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Genetic dissection of active forgetting in labile and consolidated memories in *Drosophila*

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Different memory components are forgotten through distinct molecular mechanisms. In *Drosophila*, the activation of 2 Rho GTPases (Rac1 and Cdc42), respectively, underlies the forgetting of an early labile memory (anesthesia-sensitive memory, ASM) and a form of consolidated memory (anesthesia-resistant memory, ARM). Here, we dissected the molecular mechanisms that tie Rac1 and Cdc42 to the different types of memory forgetting. We found that 2 WASP family proteins, SCAR/WAVE and WASp, act downstream of Rac1 and Cdc42 separately to regulate ASM and ARM forgetting in mushroom body neurons. Arp2/3 complex, which organizes branched actin polymerization, is a canonical downstream effector of WASP family proteins. However, we found that Arp2/3 complex is required in Cdc42/WASp-mediated ARM forgetting but not in Rac1/SCAR-mediated ASM forgetting. Instead, we identified that Rac1/SCAR may function with formin Diaphanous (Dia), a nucleator that facilitates linear actin polymerization, in ASM forgetting. The present study, complementing the previously identified Rac1/cofilin pathway that regulates actin depolymerization, suggests that Rho GTPases regulate forgetting by recruiting both actin polymerization and depolymerization pathways. Moreover, Rac1 and Cdc42 may regulate different types of memory forgetting by tapping into different actin polymerization mechanisms.

forgetting | memory | *Drosophila* | Rho GTPases | actin

Forgetting has been recently proposed to be a critical component of a healthy memory management system by providing flexibility and generalization ability (1, 2). Previous studies from invertebrates to vertebrates support that learning itself can activate signals which specifically accelerate the decay of a formed memory without affecting its acquisition (3–7). Such a process is termed active forgetting.

In *Drosophila*, 1-session training of olfactory aversive conditioning produces 2 memory forms that are distinguishable by their sensitivity to cold anesthesia (8). In wild-type flies, the cold-shock sensitive component, anesthesia-sensitive memory (ASM), lasts for up to 6 h; while the cold-shock resistant component, anesthesia-resistant memory (ARM), is more stable and lasts for over 1 d (9). Rac1, a Rho GTPase (10), regulates the rapid decay of ASM (3). Besides ASM, Rac1 also regulates a short-lived olfactory sensory memory in trace conditioning (11). In contrast, Cdc42, another Rho GTPase, regulates the forgetting of ARM. ARM is regarded as one of the consolidated memory forms in *Drosophila* (12), which gradually forms in the first hour after training, reaches a plateau at about 2 h, and undergoes slow decay thereafter (4). This later decay of ARM is modulated by repetitive training and is tied to the activity of Cdc42 (4).

Rac1 and Cdc42 are seated as signaling hubs that orchestrate actin rearrangement (13). In accordance with invertebrate studies, Rac1 was also reported to contribute to forgetting of multiple types of memories in mice (7, 14, 15) and rats (16). Still, how 2 similarly functioned Rho GTPases are involved in forgetting of different memory forms remains unknown. Rac1-mediated forgetting is partially explained by activating cofilin, an actin depolymerization factor, through a PAK/LIMK signaling cascade (3, 17). However, Rho GTPases are known to interact with numerous effectors (18, 19), and the downstream pathways that tie Rac1 and Cdc42 to actin remodeling and eventually forgetting are far from fully elucidated. Rho GTPases can also affect actin polymerization pathways. For example, 2 WASP family proteins, SCAR/WAVE and WASp, are known to transduce Rac1 and Cdc42 activity to the activation of Arp2/3 complex to promote actin polymerization (18, 20); but it is unclear how they may contribute to the forgetting functions of Rac1 and Cdc42.

Results

SCAR/WAVE Complex Regulates Labile Memory Forgetting in the MB Neurons. We first tested whether SCAR/WAVE complex affects ASM forgetting using RNAi in *Drosophila*. To avoid developmental defects, we restricted RNAi expression to the adult flies using elav-GS, a pan-neuronal conditional expression driver that depends on RU486 feeding (21). We examined memory retention curves after a 1-session olfactory aversive conditioning (9). *SCAR*-RNAi-expressing flies (RU486+) showed memory performance index (PI) similar to the uninduced controls without RU486 feeding (RU486−) shortly after training (3 min and 1 h, Fig. 1*A*), but the memory was significantly higher at later time

Significance

As a critical component of a healthy memory management system, forgetting has received increasing attention. Studies across multiple species support important roles of actin remodeling in forgetting. However, the underlying molecular mechanisms remain unclear. In *Drosophila*, Rac1 and Cdc42, 2 Rho GTPases that act as signaling hubs to coordinate actin remodeling, were reported to mediate the forgetting of labile and consolidated memories, respectively. Here, we showed that Rac1 and Cdc42 exert their effects on forgetting by acting through 2 different actin polymerization pathways, Rac1/SCAR/Dia and Cdc42/WASp/Arp2/3 complexes. These findings fill in the molecular landscape that link forgetting to actin remodeling at the cellular level and shed light on drug development that aims to tune forgetting to treat memory-related diseases.

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points (2, 3, and 6 h, Fig. 1*A*). Additional experiments using attenuated training intensity (*SI Appendix*, Fig. S1*A*) confirmed that SCAR knockdown does not affect initial learning (assayed at 3 min after training). For more stringent controls, *SCAR*-RNAi-expressing flies showed higher memory retention when compared with their parental controls (*elav-GS*/+ and *UAS-SCAR-RNAi-1*/+, RU486+, Fig. 1*B* and *SI Appendix*, Fig. S1*B*). The phenotypes were again confirmed using an independent *SCAR*-RNAi line (*SI Appendix*, Fig. S1 *C* and *D*). The higher 3-h memory performance of *SCAR*-RNAi-expressing flies was blocked by cold-shock anesthesia (Fig. 1*B* and *SI Appendix*, Fig. S1*E*), suggesting that SCAR knockdown hampers the forgetting of the ASM component.

We also tested the effect of SCAR knockdown on interference-based forgetting using a protocol as previously shown (3) (Fig. 1*C*). After an initial learning session, the trained flies were subjected to a second learning session with a novel pair of odors and the flies were evaluated for 3-h retention of the first learning. The introduction of a second learning session (interference+) lowered memory performance in control flies (RU486−), but had no significant effect in *SCAR*-RNAi-expressing flies (RU486+) (Fig. 1*C*).

We next found 3 lines of experimental evidence supporting the notion that SCAR is one of the downstream effectors of Rac1 in ASM forgetting. First, Rac1 and SCAR function in the same brain locus. Rac1-dependent forgetting has been mapped to the intrinsic mushroom body (MB) neurons (3), which play a critical role in olfactory learning and memory in *Drosophila* (22). We found that expressing SCAR-RNAi in the adult MB neurons using a MB-specific, inducible driver (MB-GS) (23) resulted in higher 3-h memory retention, but left 1-h memory intact (Fig. 1*D*). Conversely, overexpression of SCAR in the adult MB neurons did not affect 1-h memory but reduced 3-h memory retention (Fig. 1*E*), and the reduction was also blocked by cold anesthesia (*SI Appendix*, Fig. S1*F*). Second, RNAi knockdown of other proteins (Sra1, Abi, and HSPC300) in the SCAR/WAVE complex (20) confirmed the memory phenotype of SCAR at 3-h after training (Fig. 1*F*). Third, we performed a genetic epistasis experiment by combining the expression of a constitutively active mutant (Rac1-CA) and the *SCAR*-RNAi-1 in the MB neurons. Consistent with our previous report (3), Rac1-CA expression decreased 3-h memory (*MB-GS*/*UAS-Rac1-CA*, RU486+, Fig. 1*G*). The decrement was dominated by SCAR knockdown (*UAS-SCAR-RNAi-1*/+; *MB-GS*, *UAS-Rac1-CA*/+, RU486+, Fig. 1*G*). We therefore conclude that SCAR/WAVE complex functions downstream of Rac1-mediated ASM forgetting (Fig. 1*H*).

WASp Regulates ARM Forgetting in the MB Neurons. We also investigated the function of WASp in Cdc42-dependent ARM forgetting. To isolate ARM from ASM and to assay the retention of ARM (ARM PI) at different time points after training, we subjected flies to 2-min cold-shock treatment and then allowed flies to recover for 1 h at 25 °C before testing. We focus on the decay of ARM at 3 to 12 h after its formation reaches a plateau at about 2 h. Flies with conditional pan-neuronal *WASp*-RNAi expression (RU486+) showed slower ARM decay when compared with the RU486− controls. Their performance was higher when ARM was assayed at 6, 9, and 12 h after a 1-session training or at 24 h after a 4-session massed training (Fig. 2*A*). Note that the effect of WASp knockdown was specific to the later decay phase of ARM without affecting ARM retention at 2 and 3 h after a 1-session training (Fig. 2*A*) or at 2 h after attenuated training (*SI Appendix*, Fig. S2*A*), suggesting that WASp knockdown does not affect ARM formation. For more stringent controls, the higher ARM performance was confirmed by including the parental controls (Fig. 2*B*) and by using a second independent RNAi line (*SI Appendix*, Fig. S2*C*). ASM decay is likely not affected by WASp knockdown. WASp-RNAi-expressing flies had normal memory performance up to 3 h after a 1-session training (*SI Appendix*, Fig. S2*B*). Since ASM has considerable decay in the 3-h memory time window, the absence of effect of WASp knockdown

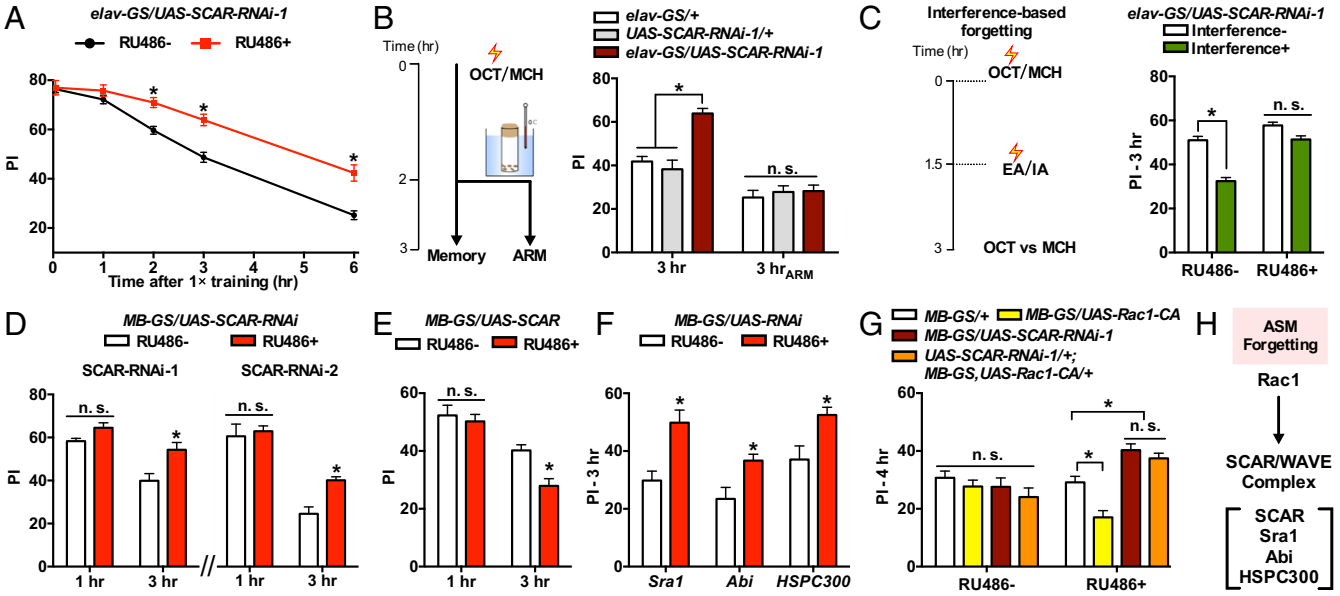

Fig. 1. SCAR/WAVE regulates ASM forgetting in the MB neurons. (*A*) Memory retention curves. *SCAR*-RNAi-expressing flies (RU486+) showed slower memory decay compared with uninduced control. $n = 8$. (*B*) SCAR knockdown increased 3-h memory without affecting ARM component (3 h_{ARM}). Lightning symbol represents electric shock reinforcement. $n = 8$. (*C*) Interference-based forgetting was suppressed by RU486-induced expression of *SCAR*-RNAi. $n = 8$. (*D* and *E*) Memory performance in flies with SCAR knockdown (*D*) and overexpression (*E*) in the adult MB neurons. $n = 8$. (*F*) Effect of RNAi knockdown of different members in the SCAR complex on 3-h memory. $n = 8$. (*G*) RNAi knockdown of SCAR dominated the effect of constitutively active Rac1 (Rac1-CA) on 4 h memory. Rac1-CA-expressing flies (yellow bar, RU486+) had lower memory performance. Flies with coexpression of both Rac1-CA and *SCAR*-RNAi (orange bar, RU486+) showed memory performance higher than controls, and the performance was not different from flies expressing SCAR-RNAi alone. $n = 8$ to 12. (*H*) Data summary. Data are means ± SEM. $^*P < 0.05$. n.s., nonsignificant.

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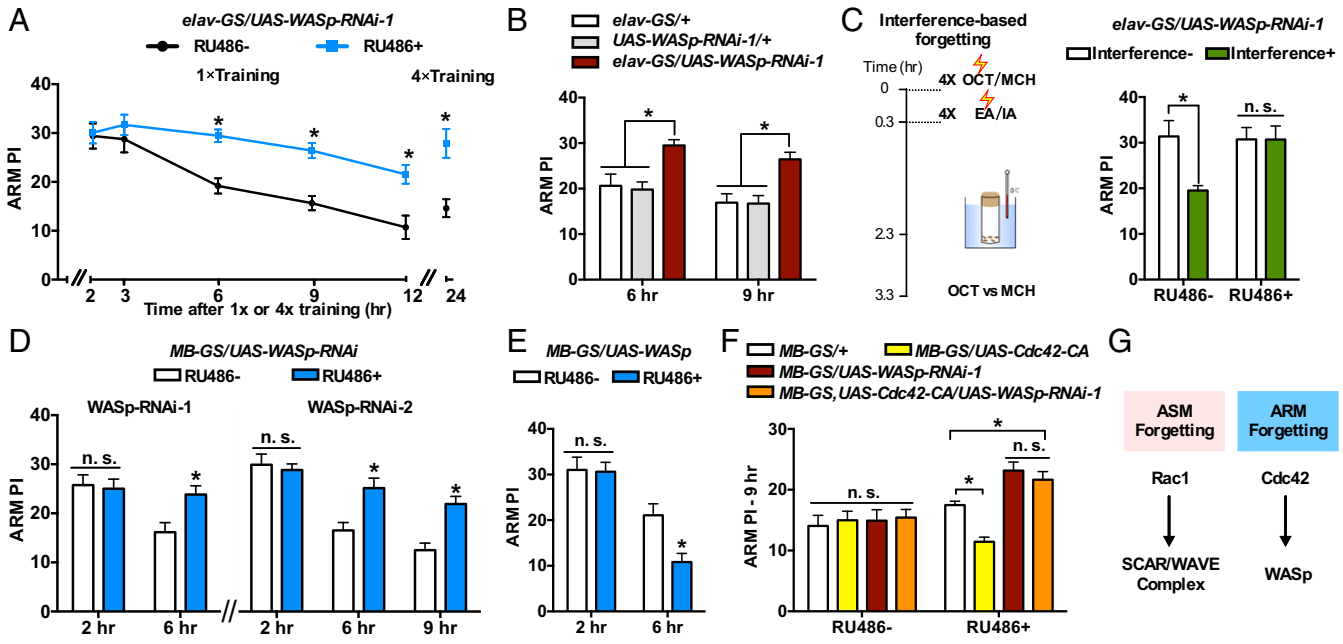

Fig. 2. WASp regulates ARM forgetting in the MB neurons. (*A*) ARM retention curves. WASp knockdown flies (RU486+) showed slower ARM decay. $n = 8$ to 11. (*B*) *WASp*-RNAi-expressing flies had higher ARM performance at 6 and 9 h after training compared with parental controls. $n = 8$. (*C*) Interference-based ARM forgetting was suppressed by RU486-induced expression of *WASp*-RNAi. Lightning symbol represents electric shock reinforcement. $n = 8$. (*D* and *E*) Memory performance in flies with WASp knockdown (*D*) and overexpression (*E*) in the adult MB neurons. $n = 8$. (*F*) RNAi knockdown of WASp dominated the effect of constitutively active Cdc42 (Cdc42-CA) on 9-h ARM. Cdc42-CA-expressing flies (yellow bar, RU486+) showed lower 9 h_{ARM}. Flies with coexpression of both Cdc42-CA and *WASp*-RNAi (orange bar, RU486+) showed memory performance higher than controls, and the performance was not different from flies expressing *WASp*-RNAi alone. $n = 8$ to 12. (*G*) Data summary. Data are means ± SEM. $^*P < 0.05$. n.s., nonsignificant.

on 3-h memory indicates that the formation and decay of ASM are independent of WASp. For better visualization, we subtracted ARM from the intact memory (without cold-shock treatment) to generate the ASM component. Despite a consistent increase of intact memory and ARM by WASp knockdown at 6 and 9 h after a 1-session training, an effect on the ASM component was not observed (Fig. 2*B* and *SI Appendix*, Fig. S2*D*).

We also tested the requirement of WASp in interference-based forgetting of ARM using a retroactive interference paradigm used in our previous study (4). Flies received 4-session massed training, and immediately following the initial training, the trained flies were exposed to a second 4-session massed training with a novel odor pair. Testing of the ARM retention of the initial learning was performed at 3.3 h after the initial training. The interference learning reduced the performance in control flies (RU486−, Fig. 2*C* and *SI Appendix*, Fig. S2*E*), while such forgetting was suppressed in *WASp*-RNAi-expressing flies (RU486+, Fig. 2*C* and *SI Appendix*, Fig. S2*E*). Together, like Cdc42 (4), WASp is required for time-based and interference-based forgetting of ARM, and on the other hand, WASp is dispensable in ARM formation and ASM decay.

There are 2 additional lines of evidence supporting the idea that WASp functions downstream of Cdc42 in ARM forgetting. First, like Cdc42 (4), WASp-dependent forgetting also takes place in the MB neurons. Knockdown (Fig. 2*D*) and overexpression (Fig. 2*E*) of WASp in the adult MB neurons led to slower and accelerated ARM decay after a 1-session training. Second, we combined Cdc42 activation and WASp knockdown to test genetic interaction (Fig. 2*F*). Consistent with our previous finding (4), flies expressing constitutively active Cdc42 (*MB-GS*/*UAS-Cdc42-CA*, RU486+) had reduced ARM performance at 9 h after a 1-session training when compared with control flies (*MB-GS*/+, RU486+). The reduction was reversed by coexpression of WASp-RNAi (*MB-GS*, *UAS-Cdc42-CA*/*UAS-WASp-RNAi-1*, RU486+). Flies expressing both Cdc42-CA and *WASp*-RNAi-1 had a performance level similar to flies expressing *WASp*-RNAi alone, and both groups were higher than the *MB-GS*/+ control. The data suggest that WASp acts as a downstream effector of Cdc42 in ARM forgetting (Fig. 2*G*).

Arp2/3 Complex Is only Required in Forgetting of ARM but Not ASM. Arp2/3 complex is a known downstream effector that ties the Rac1/SCAR pathway and Cdc42/WASp pathway to actin polymerization (18, 20). And it has been reported to be important for forgetting in *Caenorhabditis elegans* (24). We next tested the role of Arp2/3 complex in forgetting by knocking down Arp2 and Arp3, 2 major members of this complex (25), and by feeding flies with 20 μM of CK666, a specific inhibitor of Arp2/3 complex that stabilizes the inactive state of the complex (26). The inhibition of Arp2/3 complex by both genetic and pharmacological methods led to higher 6-h memory (Fig. 3 *D*–*F*), but the 3-h memory was not affected (Fig. 3 *A*–*C*). The higher memory retention at 6 h is specific to ARM, while the ASM component is spared (Fig. 3 *D*–*F*). We additionally tested the dosage-dependent effect of CK666 feeding. Increased memory retention at 12 h was observed when flies were fed with CK666 higher than 5 μM (*SI Appendix*, Fig. S3*B*), while no effects were observed for memory retention at 3 h for all of the concentrations tested (up to 20 μM, *SI Appendix*, Fig. S3*A*). The specific effect on ARM forgetting indicates that Arp2/3 complex functions downstream of the Cdc42/WASp pathway. To test this, we combined the expression of constitutively active Cdc42-CA and *WASp*-RNAi with the pharmacological inhibition of Arp2/3 complex using CK666. ARM forgetting was accelerated by Cdc42-CA expression and slowed down by *WASp*-RNAi expression (Fig. 3*G*, CK666−). However, in the presence of CK666 feeding, there are no differences among control flies and flies expressing Cdc42-CA and *WASp*-RNAi, suggesting that the effect of Arp2/3 complex inhibition dominates those induced by Cdc42-CA and *WASp*-RNAi expression. These data support the idea that Arp2/3 complex is specifically

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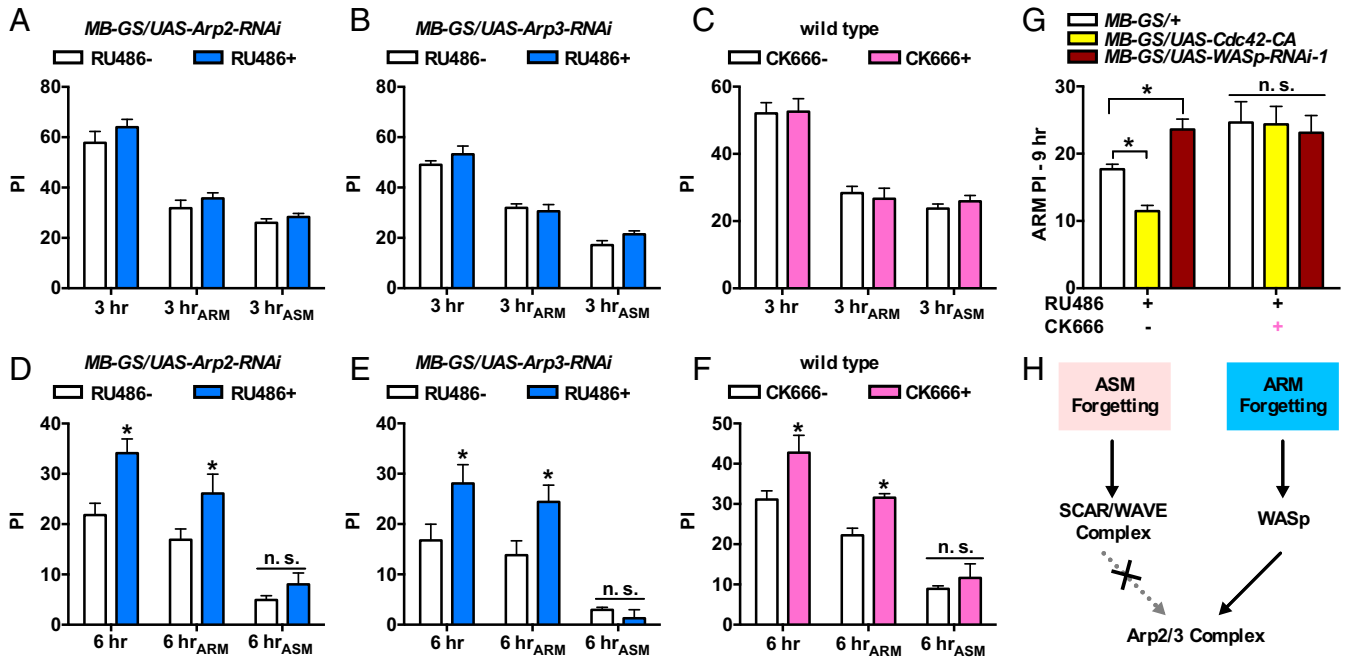

Fig. 3. Arp2/3 complex functions in ARM forgetting but not ASM forgetting. (*A* and *B*) Knockdown of Arp2 (*A*) or Arp3 (*B*) in the adult MB neurons did not affect memory retention at 3 h. $n = 8$. (*C*) Pharmacological inhibition of the Arp2/3 complex (CK666+) did not affect 3-h retention of either ARM or ASM. $n = 8$. (*D* and *E*) Knockdown of Arp2 or Arp3 in the adult MB neurons resulted in higher ARM retention, but not ASM, at 6 h. $n = 8$. (*F*) The group with CK666 feeding showed higher 6-h retention of ARM but not ASM. $n = 8$. (*G*) Pharmacological inhibition of the Arp2/3 complex dominated Cdc42-CA- and *WASp*-RNAi-dependent effect in ARM forgetting. Without CK666 feeding (CK666–) and when compared with *MB-GS*/+ control group, Cdc42-CA-expressing flies showed lower 9 h_{ARM}, while *WASp*-RNAi-expressing files showed higher 9 h_{ARM}. Such effects were masked by CK666 feeding (CK666+). $n = 8$ to 10. (*H*) Data summary. Data are means ± SEM. $^*P < 0.05$. n.s., nonsignificant.

required in Cdc42/WASp-mediated ARM forgetting but not in Rac1/SCAR-mediated ASM forgetting (Fig. 3*H*).

Formin Dia Functions with Rac1/SCAR in ASM Forgetting. To gain a better understanding of Rac1/SCAR-mediated ASM forgetting, we examined a number of interacting proteins of SCAR/WAVE complex (27–34) in a small-scale RNAi screen by knocking down these proteins in the MB neurons (*SI Appendix*, Fig. S4*A*). We found higher 3-h memory performance in flies expressing the RNAi of *Diaphanous* (*dia*), which encodes a formin family protein that induces linear actin polymerization (35).

We further tested Dia's role in ASM forgetting. Compared with the RU486– control, *dia*-RNAi-expressing flies (RU486+) showed normal memory performance at 3 min after a 1-session training, but memory decay at 3 and 6 h was slower (Fig. 4*A*). Such Dia-dependent slower memory decay was abolished by cold anesthesia (Fig. 4*A*), suggesting that Dia is required only for ASM forgetting. We also fed flies with 2.5 μM SMIFH2, a small molecule inhibitor of formin-dependent but not Arp2/3 complex-dependent actin polymerization (36). SMIFH2 also led to higher memory at 6 h after training and the phenotype is sensitive to cold anesthesia (*SI Appendix*, Fig. S4*B*), which is consistent with RNAi knockdown of Dia. Conversely, acute expression of a constitutively active form of Dia (*UAS-dia-CA*), which lacks the N-terminal regulatory sequence and the C-terminal autoinhibitory domain (37), reduced 3-h memory. The effect of Dia-CA was again blocked by cold anesthesia (Fig. 4*B*). Like SCAR, Dia is also required for interference-based forgetting (Fig. 4*C*). Thus, Dia bidirectionally regulates ASM forgetting in the MB neurons.

We next sought to determine the relationship between Dia- and Rac1/SCAR-mediated ASM forgetting using both genetic and pharmacological manipulations. The accelerated memory forgetting observed in Rac1-CA-expressing flies (yellow bar, RU486+) was dominated by the Dia effect (red bar, RU486+) in flies expressing both Rac1-CA and *dia*-RNAi (orange bar, RU486+) (Fig. 4*D*). Consistently, pharmacological inhibition of Dia also slowed down memory decay and masked the accelerated forgetting induced by Rac1-CA and SCAR overexpression (Fig. 4*E*). These data indicate that Dia functions downstream of Rac1/SCAR-mediated ASM forgetting in the MB neurons (Fig. 4*F*).

Rac1/SCAR/Dia-Dependent Forgetting Functions in the MB γ-Neurons. The MB neurons are divided into 3 major types: the γ-, α′/β′-, and α/β-neurons (*SI Appendix*, Fig. S5*A*), which have distinct roles in different phases and processes of olfactory memory (22). The MB-GS is a broad MB driver, which covers both the γ- and α/β-neurons (38) and is not suitable for differentiating different MB types. We hereby turned to the TARGET system (39) and used a temperature shift from 18 °C to 30 °C to inactivate the $Gal80^{ts}$, a temperature-sensitive Gal4 inhibitor, and switched on the expression of a dominant-negative mutant (Rac1-DN). Limited by Gal4 lines, the initial Rac1 study narrowed down Rac1 forgetting the α/β- and γ-neurons (3). With the help of additional MB Gal4 lines (*SI Appendix*, Fig. S5*B*), we recently found that, besides the 2 pan-MB Gal4 drivers, OK107 and R13F02 (40), a γ-neuron driver, 5-HT1B-GAL4 (41), also led to slower memory decay that lasted up to 24 h (Fig. 5*B* and *SI Appendix*, Fig. S5*C*). In the MB, 5-HT1B-GAL4 drives expression exclusively in the γ-neurons; whereas weak expression can still be found elsewhere in the ellipsoid body and some scattered neurons in the antennal lobe and the optical lobe (Fig. 5*A*). However, the integration of a MB-Gal80 transgene (42) suppressed the expression specifically in the MB (Fig. 5*A*) and also blocked the memory increment at 24 h (Fig. 5*B*). We note that 5-HT1B-GAL4 has a higher expression level in the γ-neurons, which may explain the discrepancy as to why similar phenotypes were not observed in the initial study with 3 other γ-neuron drivers, 1471, NP1131, and 201Y. Consistent with the previous data, we did not detect a phenotype even when a

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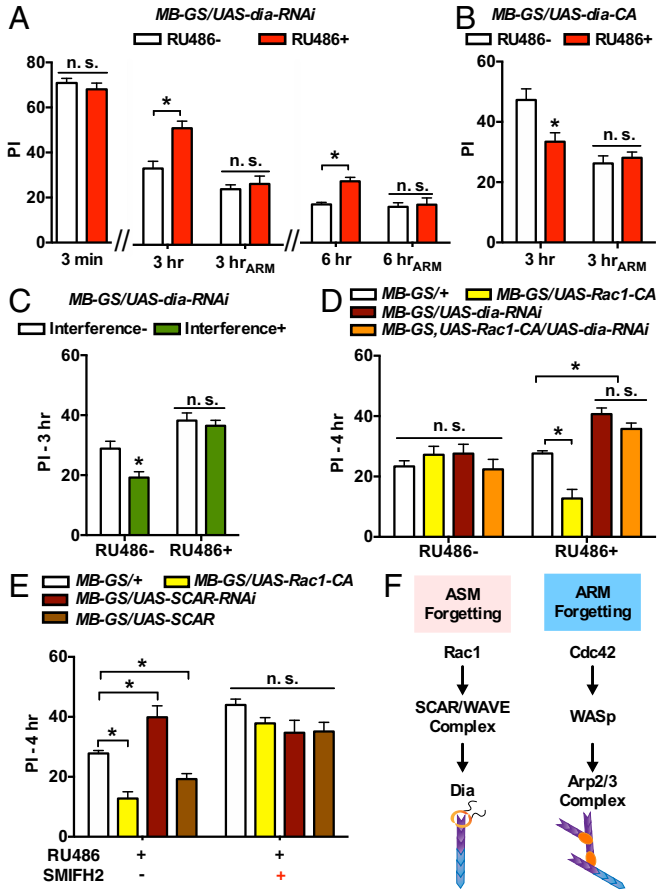

Fig. 4. Identification of Dia as a downstream effector of Rac1/SCAR in ASM forgetting. (*A*) Knockdown of Dia in the adult MB neurons using RNAi (RU486+) did not affect immediate memory (3 min), but slowed down memory decay at 3 and 6 h. $n = 8$. (*B*) Expression of Dia-CA in the adult MB neurons (RU486+) reduced 3-h memory without affecting ARM. $n = 8$. (*C*) Interference-based forgetting of 3-h memory was suppressed in *dia*-RNAi-expressing flies (RU486+). $n = 8$. (*D*) Knockdown of Dia dominated Rac1-induced forgetting in 4-h memory. Flies with coexpression of both Rac1-CA and *dia*-RNAi (orange bar, RU486+) showed memory performance higher than controls, and the performance was not different from flies expressing *dia*-RNAi alone. $n = 8$ to 12. (*E*) Pharmacological inhibition of Dia dominated Rac1- and SCAR-induced forgetting. Pharmacological inhibition of Dia had no additive effect compared with knockdown of SCAR and brought decreased memory performance in Rac1-CA-expressing and SCAR-overexpression flies to a similar level. $n = 8$ to 12. (*F*) Working model. Data are means ± SEM. $^*P < 0.05$. n.s., nonsignificant.

previously used weak γ-neuron driver, 201Y, was combined with a α/β-neuron driver, C739 (*SI Appendix*, Fig. S5*C*). Besides memory decay, the inhibition of Rac1 in the γ-neurons with the strong driver 5-HT1B-GAL4 also inhibited forgetting in reversal learning and trace conditioning (*SI Appendix*, Fig. S5*D*), 2 paradigms that have previously been used to test Rac1's roles in labile memory forgetting (3, 11).

RNAi knockdown of different members in the SCAR/WAVE complex in the γ-neurons with the 5-HT1B-Gal4 driver also suppressed forgetting (Fig. 5*C*). In addition, Dia knockdown in the γ-neurons (*Gal80^{ts}/+; 5-HT1B-Gal4/UAS-dia-RNAi*, 30 °C) inhibited forgetting, compared with parental controls (Fig. 5*D*). These data further support the idea that ASM forgetting localizes in γ-neurons. We also explored WASp knockdown with the γ-neuron driver, 5-HT1B-Gal4, and the α/β-neuron driver, C739. However, we did not observe a phenotype in ARM at 6 h after a 1-session training (*SI Appendix*, Fig. S6). The MB neuron types required for ARM forgetting are yet to be identified.

Discussion

There are 3 major findings. First, 2 WASP family proteins, SCAR/WAVE and WASp, act as downstream effectors of Rac1-mediated ASM forgetting and Cdc42-mediated ARM forgetting, respectively. Second, although the Arp2/3 complex is a well-established effector that links activation of WASP family proteins to actin polymerization, it is only required in Cdc42/WASp-mediated ARM forgetting. Instead, formin Dia functions together with Rac1/SCAR in ASM forgetting. Third, feeding inhibitors of the Arp2/3 complex and Dia to fruit flies led to rather specific effects on ASM and ARM forgetting, raising the possibility of developing drugs on these molecular targets to treat memory-related diseases.

The effect of Rac1 on ASM forgetting has been tied to the activation of an actin depolymerization regulator cofilin presumably through a PAK/LIMK phosphorylation cascade (3, 17). However, actin dynamics is a balanced play that requires continuous turnover between polymerization and depolymerization (43). It is not known whether signaling pathways regulating actin polymerization also play a role. There are 3 families of proteins that nucleate and promote actin polymerization, Arp2/3 complex, WH2-domain proteins, and formin (18, 20). Our finding that Arp2/3 complex and formin Dia function in ARM and ASM forgetting suggests that both actin polymerization and depolymerization pathways contribute to forgetting. How Arp2/3 complex and Dia separately contribute to ARM and ASM forgetting remains an open question. It is yet to be determined whether these proteins have different expression or subcellular locations in the MB neurons. However, it is interesting that Arp2/3 complex and formins are specialized in different types of actin polymerization (18, 20, 44).

In our working model, Cdc42 activates Arp2/3 complex via a canonical pathway (Cdc42/WASp/Arp2/3 complex), while Rac1-mediated ASM forgetting depends on SCAR/WAVE complex. This complex, in addition to SCAR/WAVE, includes at least 4 other members: Sra-1, Abi, HSPC300, and Kette (20). These additional members are thought to hold SCAR/WAVE in the complex in an inactive state, until GTP-bound Rac1 binds to Sra-1 and relieves the inhibition (20). On the other hand, the intact complex is essential for the stability of the SCAR/WAVE protein as well (i.e., failure to keep the intact complex can lead to SCAR degradation) (45). This latter effect may explain our observation that RNAi knockdown of SCAR complex members has the same effect on inhibiting forgetting as the knockdown of SCAR. As a WASP family protein, SCAR/WAVE is able to associate with and activate Arp2/3 complex through its C-terminal region (46). However, RNAi knockdown of Arp2 and Arp3 and pharmacological inhibition of Arp2/3 complex specifically affects ARM forgetting, while no effects on ASM retention were observed. We therefore propose that Rac1/SCAR may function through Arp2/3 complex-independent mechanisms (47). SCAR/WAVE complex is reported to physically associates with Dia through one of its members, Abi, to regulate actin dynamics (48, 49). Our behavioral characterization of Dia knockdown and overexpression, as well as the genetic epistasis experiment, support the idea that Dia could be downstream of Rac1/SCAR in ASM forgetting. Details about the functional coordination between SCAR/WAVE and Dia therefore await further clarification.

Materials and Methods

Fly Strains. Flies were reared at 25 °C and 60% relative humidity on a cornmeal medium under a 12/12 h light/dark cycle, except that flies in experiments using the TARGET system were reared at 18 °C. For details, see *SI Appendix, SI Materials and Methods*.

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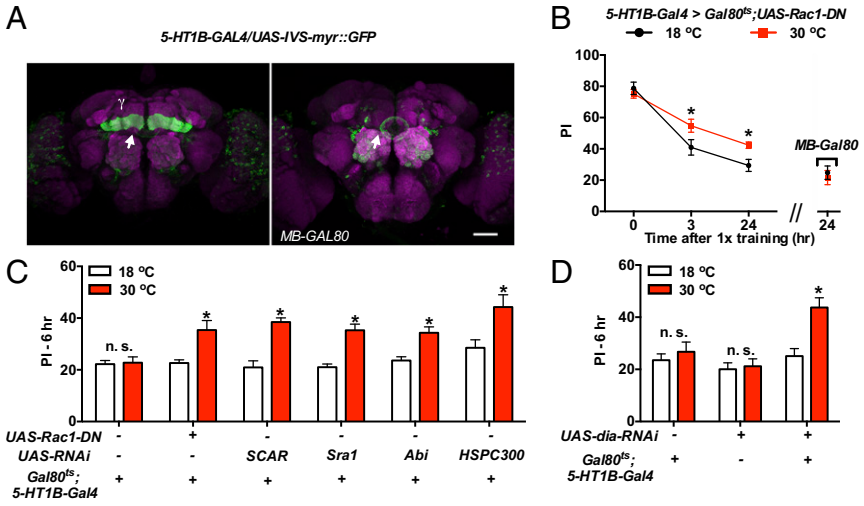

Fig. 5. Function of Rac1/SCAR/Dia forgetting pathway in the MB γ-neurons. (*A*) Expression patterns of 5-HT1B-GAL4. Besides the strong expression in the MB γ-neurons, the Gal4 also labels neurons outside the MB, including the ellipsoid body in the central complex (arrow). MB-Gal80 suppressed the expression of 5-HT1B-GAL4 in the MB neurons specifically. Green, mCD8-GFP reporter; magenta, nc82. (Scale bar, 50 μm.) (*B*) Memory retention curves. Conditional expression of Rac1-DN in the adult MB γ-neurons with the 5-HT1B-Gal4 driver was sufficient to slow down memory decay, and the effect was blocked by MB-Gal80 (24 h). $n = 6$ to 10. (*C*) Consistent with Rac1-DN, RNAi knockdown of SCAR/WAVE complex members in the MB γ-neurons led to higher memory retention at 6 h. $n = 8$ to 10. (*D*) Knockdown of Dia in the adult MB γ-neurons resulted in higher memory retention at 6 h. $n = 6$. Data are means ± SEM. $^*P < 0.05$. n.s., nonsignificant.

Behavioral Assays and Related Treatments. Aversive olfactory conditioning was performed as previously described (3, 4). For details, see *SI Appendix, SI Materials and Methods*.

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