| CRLY12   | BY4741 gid4Δ-KANMX | Sherpa et al (2021) |
| CRLY13   | BY4741 gid5Δ-KANMX | This study |
| CRLY14   | BY4741 gid7Δ-KANMX | Sherpa et al (2021) |
| CRLY15   | BY4741 gid8Δ-KANMX | This study |
| CRLY16   | BY4741 gid9Δ-KANMX | This study |
| CRLY17   | BY4741 gid10Δ-KANMX | This study |
| CRLY30   | BY4741 gid2Δ-KANMX | This study |
| CRLY68   | BY4741 gid4Δ3xFLAG-Gid4 | Qiao et al (2020) |
| CRLY74   | BY4741 gid10Δ3xFLAG-Gid10 | Qiao et al (2020) |
| CRLY186  | BY4741 gid1Δ-KANMX | This study |
| CRLY296  | BY4741 gid10Δ-NATNT2-Puro-Gid10 | This study |
| CRLY298  | BY4741 gid4Δ-KANMX gid10Δ-NATNT2-pGPD-Gid10 | This study |
| CRLY901  | BY4741 gid4Δ-KANMX gid10Δ3xFLAG-Gid10 | This study |
| CRLY314  | BY4741 gid10Δ3xFLAG-Gid10 gid5Δ-Gid5S-3xHA-KANMX | This study |
| CRLY326  | BY4741 gid4Δ3xFLAG-Gid4 gid5Δ-Gid5S-3xHA-KANMX | This study |
| CRLY353  | BY4741 art2ΔART2-3xFLAG-HPHNT1 | This study |
| CRLY365  | BY4741 gid10Δ3xFLAG-Gid10 gid8Δ-Gid8S-3xHA-KANMX gid4Δ-NATNT2 | This study |
| CRLY382  | BY4741 lyp1Δ-LYP1-GFP-HPHNT1 | This study |
| CRLY384  | BY4741 gid4Δ-KANMX lyp1Δ-LYP1-GFP-HPHNT1 | This study |
| CRLY386  | BY4741 gid10Δ-KANMX lyp1Δ-LYP1-GFP-HPHNT1 | This study |
| CRLY388  | BY4741 gid2Δ-KANMX lyp1Δ-LYP1-GFP-HPHNT1 | This study |
| CRLY407  | BY4741 art2ΔART2-3xFLAG-HPHNT1 | This study |
| CRLY431  | BY4741 art2Δ-KANMX | This study |
| CRLY434  | BY4741 art1Δ-NATNT2 | This study |
| CRLY435  | BY4741 art1Δ-NATNT2 can1Δ-CAN1-GFP-HPHNT1 | This study |
| CRLY451  | BY4741 art1Δ-NATNT2 lyp1Δ-LYP1-GFP-HPHNT1 | This study |
| CRLY453  | BY4741 art2Δ-KANMX art1Δ-NATNT2 lyp1Δ-LYP1-GFP-HPHNT1 | This study |
| CRLY458  | BY4741 art1ΔART2(P25) | This study |
| CRLY460  | BY4741 art2ΔART2(YP74AYP74AY750A) | This study |
| CRLY468  | BY4741 mdh2Δ-MDH2-3xFLAG-HPHNT1 | This study |
| CRLY473  | BY4741 gid5Δ-KANMX lyp1Δ-LYP1-GFP-HPHNT1 | This study |
| CRLY487  | BY4741 art2ΔART2(P25)-3xFLAG-HPHNT1 | This study |
| CRLY490  | BY4741 art2ΔART2(YP74AYP74AY750A) | This study |
Table 2 (continued)

| Strain         | Genotype                                                                 | Reference |
|----------------|--------------------------------------------------------------------------|-----------|
| CRLY492        | BY4741::art2::ART2(P748A,P749A,Y750A) gid5::KANMX                     | This study |
| CRLY507        | BY4741::art2::NATNT2-P_cpc::ART2 art1::KANMX                           | This study |
| CRLY517        | BY4741::art2::KANMX art1::NATNT2                                       | This study |
| CRLY520        | BY4741::gid5::KANMX art2::NATNT2                                       | This study |
| CRLY527        | BY4741::gid2::KANMX art2::NATNT2                                       | This study |
| CRLY569        | BY4741::gid4::3xFLAG-Gid4 gid10::NATNT2-P_cpc::GID10                   | This study |
| CRLY593        | BY4741::rsps::RSPS(F618A) gid2::HPHNT1                                  | This study |
| CRLY595        | BY4741::rsps::RSPS(F618A) gid5::HPHNT1                                 | This study |
| CRLY609        | BY4741::rsps::RSPS(W415F, P418A)                                        | This study |
| CRLY626        | BY4741::rsps::RSPS(W415F, P418A) gid2::HPHNT1                           | This study |
| CRLY628        | BY4741::rsps::RSPS(W415F, P418A) gid5::HPHNT1                           | This study |
| LHY146         | BY4741::gid4::NATNT2 gid10::KANMX                                      | This study |
| VBY104         | BY4741::gid4::3xFLAG-Gid4 gid5::GID5 (W606A,Y613A,Q649A)-3xHA-KANMX     | This study |
| VBY105         | BY4741::gid10::3xFLAG-GID10 gid5::GID5 (W606A,Y613A,Q649A)-3xHA-KANMX   | This study |
| VBY106         | BY4741::gid4::3xFLAG-Gid4(F359AF361A) gid5::GID5-3xHA-KANMX             | This study |
| VBY107         | BY4741::gid10::3xFLAG-GID10(A289-292) gid5::GID5-3xHA-KANMX             | This study |
| VBY109         | BY4741::gid4::GID4(F359AF361A) gid10-3xFLAG-GID10                        | This study |
| VBY110         | BY4741::gid4::3xFLAG-Gid4 gid2::KANMX                                  | This study |
| VBY111         | BY4741::gid4::3xFLAG-GID10 gid10::KANMX                                 | This study |
| VBY119         | BY4741::gid2::KANMX art1::HPHNT1                                       | This study |
| VBY120         | BY4741::gid4::KANMX art1::HPHNT1                                       | This study |
| VBY121         | BY4741::gid5::KANMX art1::HPHNT1                                       | This study |
| VBY124         | BY4741::gid10::KANMX art1::HPHNT1                                      | This study |
| VBY130         | BY4741::gid4::KANMX art1::NATNT2 lyp1::LYP1-GFP-HPHNT1                   | This study |
| VBY131         | BY4741::gid10::KANMX art1::NATNT2 lyp1::LYP1-GFP-HPHNT1                  | This study |
| VBY132         | BY4741::gid2::KANMX art1::NATNT2 lyp1::LYP1-GFP-HPHNT1                   | This study |
| VBY133         | BY4741::gid5::KANMX art1::NATNT2 lyp1::LYP1-GFP-HPHNT1                   | This study |
| VBY135         | BY4741::rsps::RSPS(F618A)                                               | This study |
| VBY155         | BY4741::gid4::KANMX gid10::NATNT2 art1::HISMX6 lyp1::LYP1-GFP-HPHNT1     | This study |
| VBY156         | BY4741::gid4::KANMX art1::NATNT2 can1::CAN1-GFP-HPHNT1                  | This study |
| VBY157         | BY4741::gid10::KANMX art1::NATNT2 can1::CAN1-GFP-HPHNT1                 | This study |
| VBY158         | BY4741::gid2::KANMX art1::NATNT2 can1::CAN1-GFP-HPHNT1                  | This study |

Table 2 (continued)

| Strain         | Genotype                                                                 | Reference   |
|----------------|--------------------------------------------------------------------------|-------------|
| VBY159         | BY4741::gid5::KANMX art1::NATNT2 can1::CAN1-GFP-HPHNT1                   | This study  |
| Y2HGOLD        | MA7a, trpl-901, leu2-3, 112, ura3-52, his3-200, gal4-1, gal80-1, lys2:GAL1::GAL2::His3, GAL2::GAL2::HIS3, -Me11::AUR1-C::MEL1 | Takara Bio  |

This study

Table (continued)

1.0 in SD media lacking histidine and allowed to recover for the indicated timepoints. At each timepoint, 1 OD of yeast cells was pelleted, supernatant removed, flash-frozen in liquid nitrogen and stored at —80°C until lysis.

For lysis, yeast cells were resuspended in 0.8 ml 0.2 M NaOH, incubated 20 min on ice, and then pelleted by centrifugation at 11,200 g for 2 min. The supernatant was removed and the pellet was resuspended in 50 μl HU buffer and heated at 70°C for 10 min. Lysates were then pre-cleared by centrifugation at 11,200 g for 5 min and loaded onto a 12% SDS-PAGE gel. Samples were transferred to nitrocellulose membranes and visualized by Western blot using αFLAG (Sigma, F1804) and αHA (Sigma H6908) primary antibodies, and Dylight 633 goat anti-Mouse (Invitrogen 35512) and Dylight 488 goat anti-rabbit (Invitrogen 35552) secondary antibodies. Proteins were imaged on an Amersham typhoon scanner (GE Lifesciences), and bands were quantified with ImageStudio software (Licor).

For visualization of 3xFLAG-Gid4, 3xFLAG-Gid10, and Lyp1-GFP (where protein levels are not quantified), cells were grown under the indicated conditions, 5 ODs for 3xFLAG-Gid4 and 3xFLAG-Gid10 or 1.5 ODs for Lyp1-GFP of yeast cells were pelleted at each timepoint, and lysed as described above. Samples were run on a 12% SDS-PAGE gel, transferred to nitrocellulose membrane, and visualized by Western blot using αFLAG (Sigma, F1804) or αGFP (Roche 1181446001), and on a separate blot αPKG (Invitrogen 459250) primary antibodies, and goat anti-mouse peroxidase secondary antibody (Sigma A4416). Proteins were visualized on Amersham ImageQuant800 (GE Lifesciences). For visualization of 3xFLAG-Gid4 (where protein levels are quantified), 1.5 OD of yeast cells were pelleted at each timepoint and lysed as described above. Samples were run on a 12% SDS-PAGE gel, transferred to nitrocellulose membrane, and visualized by Western blotting using αFLAG (Sigma, F1804) and αPKG (Invitrogen 459250) primary antibodies on the same blot, and Dylight 633 goat anti-Mouse (Invitrogen 35512) secondary antibody.

Preparation of plasmids for recombinant protein expression

All constructs for bacterial protein expression were prepared by Gibson assembly method (Gibson et al., 2009). For generation of mutant versions of the genes, the QuickChange mutagenesis protocol was applied (Stratagene). All coding sequences used for protein expression were verified by DNA sequencing. To express the GID complex in insect cells from a single baculoviral expression vector, genes encoding GID subunits were combined by the biGBac assembly method (Weissmann et al., 2016).
Recombinant protein expression and purification

Both WT and mutant versions of the GID complex used for biochemical assays were expressed in Hi-5 insect cells transfected with recombinant baculovirus variants in EX-CELL 420 Serum-Free Medium. After 72 h at 27°C, the cultures were harvested and resuspended in a lysis buffer containing 50 mM HEPES pH 7.5, 200 mM NaCl, 5 mM DTT, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 2 mM benzamidine, EDTA-free complete protease inhibitor tablet (Roche, 1 tablet per 50 ml of buffer) and 1 mM PMSF. The complex was first affinity purified via a twin-Strep tag appended to Gid8 C-terminus. Further purification was performed by anion exchange chromatography and size exclusion chromatography (SEC) in the final buffer containing 25 mM HEPES pH 7.5, 200 mM NaCl and 1 mM DTT. For substrate receptor exchange experiment, the GID<sup>SR10</sup> complex was affinity purified via a twin-Strep tag appended to Gid10 N-terminus. After pull-down, the tag was cleaved off by overnight 3c-protease cleavage. Similarly, Gid4 was expressed as an N-terminal twin-Strep tag fusion. The final purification was performed with SEC.

Aside from the GID complex and 2xS-Gid4, all recombinant proteins were expressed in <i>E. coli</i> BL21 (DE3) RIL. Cells transformed with an appropriate expression plasmid were grown in Terrific Broth (TB) medium at 37°C to an OD<sub>600</sub> of 0.6 and cooled down to 18°C. Then, overnight expression of proteins was induced by addition of 0.4 mM IPTG. All versions of Gid4 and Gid10 were expressed as GST-TEV fusions. After harvesting, cell pellets were resuspended in the lysis buffer containing 50 mM HEPES pH 7.5, 200 mM NaCl, 5 mM DTT and 1 mM PMSF. GST-tagged proteins were purified from bacterial lysates by glutathione affinity chromatography, followed by overnight digestion at 4°C with tobacco etch virus (TEV) protease to cleave off the GST tag. Further purification was carried out with SEC in a buffer containing 25 mM HEPES pH 7.5, 150 mM NaCl and 1 mM DTT, 5 mM DTT or 0.5 mM TCEP for biochemical assays, crystallization and ITC binding test, respectively. At the end of the pass-back over glutathione affinity resin was performed to get rid of the remaining uncleaved GST-fusion protein and free GST. Ubc8 was expressed with a C-terminal 6xHis-tag. After harvesting, the cell pellet was resuspended in lysis buffer containing 50 mM HEPES pH 7.5, 200 mM NaCl, 5 mM β-mercaptoethanol, 10 mM imidazole and 1 mM PMSF. Ubc8-6xHis was purified by nickel affinity chromatography, followed by anion exchange and SEC. Untagged WT ubiquitin was purified via glacial acetic acid method (Kaiser et al., 2011), followed by gravity S column ion exchange chromatography and size exclusion chromatography.

Immunoprecipitation and ubiquitination assay

For Art2 ICs, yeast cells were grown in YPD at 30°C to an OD<sub>600</sub> of 1.0–2.0. For Mdh2 ICs, yeast cells were grown in YPD to an OD<sub>600</sub> of 1.0–1.5, pelleted by centrifugation at 3,000 rpm for 3 min, washed once with pre-warmed YPE, resuspended to an OD<sub>600</sub> of 1.0 in fresh, pre-warmed YPE, and grown at 30°C for 19 h. 100 ODs of cells were pelleted by centrifugation at 3,000 rpm for 3 min, washed with 14H<sub>2</sub>O, resuspended in 1 ml lysis buffer (50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1% SDS, 1% NP-40, 0.5% Na-deoxycholate, 20 mM NEM, 1% glycerol, and complete EDTA-free protease inhibitor tablets (Roche, 1 tablet per 10 ml buffer)), and transferred to a 2 ml tube containing lysing matrix C (MP Biomedicals). Cells were lysed by three rounds of 20 s in a Fast-Prep24 instrument (MP Biomedicals), resting on ice for 5 min in between each round. Lysates were then pre-cleared by centrifugation at 4,000 g for 10 min. The supernatant was added to 50 μl pre-washed anti-DYKDDDDK magnetic agrose beads (ThermoFischer A36797) and nutated at 4°C for 2 h. Beads were then separated on a magnetic rack, and supernatant was discarded. Beads were washed twice with wash buffer 1 (50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1% SDS, 1%NP-40, 0.5% Na-deoxycholate, 20 mM NEM, 1% glycerol), twice with wash buffer 2 (25 mM HEPES, pH 7.5, 100 mM NaCl), and resuspended in 100 μl of wash buffer 2. For each ubiquitination reaction, 25 μl of this suspension were pelleted on a magnetic rack, and supernatant removed. Ubiquitination reaction mix (1 μM E2 Ubc8-6xHis, 0.5 μM GID<sup>Ant</sup>, 30 μM Ubiquitin, 0.5 μM substrate receptor (none, Gid10<sup>57-292</sup>), Gid10<sup>57-288</sup>, or Gid4<sup>362-362</sup>, as indicated), 25 mM HEPES, pH 7.5, 100 mM NaCl, 2.5 mM MgCl<sub>2</sub>, and 1 mM ATP was added to the beads. The reaction was started by the addition of 0.2 μM E1 Ub1a and incubated at room temperature for the indicated timepoints. Beads were then pelleted on a magnetic rack, washed with wash buffer 1, resuspended in 30 μl 2X sample buffer, and heated at 95°C for 5 min to elute the protein. For Art2-3xFLAG blots, the eluate was loaded on 4–12% SDS-PAGE gels, run at 200 V for 80 min, and transferred to PVDF membrane at 100 V for 90 min. For Mdh2-3xFLAG blots, eluate was loaded on a 12% SDS-PAGE gel, run at 200 V for 50 min, and transferred to nitrocellulose membrane at 100 V for 60 min. Samples were then visualized by immunoblotting with anti-FLAG (Sigma F1804) primary antibody and goat anti-mouse peroxidase secondary antibody (Sigma A4416), and imaged on an Amersham ImageQuant800 (GE Lifesciences).

In vitro binding assay

To test if the GID complex binds Gid10 in a manner similar to Gid4, WT and mutant versions (Gid5 W606A/Y613A/Q649A) of GID<sup>Ant</sup> were mixed with 2-fold molar excess of Gid10<sup>57-292</sup>, Gid10<sup>57-288</sup>, Gid4<sup>362-362</sup> and Gid4<sup>117-362</sup>. After incubating the proteins for 30 min on ice, 20 μl of Strep-Tactin resin was added to the mixture and further incubated for 30 min. After thorough wash of the resin, proteins were eluted and analyzed with SDS-PAGE.

In vitro competition binding assay

To test the swappable nature of GID substrate receptors, we competed binding of Gid10 to the GID<sup>SR10</sup> complex with increasing concentrations of 2xS-Gid4. Briefly, 10 μM of untagged GID<sup>SR10</sup> was mixed with strep-tactin either alone or after its 30-min incubation with 2xS-Gid4 (equimolar or added at 2- and 4-fold molar excess). After 30-min incubation, the resin was thoroughly washed with the wash buffer (25 mM HEPES pH 7.5, 150 mM NaCl and 5 mM DTT) and proteins were eluted. Substrate receptor exchange was assessed by inspecting the presence of the GID<sup>SR4</sup> complex and absence of Gid10 in the strep pull-down fractions visualized by Coomassie-stained SDS-PAGE.

Isothermal titration calorimetry (ITC)

The ITC measurements were carried out with MicroCal PEAQ-ITC instrument (Malvern Pananalytical) at 25°C. The Art2 degron peptide
was dissolved in the Gid10 SEC buffer containing 25 mM HEPES pH 7.5, 150 mM NaCl and 0.5 mM TCEP and their concentration was measured by absorbance at 280 nm. Binding experiments were carried out by titrating 450 µM peptide PFITSRPVAW to Gid1057–292 at 42 µM. Peptide was added to Gid10 by nineteen 2 µl injections, with 4 s injection time and 150 s equilibration between the injections. The reference power was set to 10 µcal/s. Raw ITC data were analyzed using one site binding mode in MicroCal ITC analysis software (Malvern Panalytical) to determine $K_D$ and stoichiometry of the binding reaction. All plots were prepared in GraphPad Prism.

**In vitro ubiquitylation assay**

To verify whether Art2 N-terminus can be ubiquitinated by GIDSR10, we performed an in vitro activity assay with Art2 $^{2–29}$ WT and P25 mutant peptides, with fluorescein appended to their C-termini. Ubiquitination reaction was performed in a multi-turnover format in a buffer containing 25 mM HEPES pH 7.5, 150 mM NaCl, 5 mM ATP and 10 mM MgCl2. To start the reaction, 0.2 µM E1 Uba1, 1 µM E2 Ubch6-6xHs, 0.5 µM E3 Gid5–64, 20 µM Ub, 0 or 1 µM Gid417–362 or Gid1057–292 and 1 µM peptide substrate were mixed and incubated at room temperature. At indicated timepoints, an aliquot of the reaction mix was mixed with SDS-PAGE loading buffer. The outcome of the activity assay was visualized with a fluorescent scan of an SDS-PAGE gel with Typhoon imager (GE Healthcare).

**Gid10 crystallization, data collection and structure determination**

Crystallization trials were carried out in the MPIB crystallography facility. Before setting up crystallization trays, Gid1055–294 was concentrated and mixed with PFITSRPWP peptide to obtain final concentration of protein and peptide of 262 and 760 µM, respectively (3-fold molar excess of the peptide). The crystal that gave rise to the final structure was grown at room temperature in the buffer containing 18.5% PEG3350, 0.1 M Bis-Tris propane pH 6.0 and 0.2 M potassium chloride by vapor diffusion in a sitting-drop format. Before data collection, crystals were cryoprotected in 20% ethylene glycol and flash-frozen in liquid nitrogen.

Diffraction data set was recorded at X105A beam line, Swiss Light Source (SLS) in Villingen, Switzerland. Data were recorded at 0.5-degree rotation intervals using Dectris Eiger II 16 M detector. Data were indexed, integrated, and scaled using XDS package to a resolution limit of 1.3 Å. Phasing was performed through molecular replacement using a structure of yeast Gid4 (extracted from PDB: 7NS3) with Phaser integrated into the PHENIX software suite (Malvern Panalytical) to determine $K_D$ and stoichiometry of the binding reaction. All plots were prepared in GraphPad Prism.

**Proteomics sample preparation**

Samples were prepared and analyzed as previously described (Karayel et al., 2020). Briefly, sodium deoxycholate (SDC) lysis buffer (1% SDC and 100 mM Tris, pH 8.5) were added to the frozen cell pellets. Lysates were immediately boiled for 5 min at 95°C and homogenized with sonication. Protein concentrations were estimated by tryptophan assay. Equal protein amounts were reduced and alkylated using CAA and TCEP, final concentrations of 40 and 10 mM, respectively, for 5 min at 45°C. Samples were digested overnight at 37°C using trypsin (1:100 wt/ wt, Sigma-Aldrich) and LysC (1:100 wt/wt; Wako). Next, peptides were desalted using SDB-RPS StageTips (Empore). Samples were first diluted with 1% trifluoroacetic acid (TFA) in isopropanol to a final volume of 200 µl and loaded onto StageTips and subsequently washed with 200 µl of 1% TFA in isopropanol twice and 200 µl of 0.2% TFA/2% ACN (acetonitrile). Peptides were eluted with 80 µl of 1.25% Ammonium hydroxide (NH4OH)/80% ACN, dried using a SpeedVac centrifuge (Concentrator Plus; Eppendorf) and resuspended in buffer A* (0.2% TFA/2% ACN) prior to LC-MS/MS analysis. Peptide concentrations were measured optically at 280 nm (Nanodrop 2000; Thermo Scientific) and subsequently equalized using buffer A*. Three hundred nanograms of peptide was subjected to LC-MS/MS analysis.

**LC-MS/MS measurements**

DIA is a discovery-oriented acquisition method in which the quadrupole cycles through large $m/z$ windows across the entire mass range to generate comprehensive fragment ion maps, covering nearly all detected precursors, and increasing the reproducibility, quantitative accuracy, and depth of proteome analysis in single runs compared with the conventional data-dependent acquisition (DDA) method, which takes only a selection of peptide signals forward for fragmentation.

Samples in panels 3A-B and EV2A were loaded onto a 20-cm reversed-phase column (75-µm inner diameter, packed in-house with ReproSil-Pur C18-AQ, 1.9 µm resin [Dr. Maisch GmbH]). The column temperature was maintained at 60°C using a homemade column oven. An EASY-nLC1200 system (Thermo Fisher Scientific), coupled with the mass spectrometer (Q Exactive HF-X, Thermo Fisher Scientific) via a nano-electrospray source, was employed for nano-flow liquid chromatography. A binary buffer system, consisting of buffer A (0.1% formic acid [FA]) and buffer B (0.1% FA and 80% ACN), was used for peptide separation, at a flow rate of 450 nl/min. We used a gradient starting at 5% buffer B, increased to 35% in 18.5 min, 95% in a minute, and stayed at 95% for 3.5 min. The mass spectrometer was operated in data independent acquisition mode (DIA). Full MS resolution was set to 120,000 with a full scan range of 300 to 1,650 $m/z$, a maximum fill time of 60 ms, and an AGC target of 3×e6. One full scan was followed by 12 windows with a resolution of 30,000 in profile mode. Precursor ions were fragmented by stepped HCD (NCE 25.5, 27, and 30%).

Samples in panels 2C and EV2B were processed similarly except that peptide separation was at a flow rate of 300 nl/min and an EASY-nLC1200 system (Thermo Fisher Scientific) system was coupled with the mass spectrometer Orbitrap Exploris 480 (Thermo Fisher Scientific) via a nano-electrospray source. We used a gradient starting at 5% buffer B, increased to 30% in 45 min, 65% in 5 min, 95% in 5 min and stayed at 95% for 5 min. The mass spectrometer was operated in data independent acquisition mode (DIA). Full MS resolution was set to 120,000 with a full scan range of 300 to 1,650 $m/z$, a maximum fill time of 20 ms, and an AGC target of 3×e6. One full scan was followed by 32 windows with a resolution of 30,000 in profile mode. Precursor ions were fragmented at NCE of 28%.
Data processing and bioinformatics analysis

The DIA files in panels 3A and B, and EV2A were analyzed using the proteome library previously generated (Karayel et al., 2020) with default settings and enabled cross-run normalization using Spectronaut version 13 (Biognosys AG). The files in panels 2C and EV2B were processed in Spectronaut version 15 (Biognosys AG). In both searches, we used the UniProt S. cerevisiae reference proteome of canonical and isoform sequences with 6,077 entries for final protein identification and quantification. We set carbamidomethylation as fixed modification and acetylation of the protein N-terminus and oxidation of methionine as variable modifications. Trypsin/P proteolytic cleavage rule was used with a maximum of two missed cleavages permitted and a peptide length of 7–52 amino acids. A protein and precursor FDR of 1% were used for filtering and subsequent reporting in samples (q-value mode with no imputation). The Perseus software package versions 1.6.0.7 and 1.6.0.9 (Tyanova et al., 2016) and GraphPad Prism version 7.03 were used for the data analysis. Protein intensities were log2-transformed, and the data sets were filtered to make sure that identified proteins showed expression or intensity in all biological triplicates of at least one condition and the missing values were subsequently replaced by random numbers that were drawn from a normal distribution (width = 0.3 and downshift = 1.8) in Perseus. To determine significantly different proteins, two sample t-test was applied, assuming that variance within the groups of replicates was equal. Data represent means ± SD (n = 3) and quantification was done by one-way ANOVA with Tukey’s multiple comparisons test. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001 to the corresponding legends and In panel 2C and EV2A, quantification was based on one-way ANOVA (unpaired), which is followed by Tukey’s multiple comparison test to compare every mean with every other mean. The corrected P-values were shown in the panels.

Data availability

The structure of Gid10 bound to Art2 Pro/N-degron is available in RCSB under the accession code (PDB ID): 7QOY (http://www.rcsb.org/pdb/explore/explore.do?structureId=7QOY). The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE database with the dataset identifier PXD030902 (https://www.ebi.ac.uk/pride/archive/projects/PXD030902).

Expanded View for this article is available online.

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Christine R Langlois: Conceptualization; Data curation; Formal analysis; Supervision; Investigation; Methodology; Writing—original draft; Writing—review & editing. Viola Beier: Investigation; Methodology; Writing—review & editing. Ozge Karayel: Investigation; Methodology; Writing—review & editing. Jakub Chrustowicz: Investigation; Methodology; Writing—review & editing. Dawafuti Sherpa: Investigation; Methodology; Writing—review & editing. Matthias Mann: Supervision; Funding acquisition; Writing—review & editing. Brenda A Schulman: Conceptualization; Supervision; Funding acquisition; Writing—review & editing.

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The authors declare that they have no conflict of interest.

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