Neddylation requires glycyl-tRNA synthetase to protect activated E2

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Neddylation— the conjugation of the ubiquitin-like protein NEDD8 to its target proteins—is an essential biological process that critically regulates cell-cycle progression in eukaryotes including yeast and humans\textsuperscript{1–4}. Like ubiquitination, neddylation is achieved through a sequential enzymatic cascade involving an activating enzyme (E1), a conjugating enzyme (E2), and a ligase (E3) (Fig. 1a). To date, one E1 enzyme (APPPB1–UBA3), two E2 enzymes (Ubc12, also known as Ubc2M, and Ube2F), and many E3 ligases have been identified to act in neddylation\textsuperscript{5–8} (Fig. 1a). Although numerous NEDD8 targets have been reported\textsuperscript{9}, the biological functions of neddylation have primarily been characterized to date in the context of its main targets—the cullin proteins, key components of the ubiquitin E3 cullin–RING ligase family. Neddylation of cullin activates the E3 ligases for ubiquitination and promotes the degradation of their downstream targets, including key regulators of the cell cycle\textsuperscript{10}.

The initial interest in finding a connection between ubiquitination (or ubiquitin-like modifications) and GlyRS was based on molecular considerations. Ubiquitin and most ubiquitin-like-modifier proteins, including NEDD8, have a conserved C-terminal glycine that is used to activate, conjugate, and finally attach the modifiers to their targets\textsuperscript{11}. Structurally, the glycine residue is located at the tip of a flexible 'tail' that protrudes from the central ubiquitin fold and provides accessibility\textsuperscript{12}. The well-known function of GlyRS is to catalyze the formation of glycyl-tRNA\textsuperscript{Gly} as a substrate for ribosomal protein synthesis in a two-step reaction\textsuperscript{13}; first, GlyRS activates glycine with ATP, thus generating Gly-AMP; second, the glyoy moiety is transferred from Gly-AMP to the 3' end of tRNA\textsuperscript{Gly}. Interestingly, this first-step reaction catalyzed by GlyRS is chemically equivalent to that catalyzed by E1 enzymes in the activation of ubiquitin and ubiquitin-like proteins. Moreover, our previous work has indicated that the specific amino acid-binding pocket of a tRNA synthetase, and its binding to a cognate amino acid residue on a protein, can be exploited to develop new functions\textsuperscript{14}.

We set out to test whether human GlyRS interacts with ubiquitin or ubiquitin-like proteins such as NEDD8 and SUMO1 and found that GlyRS specifically binds NEDD8. Although we did not find an E1-like activity in GlyRS as initially speculated, we did find that GlyRS binds both E1 and E2 (Ubc12) enzymes functioning in neddylation. Moreover, when Ubc12 was conjugated with NEDD8, its affinity for GlyRS was substantially enhanced, thus suggesting that GlyRS may protect activated E2 before the E2 finds its correct downstream targets. Indeed, knockdown of GlyRS, but not knockdown of another tRNA synthetase, substantially decreased the level of activated Ubc12 and the level of cullin neddylation in human cells. Moreover, knockdown of GlyRS mimicked the effect of MLN4924, a specific inhibitor of neddylation E1, and caused cell-cycle arrest. Therefore, our study reveals a chaperone-like function of GlyRS that critically supports neddylation.

RESULTS

GlyRS specifically binds NEDD8 and enhances neddylation

Using purified recombinant proteins, we found that human GlyRS specifically bound NEDD8 but did not bind ubiquitin or SUMO1 (Fig. 1b). We also tested two other human tRNA synthetases (SerRS and TrpRS) side by side and found that neither interacted with NEDD8, ubiquitin, or SUMO1 (Fig. 1b). We further verified the GlyRS-NEDD8 interaction in HEK293 cells by coimmunoprecipitation (Fig. 1c). By using

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truncated recombinant proteins, we mapped the interaction to the catalytic domain of GlyRS (Fig. 1d,e). Hydrogen/deuterium exchange (HDX) analysis confirmed that the catalytic domain was the site of interaction with NEDD8 (Supplementary Fig. 1a).

To test the effects of GlyRS on neddylation, first we ectopically expressed GlyRS in HEK293 cells. Overexpression of GlyRS (but not TrpRS) increased the amount of NEDD8-conjugated Ubc12 (a neddylation E2) but not the amount of ubiquitin-conjugated UbcH7 (a ubiquitination E2 (ref. 6)) or SUMO-conjugated Ubc9 (a SUMOylation E2 (ref. 7)) (Fig. 1f). Removal of the metazoan-specific WHEP domain from GlyRS (ΔWHEP) did not substantially affect the activity of GlyRS, whereas the anticodon-binding domain (ABD) alone had no activity (Fig. 1f), thus further indicating the involvement of the catalytic domain. In addition, knockdown of GlyRS expression in HeLa cells led to a substantial decrease in NEDD8-conjugated Ubc12 (Ubc12N8) (Fig. 1g) but not ubiquitinated UbcH7 and SUMOylated Ubc9 (Fig. 1h,i). We obtained a similar result in HEK293 cells (data not shown).
Knockdown of a different tRNA synthetase (SerRS) had no effect on all of the E2s tested (Fig. 1g–i). Moreover, knockdown of GlyRS, but not SerRS, also substantially decreased the global level of neddylation, including neddylation of cullin proteins (Fig. 1g). We observed that GlyRS knockdown had no effect on E1 neddylation (forming UBA3N8) or the level of free NEDD8 (Fig. 1g). Additionally, GlyRS knockdown did not affect Ube2F (Supplementary Fig. 2a), the other neddylation E2 that is evolutionarily more recent and is less prevalent than Ubc12 (ref. 8). These results suggest that GlyRS is required for neddylation and that GlyRS probably functions through promoting or preserving Ubc12N8.

GlyRS preferentially binds and protects Ubc12N8
Ubc12 exists in two forms in the cell: the free form and the conjugated form Ubc12N8, which is generated by transfer of a NEDD8 molecule from E1 to the catalytic cysteine (Cys111) of Ubc12 (E2) via a thioester bond15. Importantly, once NEDD8 is transferred from E1 to E2, E2N8 is released from E1 (ref. 16). We speculate that GlyRS may increase the cellular level of Ubc12N8 by facilitating the formation of Ubc12N8 or by protecting Ubc12N8 after it is released from E1. Two separate in vitro NEDD8 conjugation assays using either radioactive or fluorescence-labeled NEDD8 consistently indicated that GlyRS did not affect the production of Ubc12N8 (Supplementary Fig. 3a,b). In contrast, we found that GlyRS substantially enhanced the stability of Ubc12N8 in vitro (Supplementary Fig. 4); this result may explain the activity of GlyRS in increasing the cellular levels of Ubc12N8.

We hypothesized that the protection might arise from a strong interaction between GlyRS and Ubc12N8. We first investigated whether GlyRS, in addition to interacting with NEDD8, might also interact with Ubc12. Interestingly, both glutathione S-transferase (GST) pull-down and HDX analysis confirmed that GlyRS indeed binds Ubc12 and that the catalytic domain of GlyRS is primarily responsible for the interaction (Supplementary Fig. 3c,d). To quantify the binding, we used biolayer interferometry and immobilized GlyRS on a sensor chip to measure its interaction with Ubc12, NEDD8, and Ubc12N8. Remarkably, GlyRS bound to Ubc12N8 with a $K_d$ of 4.09 ± 0.30 nM, which is 100-fold and 30-fold stronger than its binding to Ubc12 alone ($K_d = 488 ± 73$ nM) and to NEDD8 alone ($K_d = 126 ± 19$ nM), respectively (Fig. 2a). We also analyzed the GlyRS-Ubc12N8 interaction in vitro (Supplementary Fig. 4).
GlyRS and obtained a similar GlyRS
firming that GlyRS strongly binds to NEDD8-conjugated Ubc12 during the neddylation cascade.

Coordinates of the GlyRS-APPBP1–UBA3 model are available in Supplementary Data Set 3.

Using the crystal structures of human GlyRS and Ubc12N8 (adapted from its complex with substrate cullin and E3)\(^\dagger\), we generated a model for the GlyRS-Ubc12N8 interaction, by using the Patchdock algorithm (http://bioinfo3d.cs.tau.ac.il/PatchDock/\(^\dagger\)9,\(^\dagger\)2). The top result indicated Ubc12N8 binding to the catalytic domain of GlyRS, with NEDD8 nestled in between two β-hairpin loops (F84–L93 and I232–M238) (Fig. 2c). This model is generally consistent with the HDX analysis results (Supplementary Fig. 1a and Fig. 3d). To validate the model, we created two deletion mutants of the β-hairpin loops (Δ84–93 and Δ232–238) and found that the Δ84–93 mutant showed nine-fold-decreased binding of GlyRS to Ubc12N8 (Kd = 27.0 ± 0.4 nm), and the Δ232–238 mutant showed abolished binding (Fig. 2c–f).

In contrast, deletion of an insertion domain unique to GlyRS (insertion 1)\(^\dagger\), which showed no involvement in the GlyRS-Ubc12N8 interaction according to the model (Fig. 2d), had no effect on the binding (Kd = 4.32 ± 0.12 nm) (Fig. 2g). In accord with its loss of binding to Ubc12N8, the Δ232–238 GlyRS was inactive in protecting Ubc12N8 from degradation in vitro (Supplementary Fig. 4a) and in promoting the level of Ubc12N8 in HEK293 cells (Fig. 2h).

Interestingly, we found that GlyRS also bound the heterodimeric neddylation E1 enzyme APPBP1–UBA3 (Fig. 3a and Supplementary Fig. 5a). However, unlike NEDD8 and Ubc12, E1 bound the ABD domain of GlyRS, as revealed by both biolayer interferometry analysis and GST pulldowns (Fig. 3a and Supplementary Fig. 5a).

Furthermore, the APPBP1–UBA3 heterodimer and the APPBP1 subunit alone had similar binding affinity for ABD (Fig. 3b), thus suggesting that the APPBP1 subunit of E1 is responsible for the GlyRS interaction. We further validated the APPBP1–GlyRS interaction by coimmunoprecipitation in HEK293 cells (Fig. 3c).

A modeling study with Patchdock placed ABD in a position suitable for interacting with the APPBP1 subunit of the E1 (Supplementary Fig. 5b).

The observation that GlyRS uses different domains to interact with E1 and Ubc12N8 suggested that GlyRS might be able to bind E1 and Ubc12N8 simultaneously. As mentioned above, once NEDD8 is transferred from E1 to Ubc12, the interaction between them is weakened, thus releasing Ubc12N8 from the catalytic subunit UBA3 of E1. Perhaps, by binding the APPBP1 subunit of E1 through the ABD domain, GlyRS is positioned in proximity to capture the released Ubc12N8 and to provide protection (Fig. 3d).

We designed an experiment to test this concept. We immobilized E1 to detect its interaction with Ubc12N8 and GlyRS, both separately and simultaneously. As expected, the binding of Ubc12N8 to E1 was weak, and the dissociation was fast (Fig. 3e). In contrast, the binding of GlyRS to E1 was strong (Fig. 3e). Interestingly, when both Ubc12N8 and GlyRS were present, the overall binding was stronger than the sum of the individual bindings (Fig. 3e); this result suggests that a
ternary complex of E1–GlyRS–Ubc12N8 was formed, thus preventing Ubc12N8 from being released to the solvent. The ABD of GlyRS was used as a control for this experiment. Although the ABD domain alone interacted with the E1 as strongly as did the full length GlyRS (Fig. 3a,b), the absence of the catalytic domain of GlyRS for capturing Ubc12N8 did not permit the formation of a ternary complex. Indeed, we did not detect a synergistic effect between ABD and Ubc12N8 in binding to E1 (Fig. 3f).

GlyRS does not interfere with NEDD8 downstream transfer
Because we observed tight binding between GlyRS and Ubc12N8, it was important to confirm that the binding did not impede Ubc12N8 from passing NEDD8 to downstream targets such as cullin1. Notably, the protection of Ubc12N8 by GlyRS did not appear to require burying of the thioester linkage. In our model of the GlyRS–Ubc12N8 complex, the thioester bond between the C-terminal glycine residue Gly76 of NEDD8 and Cys111 of Ubc12 faces outward rather than being buried inside (Fig. 2d) and is fully accessible to the acceptor residue Lys720 of cullin1 (Supplementary Fig. 6a). We tested whether the E3–substrate pair of RING-box protein 1 (Rbx1)–cullin1 could bind to Ubc12N8 in the presence of GlyRS. Rbx1–cullin1 indeed bound Ubc12N8 in the presence of GlyRS, and moreover the binding appeared to release GlyRS from Ubc12N8 (Supplementary Fig. 6b), presumably allowing GlyRS to turn over. Using an in vitro neddylation assay, we further demonstrated that the presence of wild-type (WT) GlyRS, but not Δ232–238 GlyRS, which lacks Ubc12N8 binding (Fig. 2e), increased the neddylation of cullin1, presumably by protecting Ubc12N8 (Supplementary Fig. 6c).

GlyRS regulates cell-cycle progression
Cullin neddylation activates the cullin–RING ubiquitin ligases and facilitates the degradation of their downstream targets, including the cell-cycle inhibitor p27 (ref. 22 and Fig. 4a). p27 induces cell-cycle arrest by binding to cyclin–CDK complexes and inhibiting their catalytic activity23. Therefore, degradation of p27 through cullin neddylation promotes cell-cycle progression and cell proliferation. Because this is a well-established pathway, we focused on p27 degradation and cell-cycle progression to study the biological role of GlyRS in neddylation.

To evaluate p27 degradation in cells, we used cycloheximide (CHX), an inhibitor of translation, to block new protein synthesis. The level of p27 decreased rapidly after CHX treatment in untreated HeLa cells or cells transfected with empty vectors or plasmids expressing short hairpin RNAs (shRNAs) against SerRS (shSerRS) (Fig. 4b). (We included the proteasome inhibitor MG132 to confirm the ubiquitin-dependent proteolysis of p27.) However, treatment with shRNAs against GlyRS (shGlyRS) enhanced the stability of p27 (Fig. 4b), as evidenced by both the increased level of p27 before the CHX treatment and the extended half-life of p27 during the treatment. Moreover, the stabilization effect on p27 was concurrent with a decreased level of cullin8N8 (Fig. 4b). To further confirm that this effect was related to neddylation, we used the neddylation-specific inhibitor MLN4924 (ref. 24) as the positive control. Indeed, MLN4924 treatment abolished cullin neddylation and blocked p27 degradation (Fig. 4a.b). Abnormal accumulation of p27 leads to cell-cycle arrest25. Hence, compared with the control cells, cells transfected with shGlyRS or treated with MLN4924 showed a substantial decrease in the number of diploid (2n) cells and an increase in the number of tetraploid (4n) cells (Fig. 4c), thus indicating cell-cycle arrest.

Active site is not essential for GlyRS-mediated neddylation
Although our study was initially inspired by the chemical similarity between reactions catalyzed by GlyRS and E1, we have not been able to detect an E1-like activity of GlyRS toward NEDD8 (data not shown). Moreover, we found that removal of the C-terminal diglycine motif of NEDD8 had little effect on the GlyRS-NEDD8 interaction (Supplementary Fig. 1b,c). To further clarify whether the active site and the ability to synthesize Gly-AMP are important in GlyRS-mediated neddylation, we tested the GlyRS mutant G526R. The G-to-R substitution blocks the ATP-binding site of GlyRS (Fig. 5a) and completely abolishes the aminoacylation activity17. However, despite having reduced activity, G526R GlyRS was active in increasing the cellular level of Ubc12N8 (Fig. 5b) and in restoring p27 degradation.
and cell-cycle progression in the presence of a low dose of the neddylation inhibitor MLN4924 (Fig. 5c,d), thus suggesting that the active site or the ability to synthesize Gly-AMP may affect, but is not essential for, the role of GlyRS in neddylation. Interestingly, we found that G526R GlyRS had decreased (approximately ten-fold) binding to Ubc12N8 ($K_d = 39.5 \pm 1.2$ nM) compared with that of WT GlyRS (Fig. 5e); this result may explain the reduced activity of G526R GlyRS in increasing the cellular level of Ubc12N8 and in facilitating neddylation.

**DISCUSSION**

Human GlyRS contains a metazoan-specific WHEP domain, a catalytic domain and an ABD (Fig. 1d). Only the catalytic domain and ABD are essential for tRNA aminoacylation$^{17}$, and interestingly both domains, after Ubc12N8 is released from the E1 and before it finds the correct E3 and substrate and transfers the NEDD8 modifier. The conjugated E2 specifically binds Ubc12 but not Ube2F (Supplementary Fig. 2b), thus suggesting that the active site or the ability to synthesize Gly-AMP may affect, but is not essential for, the role of GlyRS in neddylation. Interestingly, we found that G526R GlyRS has reduced activity of G526R GlyRS in increasing the cellular level of Ubc12N8 and in facilitating neddylation.

Neddylation activates cullin proteins as molecular scaffolds in the assembly of the cullin–RING-box protein E3 ubiquitin ligase, the largest family of ubiquitination E3 ligases. In humans, one of the six cullins (cullins 1, 2, 3, 4A, 4B, and 5) assembles with one of the two RING-box proteins (Rbx1 and Rbx2) and subsequently mediates neddylation and degradation of thousands of targets, thereby regulating a vast array of biological functions$^{26,27}$. In the hierarchical cascade of cullin neddylation, selectivity is achieved through the collaboration of RING and E2. Two E2s for NEDD8, the same number as the number of RINGs, have been identified: Ubc12 specifically pairs with Rbx1 and mediates neddylation of cullin1–4, and Ube2F pairs with Rbx2 and controls cullin5 neddylation$^{8,28}$. Interestingly, Ube2F–Rbx2–cullin5 neddylation constitutes a metazoan-specific NEDD8 cascade$^8$, whereas Ubc12, Rbx1, and cullin1, along with NEDD8 and E1, are present in all eukaryotes. Although GlyRS is ubiquitously and abundantly present in all organisms, eukaryotic GlyRSs are substantially different from most of their bacterial counterparts$^{29}$. Our observation that GlyRS specifically binds Ubc12 but not Ube2F (Supplementary Fig. 2b) suggests that GlyRS is a component of the prototypic cascade of neddylation and that the role of GlyRS in protecting the conjugated Ubc12 and facilitating neddylation may have existed ever since the modification first occurred. Further observations that GlyRS interacts with multiple components of the prototypic neddylation pathway and that the metazoan-specific WHEP domain is dispensable in all these interactions (Fig. 1c, Supplementary Fig. 3c and Supplementary Fig. 5a) are consistent with this concept.

Apparently, dysregulation of neddylation can cause abnormalities in protein degradation through the ubiquitin–proteasome system and can play a pathological role in various diseases. It has been reported that high levels of NEDD8 are present in ubiquitinated inclusion bodies in neural tissues collected from patients with neurodegenerative disorders such as Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis$^{30,31}$. Interestingly, dominant mutations in GlyRS have been found to cause a neurodegenerative disorder called Charcot–Marie–Tooth disease type 2D (CMT2D) in patients$^{32,33}$. In fact, the G526R mutation that we tested is a CMT2D-causing mutation$^{34}$. Although G526R GlyRS, compared with the WT protein, exhibits less binding to Ubc12N8 (Fig. 5e), genetic studies in mouse...
models have clearly demonstrated that CMT2D is caused by a toxic gain-of-function mechanism rather than the loss of function of either canonical or noncanonical activities of GlyRS. Moreover, an aberrant interaction between neuropilin 1 and mutant, but not WT, GlyRS has recently been found to account (at least partially) for the selective motor-neuron pathology in CMT2D. Finally, no pathological aggregation or ubiquitin-positive inclusion has been detected in neural tissues from CMT2D mouse models, thus further indicating that dysregulation of neddylation is unlikely to be involved in the etiology of CMT2D.

Upregulation of neddylation is strongly associated with cancer. Many targets of neddylation are involved in regulating cancer progression, including regulators of the cell cycle (p27 (ref. 40) and cyclin E (ref. 41), transcription (NRF2 (ref. 42), HIF-1 (ref. 43), and p16 (ref. 44)), and DNA replication (CDT-1 (ref. 45)). In fact, the neddylation pathway is known to be an important anticancer target, and the selective NEDD8 E1 inhibitor MLN4924 is currently being tested in several clinical trials as a treatment for hematological malignancies and solid tumors. As a component of the translation machinery, GlyRS is expected to be associated with cancer progression, which requires robust protein-synthesis activity. However, the critical role of GlyRS in neddylation revealed in this study suggests that GlyRS might be associated with cancer for an additional reason. Notably, in an examination of the expression of aminoacyl-tRNA synthetases in previously generated microarray data sets from 3,557 breast cancer patients, we found that GlyRS showed the strongest association with cancer mortality among all 20 aminoacyl-tRNA synthetases. Following GlyRS at the top of the list were ThrRS and LysRS. Notably, ThrRS has been reported to be an angiogenic, promigratory extracellular signaling molecule, and LysRS has been found to promote cancer cell migration and metastasis by inhibiting ubiquitin-dependent degradation of the lamin receptor.

In conclusion, by using biochemical, structural, and cell-based approaches, we found that GlyRS, in addition to being an essential enzyme in protein synthesis, also acts as a chaperone that critically facilitates neddylation, including neddylation of cullin proteins of the ubiquitin–proteasome system (Fig. 6b). Through its dual functions, GlyRS supports both general protein production and selective protein degradation, and hence it may play a crucial role in maintaining the homeostasis of the eukaryotic proteome.

**METHODS**

Methods and any associated references are available in the online version of the paper.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

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**AUTHOR CONTRIBUTIONS**

X.-L.Y. and Z.M. designed experiments, analyzed data, and wrote the manuscript. Z.M. performed the molecular cloning, binding analysis, structural docking, bioinformatic analysis, and additional biochemical analysis. Z.M. and Q.Z. carried out protein purification. Z.M. and Q.Z. performed the cell-cycle analysis. J.L. and P.R.G. performed the HDX analysis. Y.S. and L.S. contributed to biochemical analysis.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS
Plasmid construction and protein purification. GST-tagged or untagged human APPBP1–UBA3, Ubc12, and NEDD8 proteins were purified as previously described52. GST-APPBP1 was obtained by injecting the purified GST-APPBP1–UBA3 onto a Superdex 200 column and collecting the fractions that contained only GST-APPBP1. The N-terminal His-SUMO-tagged or His-tagged NEDD8 construct was generated by subcloning pGEX2TK-NEDD8 into a modified pET28a vector, and the proteins were purified with an Ni–NTA column, then with a MonoQ ion-exchange column. His-NEDD8AgG was generated by site-directed mutagenesis and purified in a similar manner. Recombinant human ubiquitin and SUMO1 proteins (Boston Biochem) were purchased. His-tagged full-length and AHWEP human GlyRS, SerRS, and TrpRS were purified as previously reported53–55. The ABD (V541–E685) of GlyRS was cloned, expressed, and purified in a similar manner as for the full-length GlyRS (the yield for ABD alone was higher than that for the full-length GlyRS). Tag-free wild-type GlyRS, Δ84–93 (P84–L93 replaced by GGG), Δ232–238 (I232–M238 replaced by G), Δinsertion (F147–F224 replaced by GS overlapping), and G252R GlyRS proteins were generated with an N-terminal His-SUMO-tag, which was subsequently removed by Upl1 protease. Insertion of the GlyRS sequence into a modified pET28a vector generated N-terminal GST-tagged GlyRS, and the protein was purified by glutathione–Sepharose chromatography. GST-cullin1–Rbx1 was also purified by glutathione–Sepharose chromatography56. The Ubc2F protein was prepared as previously described55. The purity of proteins was examined by SDS–PAGE to be above 95%.

Affinity pulldown assay. Glutathione–Sepharose beads (GE Healthcare) were equilibrated with TEE buffer (50 mM Tris, pH 7.9), 1 mM EDTA, and 1 mM EGTA. GST-fusion proteins were mixed with 50 µl of glutathione–Sepharose beads, incubated for 2 h at 4 °C, and then washed with TEE buffer twice. Aliquots of the protein-bound beads were then incubated with different forms of GlyRS for 2 h at 4 °C. Finally, the beads were washed five times with washing buffer (20 mM HEPES, pH 7.9, 150 mM NaCl, 0.5 mM EDTA, 10% glycerol, 0.1% Triton X-100, and 1 mM DTT), and proteins were eluted with SDS sample buffer and analyzed by immunoblotting. Control experiments were performed with GST-coated beads. His-tag pulldown assays were carried out in a similar manner with purified His-tagged proteins and incubated with Ni–NTA beads (Qiagen).

Cell culture and shRNA knockdown. HEK293 and HeLa cells were obtained from ATCC without further authentication. Both cell lines were cultured in DMEM (11995, Gibco) supplemented with pen-strep (15140, Gibco) and 10% FBS (FB-12, Omega). shRNA sequences targeting human GlyRS (5′-GCATGGAGTATCTCACAAAGT-3′), SerRS (5′-GCATGGAGTATCTCACAAAGG-3′), or human Srs2 (5′-GGCATGGAGGATCCATCAATGTA-3′) were inserted into the pLentiLox 3.7 plasmid to drive shRNA expression. Sequences of GlyRS (full length or truncations, WT or mutants) or TrpRS or His-tagged GlyRS were equilibrated with TEE buffer (50 mM Tris, pH 8.0). The mixture was incubated at 37 °C for 1 h and washed four times with 1 mL of cold PBS buffer, pH 7.4. The bead-bound proteins were eluted and denatured with SDS loading buffer and subjected to SDS–PAGE and immunoblotting.

Immunoblotting and antibodies. Cells were washed with PBS and then lysed with either lysis buffer (cat. no. 9803, Cell Signaling Technology; 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, and 1 µg/mL leupeptin) or acidic lysis buffer (50 mM HEPES, pH 6.0, 150 mM NaCl, and 0.1% (w/v) SDS) supplemented with protease-inhibitor cocktail (Roche). The acidic lysis buffer was used to preserve the ubiquitin or ubiquitin–like conjugations (in, for example, UBA3N8, Ubc12N8, UbcH7H75, and Ubc9Ubc9) that are labile in the regular lysis buffer. The supernatants of the lysates were used for immunoblotting. The antibodies used in this study include anti-cullin1 (H213, Santa Cruz), anti-Ubc12 (F-10, Santa Cruz), anti-GlyRS (B01P, Abnova or sc-98614, Santa Cruz), anti-GlyRS (B01P, Abnova or sc-98614, Santa Cruz), anti-SerRS antibody (generates 4% CH3CN to 40% CH3CN, 0.3% formic acid) at 1 °C across a 1 mm 5× 50 mm C18 reversed-phase HPLC column (Hypersil Gold, Thermofisher) and electrosprayed directly into an orbitrap mass spectrometer (either LTQ Orbitrap or Q-Exactive, Thermo Fisher). Data were processed with in-house software58 and visualized with PyMOL (http://www.pymol.org/). To measure the difference in exchange rates, we calculated the average percentage deuterium uptake for unbound GlyRS protein at all time points. From this value, we subtracted the average percentage deuterium uptake for GlyRS protein bound to NEDD8. Negative perturbation values indicate that exchange rates are slower for GlyRS bound to NEDD8, thus suggesting that the region is less accessible to amide exchange, owing to structural alteration or direct contact between GlyRS and NEDD8. The GlyRS-Ubc12 interaction was analyzed in a similar way.

In vitro neddylation with [γ-32P]ATP-labeled NEDD8. The assay was performed as described by Huang et al5. Briefly, 20 µg NEDD8 protein was freshly labeled by [γ-32P]ATP with protein kinase A (9 U/µg) at room temperature for 2 h. Then 5 µM Ubc12 was charged at RT with [γ-32P]ATP-modified NEDD8 in the reaction buffer (50 mM Tris, 50 mM NaCl, and 5 mM MgCl2, pH 7.6) in the presence of 0.3 µM E1, 5 mM ATP, 1 mM DTT, 2 mg/mL BSA, 10 µM pyrophosphatase, 10 µM creatine kinase, and 5 µM creatine phosphate with or without 50 µM GlyRS. The reaction was then quenched after 10 min by addition of 2× SDS loading buffer, the samples were subjected to SDS–PAGE, and the gel was visualized by autoradiography.

In vitro neddylation with fluorescein-modified NEDD8. Fluorescein-modified NEDD8 (Boston Biochem, UL-830-050) was used for this assay. Briefly, the reaction was initiated by mixture of 0.3 µM APPBP1–UBA3, 5 µM Ubc12, 25 µM NEDD8, and 1 mM Mg2+-ATP with or without 5 µM GlyRS in the reaction buffer (50 mM HEPES and 50 mM NaCl, pH 8.0). The mixture was incubated at 37 °C and quenched after 30 min by addition of 2× SDS loading buffer. The samples were then prepared and subjected to SDS–PAGE. The gel was then visualized with a FluorChem M system (Proteinsimple).

Ubc12N8 preparation and stability assay. The conjugated Ubc12N8 was prepared by mixture of 0.2 µM APPBP1–UBA3, 7 µM Ubc12 (C111S, a mutant that forms a stable ester linkage to NEDD8), 10 µM His-SUMO-tagged NEDD8 in 50 mM Tris, pH 8.0, buffer supplemented with 50 mM NaCl, 1 mM MgCl2, and 2 mM ATP. The mixture was incubated at 25 °C for 16 h. Ubc12N8 was then purified by gel-filtration chromatography with a Superdex 200 column. The fractions containing only Ubc12N8 were concentrated and used for the study. The purity of proteins was examined by SDS–PAGE to be above 95%.
The transient interaction between E1 and Ubc12N8 was modeled by aligning the
Data Set 3
APPBP1-UBA3 (PDB 2NVU chains A and B) assigned as the receptor and GlyRS
set at 4.0. The GlyRS-APPBP1 interaction was modeled with similar settings, with
SDS–PAGE to be above 95%. The stability assay was carried out by incubating
concentrated and used for the study. The purity of proteins was examined by
kinetic buffer (PBS with 0.1% BSA and 0.002% Tween-20) and immobilized on
were dispensed into 96-well plates (Greiner Bio-one) at 200
biolayer interferometry with an Octet QK system (FortéBio). Samples or buffer
were subjected to SDS–PAGE and immunoblot analysis.

Cycloheximide chase assay. HeLa cells at 80% confluence were transfected with Lipofectamine
2000 with pLentiLox-hH1 vectors containing either a scrambled sequence or a
specific sequence targeting GlyRS or SerRS. Forty-eight hours after transfection, cells were treated with medium containing either 0.2 μM MLN4924 or the same
amount of DMSO. Twenty-four hours after treatment, cells were washed once
with sorting buffer (PBS with 1% FBS) and collected with 0.05% trypsin. The mix-
ture was then centrifuged at 500g to collect the cell pellets. Cells were then washed
twice with sorting buffer and then suspended and fixed with 70% EtOH at 4 °C
for 2 h. After fixation, cells were washed twice with sorting buffer and suspended
with the propidium iodide staining solution. Samples were later analyzed by flow
cytometry (BD FACs Canto, BD Bioscience). HeLa cells at 80% confluence were
transfected with Lipofectamine 2000 with pcDNA6v5c vectors or vectors contain-
ing inserts of either WT GlyRS or G526R GlyRS. Forty-eight hours after transfec-
tion, cells were treated with medium containing 0.2 μM MLN4924. Twenty-four
hours after treatment, cells were collected and processed as described above and
analyzed by flow cytometry.

Kaplan–Meier curves for human aminoacyl-tRNA synthetases. Kaplan–Meier
curves of human aminoacyl-tRNA synthetases were plotted with an online sur-
vival analysis tool (http://kmplot.com/analysis/index.php?p=service&cancer=b
reast)49 with a relapse-free survival cohort of 3,557 breast cancer patients. The
samples were divided in halves into low-expression (black) and high-expression
(red) sets, and only JetSet best probe results were used to draw the plots. The
follow-up time course was set at 17 years to calculate the P value and hazard
ratio (HR).

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