Protein–protein interactions between jasmonate-related master regulator MYC and transcriptional mediator MED25 depend on a short binding domain

Received for publication, September 13, 2021, and in revised form, December 11, 2021 Published, Papers in Press, December 18, 2021, https://doi.org/10.1016/j.jbc.2021.101504

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Edited by Joseph Jez

A network of protein–protein interactions (PPI) is involved in the activation of (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile), a plant hormone that regulates plant defense responses as well as plant growth and development. In the absence of JA-Ile, inhibitory protein jasmonate-ZIM-domain (JAZ) represses JA-related transcription factors, including a master regulator, MYC. In contrast, when JA-Ile accumulates in response to environmental stresses, PPI occurs between JAZ and the F-box protein COI1, which triggers JAZ degradation, resulting in derepressed MYC that can interact with the transcriptional mediator MED25 and upregulate JA-Ile-related gene expression. Activated JA signaling is eventually suppressed through the catabolism of JA-Ile and feedback suppression by JAZ splice variants containing a cryptic MYC-interacting domain (CMID). However, the detailed structural basis of some PPIs involved in JA-Ile signaling remains unclear. Herein, we analyzed PPI between MYC3 and MED25, focusing on the key interactions that activate the JA-Ile signaling pathway. Biochemical assays revealed that a short binding domain of MED25 (CMIDM) is responsible for the interaction with MYC, and that a bipartite interaction is critical for the formation of a stable complex. We also show the mode of interaction between MED25 and MYC is closely related to that of CMID and MYC. In addition, quantitative analyses on the binding of MYC3-JAZs and MYC3-MED25 revealed the order of binding affinity as JAZiso < MED25-CMIDM < JAZCMID, suggesting a mechanism for how the transcriptional machinery causes activation and negative feedback regulation during jasmonate signaling. These results further illuminate the transcriptional machinery responsible for JA-Ile signaling.

(+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile) is an oxylipin-derived plant hormone that regulates plant defense responses and growth (1, 2). In its absence, JA-Ile responses are restrained by a group of transcriptional repressors called “jasmonate-ZIM-domain” (JAZ) proteins. In Arabidopsis thaliana, a conserved C-terminal Jas motif of JAZ proteins physically interacts with and inhibits basic helix–loop–helix transcription factors (TFs), including MYC2, MYC3, and MYC4. In addition, JAZ proteins recruit TOPELESS (TPL) as a corepressor either through direct binding of TPL to ethylene-response factor-associated amphiphilic repression motifs at the N terminus of JAZ proteins, or indirectly through the ethylene-response factor-associated amphiphilic repression motif-containing NOVEL INTERACTOR OF JAZ (NINJA) adaptor protein that binds to the central ZIM domain of JAZ proteins (3–6). JA-Ile is quickly biosynthesized in response to internal or external cues and then induces the protein–protein interaction (PPI) between F-box protein CORONATINE INSENSITIVE 1 (COI1) and JAZ, which triggers ubiquitination and subsequent degradation of the JAZ through the 26S-proteasome (7–10). Thereafter, the derepressed MYC TFs can physically interact with the transcriptional mediator MED25, recruiting the histone acetyl transferase1 to the target promoters of MYC and bridging MYC and the RNA polymerase II for transcriptional preinitiation complex assembly, upregulating various JA-Ile-related genes (Fig. 1) (11–16). Activated jasmonate signaling is eventually suppressed through two independent mechanisms: catabolism of JA-Ile by CYP94B1/B3/C1 monooxygenases (17–19) and feedback suppression of jasmonate signaling through the expression of JAZ10 splice variants, such as JAZ10.3 (lacking the C terminus of Jas motif), JAZ10.4 (lacking the entire Jas motif), and JAZ10.1 (full-length JAZ10). These splice variants contain an N-terminal cryptic MYC-interaction domain (CMID) that interacts with MYC proteins to repress the MYC target gene expression. Because the increase of JA-Ile level causes accumulation of JAZ10.4 protein, JAZ10 splice variants are considered to be the important negative feedback regulators of JA signaling (20–23). However, with the exception of COI1-JAZ and JAZ-MYC, the detailed structural basis of PPIs involved in jasmonate signaling remains unclear (21, 24, 25). For example, 407 to 680aa of MED25 was reported to participate in the binding with MYC3, however, the detailed mode of binding has not been clarified (25).

Here, we report a biochemical analysis of the mode of PPI between MYC and MED25. We conclude that a short-binding
domain of MED25 is responsible for the interaction with MYC, and the mode of interaction between MED25 and MYC is similar to that of CMID and MYC. These findings give new insights into the structural basis of jasmonate signaling machinery.

Results

AlphaScreen assay of protein–protein interaction between MYC3 and MED25

A previous study by Zhang et al. mapped the interaction domain of MED25 with MYC3 to a large region (amino acids 407–680) and includes the MED25 activator interaction domain (25). To investigate the PPI between MED25 and MYC3, we constructed a wheat germ-derived cell-free translation system for full-length MYC proteins bearing a FLAG tag (FLAG-MYC2, 3, 4) and a MED25 (407–680 aa) protein bearing a GST tag and biotin-ligation site (Bls) sequence (GST-Bls-MED25) and studied them using the AlphaScreen technique, as previously described (Figs. 2A and S1) (26, 27). A full-length JAZ9 protein bearing a GST tag and Bls sequence (GST-Bls-JAZ9) was also constructed for comparison (Figs. 2A and S1). In the AlphaScreen assay, the reaction mixture containing FLAG-MYC and GST-Bls-MED25 or GST-Bls-JAZ9 was initially incubated for 2 h at 4 °C. Subsequently, anti-FLAG antibody-coated AlphaScreen acceptor beads and Streptavidin-coated donor beads were added to each sample, and the mixture was incubated for 5 h at room temperature. On laser excitation, a photosensitizer in the donor bead converts ambient oxygen to a more excited singlet state. In the case of interaction between two proteins and the resulting proximity of the two beads, the singlet state oxygen molecules diffuse across to react with the acceptor bead generating chemiluminescence light (Fig. S1A). In contrast, in the absence of a specific interaction between two proteins, the singlet state oxygen molecules produced by the donor bead go undetected without the close proximity of the acceptor bead, resulting in the production of a very low background signal. As shown in Figure 2B, the samples containing FLAG-MYC3 and GST-Bls-MED25 had a high signal intensity of the AlphaScreen compared with MYC3 alone, indicating that full-length MYC3 directly interacts with MED25 in vitro, as previously reported (25).

Previous studies of the crystal structure of MYC3-JAZ9 identified the amino acid residues L125, E148, F151, L152, and M155 of MYC3 as being important for interaction with JAZ9 (25). We prepared the alanine or arginine mutants of these amino acids to examine the interaction mode between MYC3 and MED25 (Fig. S2). As previously reported, the affinities of these MYC3 mutants for JAZ9 (Fig. 2C) were minimal, whereas their affinities for MED25 were similar or slightly lower than that of WT MYC3 (Fig. 2B). The affinities for MED25 of mutants incorporating alanine in place of the hydrophobic amino acids F151, L152, and M155 were slightly decreased. Therefore, we next evaluated the affinities of double mutants of these peptides, L125A/F151A, L125A/
M155A, and F151A/L152A (Fig. S3). As was the case for JAZ9, their affinities for MED25 were all significantly decreased (Fig. 2, B and C), suggesting that the hydrophobic interactions between MED25 and more than one amino acid of MYC3 are crucial for their interaction. This interaction between MED25 and MYC3 was clearly different from that of MYC3-JAZ9. However, close mode of interaction can be found in the interaction between MYC3 and JAZ10CMID, in which the CMID of JAZ10 is involved in repression of MYC (20, 21).

Identification of the interaction domain of MED25 with MYC

It is reported that the CMID of JAZ10 is also conserved in JAZ1, 2, 5, and 6 (21). Therefore, we next examined the homology between MED25 and these CMID regions. Of the MYC3-interacting domains of MED25 (amino acids 407–680) previously reported, the domain comprising amino acids 614 to 655 of MED25 is moderately homologous (21%) with the CMID regions (Fig. 3A), and so we named this domain the “CMID-like MYC-interacting domain” (CMIDM). To examine the role of CMIDM in the interaction between MED25 and MYC, we prepared MED25 mutants incorporating alanine in place of the highly conserved amino acids in the CMIDM and CMID sequences (S627A, K638A, P647A, and L653A, Fig. S4) and selected MED25 double mutants (S627A/K638A, S627A/P647A, S627A/L653A, K638A/L653A, K638A/L653A, and P647A/L653A, Fig. S5). As shown in Figure 3B, a significant decrease was observed in the signal intensities on AlphaScreen analyses, suggesting that most of the MED25 mutants have weaker affinities for MYC3 compared with WT MED25. Furthermore, a marked decrease of binding for MYC3 was observed for CMIDM-deletion mutant of MED25 (ΔCMIDM) (Figs. 3C and S6), indicating that CMIDM significantly contributes to the binding between MYC3 and MED25. Based on these findings, we prepared the biotin-conjugated CMIDM peptide of MED25 (Biotin-MED25CMIDM) (Figs. 4A and S7) for further analyses. In the AlphaScreen assay, this was demonstrated to directly interact with MYC3 (Fig. 4B). Moreover, it was revealed that Biotin-MED25CMIDM has affinity for MYC2 or MYC4 and the MED25 protein (GST-Bls-MED25 (407–680aa) Fig. 4C). These results suggested that the CMIDM is an interacting domain of MED25 with the MYC TFs.

Figure 2. AlphaScreen analyses of the protein–protein interactions between FLAG-MYC3 and GST-Bls-MED25. A, schematic representation of each protein construct used in the AlphaScreen assay; FLAG-MYC3: FLAG-tagged full-length MYC3, GST-Bls-MED25: GST- and biotin-ligase substrate sequence (Bls)-tagged MED25 (407–680) containing ACID (551–680) and CMIDM (614–655), and GST-Bls-JAZ9: GST- and Bls-tagged full length JAZ9. B and C, AlphaScreen analyses of the protein–protein interactions between (B) FLAG-MYC3 (wt or Ala mutants, 50 nM) and GST-Bls-MED25 (150 nM) or (C) FLAG-MYC3 (wt or Ala mutants, 50 nM) and GST-Bls-JAZ9 (150 nM). The experiments were performed in triplicate to obtain mean values with SD (shown as error bars). The asterisks represent significant differences between signal intensity of wt-MYC3/MED25 or wt-MYC3/JAZ9 and that of mutated-MYC3/MED25 or mutated-MYC3/JAZ9 and were evaluated by Students t test (*p < 0.01, **p < 0.005, ***p < 0.001). Bls, biotin-ligation site; CMIDM, CMID-like MYC-interacting domain; JAZ, jasmonate-ZIM-domain; JID, the JAZ-interacting domain; TAD, the transcription activation domain.
Quantitative binding affinity between MYC3 and its binding partners JAZ, MED25, and CMID

We next examined the binding affinity of MYC3 for its binding partners. As previously reported, a fluorescence anisotropy (FA) assay can be used to quantitatively assess the binding between the JAZ-binding domain of MYC3 (His6-SUMO-MYC3 (44–238)) and fluorophore-conjugated JAZ-Jas motif peptide (28). Accordingly, we prepared the fluorescein-conjugated binding domains of MED25 and CMID (Fl-MED25CMID and Fl-JAZ10CMID, Figs. 5A and S8). Briefly, MYC3 protein solution was added dropwise to the solution containing Fl-MED25CMID or Fl-JAZ10CMID, and FA values were monitored by using fluorescence spectrometer with polarizers. As the results, the FA values of these peptides were significantly increased upon addition of His6-SUMO-MYC3 in a dose-dependent manner, similar to the previously reported complex of fluorophore-conjugated JAZ9Jas (TMR-JAZ9Jas) and His6-SUMO-MYC3 (Fig. 5, B and C). The apparent $K_d$ value of Fl-MED25CMID was calculated to be 1.9 μM (Fig. 5B), which is significantly lower than that corresponding to TMR-JAZ9Jas and MYC3 (5.0 μM) (28). In contrast, the $K_d$ value of Fl-JAZ10CMID was 0.47 μM (Fig. 5C), which is 3.6-fold lower than that of Fl-MED25CMID and 10-fold lower than that of TMR-JAZ9Jas (Fig. 6). These results indicate that CMID has a stronger binding affinity for MYC than both the Jas motif of JAZ and MED25, consistent with previous reports (21).

Discussion

The mechanism by which the transcriptional activation associated with JA-Ile signaling occurs is believed to be because of the changes in the combination of the various protein–protein interactions, based on recent reports of the genetic regulators involved in JA-Ile signaling. Here, we analyzed in vitro the protein–protein interactions between MYC3 and MED25, a key step in the activation of the JA-Ile signaling (Fig. 1). Using alanine mutants of MYC3, we revealed that the PPI between MYC3 and MED25 are predominantly reliant on a different mode-of-interaction from...
those associated with the interaction between MYC3 and JAZ9 (Fig. 2) (21, 25). Furthermore, by using mutated MED25, we assigned the interaction domain of MED25 with MYC3 (CMIDM) (Figs. 3 and 4). The interaction between MYC3 and CMIDM was confirmed by canonical assays for protein–protein interaction, such as AlphaScreen (Fig. 4B), FA assay, (Fig. 5B), and pull down (Figs. S9 and S10). Our current results demonstrate that CMIDM contributes to the interaction between MED25 and MYC2/3/4 TFs (Fig. 4C) (12).

Previous reports have shown that JAZ10-CMID has a clamp-like twin-loop–helix structure bent at the proline48 (N-loop–α1–α1-2 loop–α2, Fig. S11), which interacts with both the Jas-binding groove and the backside of the Jas-interaction domain of MYC3 (21). Therefore, we prepared a biotin-conjugated partial peptide of CMIDM consisting of the part corresponding to N-loop–α1 in CMID (Biotin-MED25N-loop–α1) or α1-2 loop–α2 in CMID (Biotin-MED25α1-2 loop–α2) (150 nM each). The experiments were performed in triplicate to obtain mean values with SD (shown as error bars). In (B) and (C), significant differences were evaluated by one-way ANOVA/Tukey HSD post hoc test (p < 0.01). In (D), the asterisks represent significant differences between the signal intensity of each peptide and that of mock condition with MYC3 and were evaluated by Students’ t test (*p < 0.01, **p < 0.005). Bls, biotin-ligation site; CMID, cryptic MYC-interacting domain; CMIDM, CMID-like MYC-interacting domain; JAZ, jasmonate-ZIM-domain.

Figure 4. AlphaScreen analyses of the protein–peptide interactions between FLAG-MYC family and biotin-conjugated CMIDM peptide. A, chemical structures and amino acid sequences of JAZ10CMID and biotin-conjugated CMIDM (614–655aa, Biotin-MED25CMIDM). B, AlphaScreen analyses of FLAG-MYC3 (50 nM) with or without GST-Bl.s-MED25 (150 nM) or Biotin-MED25CMIDM (50, 150, or 250 nM). C, AlphaScreen analyses of FLAG-MYC2 or FLAG-MYC4 (50 nM) with or without GST-Bl.s-MED25 (150 nM) or Biotin-MED25CMIDM (150 nM). D, AlphaScreen signal intensity of FLAG-MYC3 (50 nM) in the absence and presence of Biotin-MED25CMIDM, Biotin-MED25N-loop–α1 or Biotin-MED25α1-2 loop–α2 (150 nM each). The experiments were performed in triplicate to obtain mean values with SD (shown as error bars). In (B) and (C), significant differences were evaluated by one-way ANOVA/Tukey HSD post hoc test (p < 0.01). In (D), the asterisks represent significant differences between the signal intensity of each peptide and that of mock condition with MYC3 and were evaluated by Students’ t test (*p < 0.01, **p < 0.005). Bls, biotin-ligation site; CMID, cryptic MYC-interacting domain; CMIDM, CMID-like MYC-interacting domain; JAZ, jasmonate-ZIM-domain.
**Protein–protein interaction analyses between MYC and MED25**

ARABIDOPSIS AP2/ERF59 (ORA59), ethylene-responsive factor (ERF), MYB, WRKY, bZIP, and zinc-finger TF families, indicating that MED25 acts as an integrator in the numerous steps in the JA-related transcriptional cascade (11, 12, 29, 30). These results provide structural insights into these complexes and could lead to elucidation of the switching mechanism between suppression and activation of JA-related transcription factors.

Furthermore, quantitative analyses by FA revealed the order of binding affinity between MYC3 and the partner proteins as JAZ<sub>jas</sub> < MED25<sub>CMIDM</sub> < JAZ<sub>CMID</sub>. In the AlphaScreen-based competitive assay for TMR-JAZ9<sub>jas</sub> with the complex of MYC3

**Figure 5. Quantitative analyses on the binding of MYC3-MED25 and MYC3-JAZ.** A, Chemical structures and amino acid sequences of fluorescein-conjugated JAZ10<sub>jas</sub> (26–59aa, Fl-JAZ10<sub>jas</sub>) and MED25<sub>CMIDM</sub> (614–655aa, Fl-MED25<sub>CMIDM</sub>). B and C, Fluorescent anisotropy experiments of Fl-MED25<sub>CMIDM</sub> (50 nM) or Fl-JAZ10<sub>jas</sub> (50 nM) with the addition of His6-SUMO-MYC3 (44–238) (0–7.5 μM). The experiments were performed in triplicate to obtain mean values with SD (shown as error bars). CMID, cryptic MYC-interacting domain; CMIDM, CMID-like MYC-interacting domain; JAZ, jasmonate-ZIM-domain.

**Figure 6. Schematic of JAZ, MED25, and CMID-mediated activation and desensitization mechanism of MYC in JA responses.** When accumulation of JA-Ile, it induces PPI between COI1/JAZ<sub>jas</sub> (upper right), which triggers degradation of JAZ and interaction of MED25 and MYC (ON, +JA). Then, subsequent expression of JAZ10.4 causes suppression of the gene expressions (desensitized and prolonged JA) and finally return to steady state (OFF, -JA). Kₐ values of these protein–peptide interactions between MYC3 and its binding partners or COI1–JA-Ile–JAZ are also included (28, 32). CMID, cryptic MYC-interacting domain; COI1, CORONATINE INSENSITIVE 1; JA-Ile, (+)-7-iso-jasmonoyl-L-isoleucine; JAZ, jasmonate-ZIM-domain; PPI, protein–protein interaction.
and MED25⁶CMIDM or biotin-MED25⁶CMID with the complex of MYC3 and JAZ10⁶CMID, these peptides competitively inhibited the corresponding complex dose-dependently (Fig. S13). These results suggested that the three binding domains belonging to JAZ (Jas motif and CMID) or MED25 (CMIDM) competitively bind to the same interacting site on MYC3. This order suggested that MED25⁶CMIDM can replace the position of JAZjas in MYC3-JAZjas complex, and the resulting MYC3-MED25⁶CMIDM can be replaced subsequently by JAZ⁶CMID to form MYC3-JAZ⁶CMID complex. These results strongly support the previously proposed picture of how the formation and transformation of transcriptional machinery in jasmonate signaling cause negative feedback regulation. The formation of COI1–JA-Ile–JAZ triggers degradation of JAZ repressor to upregulate the expression of MED25 (11–13, 15, 16), and then subsequent expression of JAZ including the CMID sequence cause suppression of the gene expressions in jasmonate signaling (Fig. 6) (20, 21). Overall, revealing the MED25-Mediated activation or desaturation mechanism of JA-Ile-related transcription factors would be an important step for not only clarifying the control mechanism of the entire jasmonate signaling, but leading to development of the specific chemical tools for JA-Ile related TFs in the future (28).

Experimental procedures

General materials and methods

All chemical reagents and solvents were obtained from commercial suppliers (Wako Pure Chemical Industries Co. Ltd, Nacalai Tesque Co., Ltd, Watanabe Chemical Industries Co. Ltd, Thermo Fisher Scientific K.K., and GE Healthcare) and used without further purification. DNA purification was accomplished using a GENE PREP STAR PI-80X DNA isolation system (KURABO). SDS-PAGE and Western blotting were performed using Mini-Protean III (Bio-Rad Laboratories, Inc.), Trans-Blot Turbo (Bio-Rad Laboratories, Inc.) and iBind Flex (Thermo Fisher Scientific) devices. Chemiluminescent images were detected using the Amersham Imager 680 detector (GE Healthcare). Reverse-phase HPLC was performed on a PU-4180 plus equipped with UV-4075 and MD-4010 detectors (JASCO). The absorbance at 220 nm and 488 nm was monitored by an MD-4010 photodiode array detector (PDA). MALDI-TOF MS analysis was performed on an Autoflex II (Bruker Daltonics Inc.).

Preparations of FLAG-MYC and GST-Bls-MED25 proteins

Standard methods for cloning were used, and all the PCR-amplified DNA fragments were sequenced after cloning into the vectors. The coding sequences of MYC2, 3, and 4 were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT/AMED, (pda00106 for MYC2 (AT1G32640), pda09611 for MYC3 (AT5G46760), and pda15392 for MYC4 (AT5G46830)). The plasmids for MYC2 and MYC4 are categorized to RIKEN Arabidopsis full-length cDNA clones (RAFL clones), which are inserted into modified pBluescript, and these plasmids were used as they were in the subsequent cell-free translation system. The plasmid for MYC3 is categorized to Arabidopsis Transcription factor clones (TF clones), which is inserted into pENTR207. Because the stop codon is missing in the TF clone, the MYC3 sequence was amplified by PCR with the addition of the stop codon and the restriction enzymes sites (EcoRV and BamHI, Table S1) and inserted into the pEU-E01-FLAG-MCS vector to generate pEU-E01-FLAG-MYC3. The coding sequences of MED25 (407–680aa) were isolated from Arabidopsis cDNA using the primers with the addition of the restriction enzymes sites (BamHI and Spel, Table S1) and inserted into pEU-E01-GST-Bls-MCS vector to generate pEU-E01-GST-Bls-MED25. Site-directed mutagenesis of FLAG-MYC3 and GST-Bls-MED25 was performed based on the conventional inverse-PCR method with each primer (Tables S2 and S3). L125A/F151A, F125A/F151A, and F151A/L152A mutants of FLAG-MYC3 were amplified based on the plasmid of FLAG-MYC3 (F151A) mutant with corresponding primers. S627A/K638A and S627A/P647A mutants of GST-Bls-MED25 were amplified based on the plasmid of GST-Bls-MED25 (S627A) mutant with corresponding primers. K638A/P647A mutant of GST-Bls-MED25 was amplified based on the plasmid of GST-Bls-MED25 (K638A) mutant with corresponding primers. S627A/L653A, K638A/L653A, and P647A/L653A mutants of GST-Bls-MED25 were amplified based on the plasmid of GST-Bls-MED25 (L653A) mutants with corresponding primers. These proteins were expressed in wheat germ-derived cell-free protein expression system according to the previous report (26). The obtained protein mixture was centrifuged (20,000 rpm for 15 min at 4 °C), and the supernatant was used for the SDS-PAGE or AlphaScreen experiments without any purification. In the immunoblotting analyses, FLAG-tagged proteins were detected using anti-FLAG antibody (Sigma Aldrich, F1804, clone M2, 2500-fold dilution in blocking buffer) and anti-GST-HRP conjugate (RPN1236, GE Healthcare, 5000-fold dilution in blocking buffer), and mouse IgG-HRP antibody (Southern Biotech. Inc., 1031–05, 20,000-fold dilution in blocking buffer), shown in Figures S1A–S3. GST-tagged proteins were detected using anti-GST-HRP conjugate (RPN1236, GE Healthcare, 5000-fold dilution in blocking buffer), shown in Figure S1B. Biotin-tagged proteins were detected using streptavidin-HRP conjugate (RPN1231V, GE Healthcare, 5000-fold dilution in blocking buffer), shown in Figures S4–S6.

AlphaScreen assay

AlphaScreen experiments were performed at 25 °C in the incubation buffer (50 mM Mops, pH 7.4, 50 mM NaF, 50 mM CHAPS, 0.1 mg/ml bovine serum albumin) (31). 15 μl of the reaction mixture in the incubation buffer containing 50 nM FLAG-MYC and 150 nM GST-Bls-MED25 (or GST-Bls-JA29) was added to a 1/2 Area AlphaPlate-96 (PerkinElmer) and incubated for 2 h at 4 °C. Subsequently, 10 μl of a detection mixture in the incubation buffer containing 0.1 μl of anti-FLAG antibody-coated AlphaScreen acceptor beads and 0.1 μl of Streptavidin-coated donor beads was added to each well. In Figure S13B, anti-FITC antibody-coated donor beads were used instead of Streptavidin-coated donor beads. Thereafter, the mixture was incubated for 5 h at room
temperature, and the luminescence signals were detected using the Envision 2105 Multimode Plate Reader (PerkinElmer, Inc.). The experiment was repeated three times, and the data are presented as average values with SD.


The peptides were prepared using the fully automated microwave peptide synthesizer Initiator+ Alstra (Biotage Ltd) starting from Fmoc protected amino acid-tethered Wang resin (90 μm) as previously reported, with minor modifications (32, 33). The resin was swollen in N,N-dimethylformamide (DMF) at 70 °C for 20 min. The Fmoc protecting group was removed by treating with 20% piperidine in DMF twice. Amino acid coupling was accomplished by mixing the resin with Fmoc protected amino acids (3 eq), 1-(7-aza-1H-benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphat (3 eq), 1-hydroxy-7-azobenzotriazole (3 eq), and DIPEA (12 eq) in DMF and subjecting it to microwave irradiation at 50 °C for either 30 min (Fmoc-Arg-OH) or 10 min (all others). After the peptide had been fully elongated, the resin was mixed with biotin or 5-carboxy-fluorescein diacetate (2 eq), 1-(7-aza-1H-benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphat (4 eq), and DIPEA (8 eq) in DMF and incubated at r.t. for 3 h. After the reaction, the peptide was deprotected by stirring in TFA solution containing thioanisole, 1,2-ethanedithiol at r.t. for 1.5 h. The reaction mixture was purified by HPLC using a Develosil ODS-HG-5 column (Φ 4.6 × 250 mm) eluting with a linear gradient (CH₂CN (0.05% TFA):H₂O (0.05% TFA) = 20:80 (5 min) to 60:40 (35 min)) to afford biotin-conjugated MED25N-loop-α1 peptide. After lyophilization, the peptide was dissolved in sterilized water. The concentration of the stock solution of Biotin-MED25^N-loop-α1 was determined by BCA assay. The purity of the peptide was confirmed by HPLC analyses, and it was characterized by MALDI-TOF MS as follows: Biotin-MED25^N-loop-α1, m/z [M + H]^+ calcd for 4934.49, and Fl-MED25^571-612, m/z [M + H]^+ calcd for 5066.46, found 5066.46.

**Fluorescent anisotropy assay**

Fluorescent anisotropy experiments were performed at room temperature in 20 mM Tris-HCl (pH 8.0, 200 mM NaCl, and 10% Glycerol) using a quartz cell (150 μl), as previously described. His6-SUMO-tagged MYC3 (44–238) was added dropwise to the solution containing fluorophore-conjugated peptides (Fl-MED25^CMIDM or Fl-JAZ10^CMID, 100 nM). Fluorescence anisotropy was recorded on a FP-8500 (Jasco). Excitation and emission wavelengths for fluorescein are 485 nm and 516.5 nm, respectively. Fluorescence anisotropy values (r) were calculated using the following equation: \( r = (I_{HV} - G × I_{VH})/(I_{HV} + 2G × I_{VH}) \), where \( I_{HV} \) and \( I_{VH} \) are the fluorescence intensities observed through polarizers parallel and perpendicular to the polarization of the exciting light, respectively, and G is a correction factor to account for instrumental differences in detecting emitted compounds (G = \( I_{HV}/I_{VH} \)). An average value of three independent measurements was calculated for each point. Anisotropy titration curves were analyzed with the nonlinear curve-fitting analysis to evaluate apparent \( K_a \) and \( K_d \) values.

**Pull down assay**

For the pull down assay, FLAG-MYC3 (0.5 μM) and Fl-MED25^CMIDM peptide (5 μM) or Fl-MED25^571-612 peptide (5 μM, as the negative control peptide that is another domain with the same length as CMIDM in MED25) in 70 μl of incubation buffer (50 mM Mops, pH 7.4, 50 mM NaF, 50 mM CHAPS, 0.1 mg/ml bovine serum albumin) was incubated at 4 °C for 2 h, and then mixed with anti-fluorescein antibody (Genetex, GTX26644, 0.5 μl). After 10 h incubation at 4 °C, the samples were mixed with Protein G magnetic beads (Bio-Rad, SureBeads Protein G Magnetic Beads, 10 μl in 50%...
incubation buffer slurry). After 3 h incubation at 4 °C, the samples were washed three times with 200 μl of the incubation buffer. The washed beads were resuspended in 50 μl of SDS-PAGE loading buffer containing DTT (100 mM). After boiling for 10 min at 60 °C, the samples were subjected to SDS-PAGE and analyzed by Western blotting. The bound FLAG-MYC3 proteins were detected using anti-FLAG antibody (Sigma Aldrich, F1804, clone M2, 2500-fold dilution in blocking buffer) and anti-mouse IgG-HRP antibody (Southern Biotech, Inc., 1031–05, 20,000-fold dilution in blocking buffer), shown in Figure S10.

Data availability

All relevant data are contained in the article.

Supporting information—This article contains supporting information.


Funding and additional information—This work was financially supported by a Grant-in-Aid for Scientific Research from JSPS, Japan (Nos. 17H06407, 18K0162, and 20H00402 for M.U., nos. 18H02101, 19H05283, and 21H00270 for Y.T., and 21H00287 for H. T.), JSPS A3 Foresight Program (M. U.), JSPS Core-to-Core Program Asian Chemical Biology Initiative (M. U.), and Takeda Science Foundation (Y. T.).

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: Bls, biotin-ligation site; CMID, cryptic MYC-interacting domain; CMDM, CMID-like MYC-interacting domain; COI1, CORONATINE INSENSITIVE 1; DMF, N,N-dimethylformamide; FA, fluorescence anisotropy; JA-Ile, (+)-7-iso-jasmonoyl-L-isoleucine; JAZ, jasmonate-ZIM-domain; PDA, photodiode array detector; PPL, protein–protein interaction; TFs, transcription factors.

References

Protein–protein interaction analyses between MYC and MED25


