Single-particle analysis reveals shutoff control of the *Arabidopsis* ammonium transporter AMT1;3 by clustering and internalization

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Ammonium is a preferred source of nitrogen for plants but is toxic at high levels. Plant ammonium transporters (AMTs) play an essential role in NH4+ uptake, but the mechanism by which AMTs are regulated remains unclear. To study how AMTs are regulated in the presence of ammonium, we used variable-angle total internal reflection fluorescence microscopy and fluorescence cross-correlation spectroscopy for single-particle fluorescence imaging of EGFP-tagged AMT1;3 on the plasma membrane of *Arabidopsis* root cells at various ammonium levels. We demonstrated that AMT1;3-EGFP dynamically appeared and disappeared on the plasma membrane as moving fluorescent spots in low oligomeric states under N-deprived and N-sufficient conditions. Under external high-ammonium stress, however, AMT1;3-EGFPs were found to amass into clusters, which were then internalized into the cytoplasm. A similar phenomenon also occurred in the glutamine synthetase mutant gln1;2 background. Single-particle analysis of AMT1;3-EGFPs in the clathrin heavy chain 2 mutant (ch2;2 mutant) and Flotillin1 artificial microRNA (Flot1 amiRNA) backgrounds, together with chemical inhibitor treatments, demonstrated that the endocytosis of AMT1;3 clusters induced by high-ammonium stress could occur mainly through clathrin-mediated endocytic pathways, but the contribution of microdomain-associated endocytic pathways cannot be excluded in the internalization. Our results revealed that the clustering and endocytosis of AMT1;3 provides an effective mechanism by which plant cells can avoid accumulation of toxic levels of ammonium by eliminating active AMT1;3 from the plasma membrane.

VA-TIRFM | FCS

Ammonium (NH4+) and nitrate (NO3−) are the primary sources of nitrogen (N) for most plants growing in agricultural soils. Ammonium assimilation requires less energy than nitrate assimilation, and, thus, ammonium is absorbed preferentially when plants are N-deficient. However, high concentrations of ammonium can be toxic (1); therefore, ammonium absorption and metabolism must be strictly controlled. Understanding the mechanisms by which plant cells regulate ammonium uptake and translocation is of critical importance for agricultural improvements in N-use efficiency and avoiding ammonium toxicity.

Evidence suggests that membrane ammonium transporters (AMTs) act in NH4+ uptake into plant cells, serving as the major transporters for high-affinity ammonium uptake (2). In *Arabidopsis thaliana*, the AMT family comprises six isoforms, of which three (AtAMT1;1, AtAMT1;2, and AtAMT1;3) are responsible for about 90% of the total high-affinity N uptake in roots (3). AMT gene expression in *Arabidopsis* roots is generally repressed by high N and induced by N deficiency (4). In addition to transcriptional mechanisms, regulation of membrane transporter activity is also involved in the plant’s responses to changing nutrient supplies (1). Although posttranscriptional regulation of AMT appears to be N-dependent (5), the question of how ammonium regulates AMT transporter activity, particularly the initial events that take place immediately after the addition of ammonium, remains to be characterized.

It is difficult to unambiguously determine the behavior and characteristic of individual molecules in living cells by means of bulk methods because these can only provide “average data” across many millions of molecules. However, single-molecule techniques have been recently used to reveal new information that would otherwise be lost in averages (6, 7). For example, variable-angle total internal reflection fluorescence microscopy (VA-TIRFM) can detect individual molecules of membrane-associated proteins with fast dynamics in intact plant cells (8). Fluorescence-correlation spectroscopy (FCS) allows direct measurements in living cells to estimate densities of live cell membrane proteins in their native environment without affecting protein function (9). Dual-color fluorescence cross-correlation spectroscopy (FCCS) is an extension of FCS that can quantitatively estimate molecule–molecule interactions in living cells (10). The combination of these single-molecule techniques greatly facilitates identification and characterization of single-membrane protein molecules.

In this investigation, we used these single-particle approaches to investigate the behavior of individual molecules of AMT1;3 in living roots of transgenic *Arabidopsis* expressing an AMT1;3-EGFP construct. We found that cells respond to high-ammonium stress by clustering these transporter proteins and subsequently internalizing the transporters, thereby likely reducing their ammonium-transport capacity. Clathrin-dependent and microdomain-associated pathways are involved in this internalization. Our single-particle analyses offer insights into the shutoff regulation of AMT1;3 transporter to protect against toxic ammonium accumulation under excess-ammonium conditions and may also serve as a model of how membrane transporters control substrate transport.

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Results and Discussion

In plant roots, transport of ammonium across membranes is mainly mediated by the AMTs, and AMT expression and activity are regulated in response to the changing ammonium supply (5, 11). Evidence suggests that the behavior of individual molecules is of particular importance because it can be correlated to the environmental conditions (12). It has been reported that AMT1;3 is widely distributed in the epidermal cells of Arabidopsis roots and makes an important contribution to the absolute ammonium uptake capacity (2, 3). By contrast, AMT1;1 is mostly localized in the pericycle cells, and AMT1;2 is found in endodermis and cortex cells. Because AMT1;3 is expressed in the epidermal cells of Arabidopsis roots (2, 3, 13), we, therefore, selected AMT1;3 as a representative protein to track the dynamic behavior of ammonium transporters under different external ammonium conditions. We first confirmed that AMT1;3-EGFP is functional by a complementation assay in yeast (Fig. S1) and also showed that it localized at the plasma membrane in root epidermal cells (Fig. S2 A–L).

At low or null ammonium concentrations, the fluorescence of AMT1;3-EGFP spots exhibited heterogeneous dynamic modes with different surface residence times (Fig. 1A). One typical dynamic mode is the spots appeared and then immediately disappeared from the cell cortex (Fig. 1B, M1 mode). In another common dynamic mode, the spots had a long membrane residence time before they completely disappeared (Fig. 1B, M2 mode). Defining a lifetime of <1 s as short-lived and anything above this threshold as long-lived, our statistical analysis indicated that, at low ammonium concentration (including 2 mM NH₄NO₃, regarded as N-sufficient conditions), 44.5% of AMT1;3-EGFP spots were short-lived and 55.5% of the spots were long-lived (Fig. 1C and Movie S1). However, in N-deprived conditions, the long-lived spots increased significantly to 78% (Fig. 1D and Movie S2), suggesting that the distribution of surface-residence time of AMT1;3 spots depended on ammonium conditions.

Crystal structure studies have shown that AMT transporters exist as trimers (14); however, because the structure of detergent-isolated proteins may not always reflect the physiologically relevant state (15), it is necessary to directly analyze the oligomeric state of AMTs in living cells. Recent advances in single-molecule fluorescence imaging of living cells has provided a new way to analyze protein structures under physiological conditions (7, 16). Here, we report the native oligomeric status of AMT1;3 based on single-particle fluorescence imaging. We found that, under low or null ammonium concentrations, AMT1;3-EGFP exhibited a broad asymmetric distribution of intensities, with the majority of spots ranging from 500 to 1,000 counts (Fig. 2A), which were approximately one- or two-fold that of purified of EGFP monomers (peak intensity at 545; Fig. S3A), suggesting that these spots included one or two AMT1;3-EGFP molecules. To circumvent the signal fluctuation attributable to the dynamics of AMT1;3-EGFP on living cell surfaces, we then confirmed the subunit number of AMT1;3 by counting bleaching steps of GFP tags fused to the proteins in fixed cells (Fig. 2B and Fig. S3B), through a single-molecule photobleaching approach (16). As shown in Fig. 2 C–E, the number of elemental EGFP photobleaching steps in an individual spot was not uniform, ranging from one to three. Because the maximum number was three, the most conservative explanation is that AMT1;3 exists on the membrane as a trimer, in agreement with previous reports (14). The one- or two-step photobleaching may result from oligomers that incorporate endogenous unlabeled AMTs or some AMT1;3-EGFP with an immature EGFP tag. However, another possibility that cannot be excluded is that besides existing as a trimer on the plasma membrane, AMT1;3 may also exist as a mixture of various multimeric forms including monomers, dimers, and trimers.

Ammonium provides an essential plant nutrient but is toxic when present in excess. When plants are exposed to high-ammonium conditions, roots undergo a rapid decrease in N-uptake capacity to avoid cellular ammonium toxicity (3). Previous studies reported that the spatial organization of transmembrane proteins is a critical step in signal transduction and protein trafficking, and different multimeric states of the protein complex can result in distinct cellular responses (17). To test whether high-ammonium stress changes AMT1;3 protein organization, we compared the state of individual AMT1;3-EGFP spots under high-ammonium stress (30 mM NH₄⁺) and under N-sufficient conditions (2 mM NH₄⁺). Our results showed that, under N-sufficient conditions, most of the AMT1;3 transporters existed in low oligomeric states with the average size 2.32 ± 0.23 ± 0.25 pixels and fluorescence intensity of 600.5 ± 75 counts per pixel. However, 30 min after transfer to high-ammonium medium, the average size and fluorescence intensity of AMT1;3-EGFP spots

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**Fig. 1. Dynamic analysis of AMT1;3-EGFP spots in the plasma membrane under N-sufficient conditions, based on VA-TIRFM observation of a total of 200 spots from five representative living Arabidopsis roots.** To calculate the surface residence time, we measured the changes of fluorescence intensity of AMT1;3 spots over an interval of fixed duration (12 s), with the interval starting first at 0 ms after the beginning of the recording, then 200 ms, 400 ms, and so on until 12 s. (A) Typical image showing diffraction-limited fluorescent spots of AMT1;3-EGFP in living expanding Arabidopsis root epidermal cells, imaged with VA-TIRFM. (Scale bar: 1 μm.) (B) The fluorescence intensity over time of AMT1;3-EGFP spots in two typical dynamic modes. M1, spots that appeared and then immediately disappeared. On first appearance, these spots had low but detectable fluorescence that increased steadily. After reaching a peak fluorescence intensity, the fluorescence rapidly decreased to background levels and the spots disappeared from the cell cortex. M2, spots that disappeared after long-lasting residence in the plasma membrane. (C) Surface residence time of 200 AMT1;3-EGFP spots in the plasma membrane in N-sufficient seedlings. A surface residence time of <1 s was defined as short-lived, and anything above this threshold was defined as long-lived (n = 200). The data came from three independent replicates. (D) Surface residence time of 200 AMT1;3-EGFP spots in the plasma membrane in N-deprived seedlings (n = 200). The data came from three independent replicates.
and dynamics of AMT1;3 proteins are specific to the ammonium ion rather than a result of its action as a weak base. In addition, treatments with 2 and 30 mM NH₄Cl, KNO₃, KCl further confirmed that the change of AMT1;3-EGFP behavior did not result from the effects of NO₃⁻ or osmotic potential (Fig. S5 A–C). Moreover, we examined the effect of ammonium availability on localization of GFP-PIF2;1 (a plasma membrane aquaporin transporter) and clathrin light chain (CLC)-GFP by confocal microscopy. Although the distribution of GFP-PIF2;1

significantly increased to 5.38 ± 1.8 pixels and 3,046 ± 502 counts per pixel (P < 0.01), respectively, suggesting that AMT1;3 molecules amassed into protein clusters (Fig. 3 A and B). Additionally, representative time courses of EGFP emission of spots under high ammonium showed that most of the bleaching steps exhibited an exponential decay without discrete steps (Fig. 3C), suggesting that each fluorescent spot was a cluster of several AMT1;3-EGFP molecules. Because trimers are believed to be the functional units (14) and abnormal protein complex organization can result in dysfunction (18), these results suggest that the clustering of AMT1;3 induced by high ammonium may play an important role in regulating transporter activity.

It has been reported that clustering can promote subsequent internalization of membrane proteins (19). Thus, we further investigated the fate of the AMT1;3 clusters. Compared with N-sufficient conditions (Fig. 3D), whereas the size and fluorescence intensity of spots increased under high-ammonium conditions, the overall number of spots and their residence time dramatically decreased (Fig. 3E). During 30 min of high-ammonium supply, only 31.6 ± 1.6% of AMT1;3-EGFP surface fluorescence remained (Fig. 3F). This observation, together with the disappearance of individual AMT1;3 spots from the plasma membrane (Movie S3), suggests that at high-ammonium stress, AMT1;3 protein clusters are rapidly internalized. To investigate whether ammonium exerts a specific effect on AMT1;3 localization, we analyzed the cytoplasmic pH of root cells using the Oregon Green 488 fluorescent probe (20) and found that there was no significant difference in cytoplasmic pH in root cells between N-sufficient and high-ammonium conditions (Fig. S4 A–C; P > 0.05), indicating that the effects of ammonium on the localization

Fig. 2. Analysis of oligomeric states of AMT1;3-EGFP in Arabidopsis root cells. (A) Distribution of fluorescence intensities of the diffraction-limited single AMT1;3-EGFP spots (n = 200) in five live cells from five representative Arabidopsis roots. (B) Typical image showing diffraction-limited fluorescent spots of AMT1;3-EGFP on fixed cell membrane of Arabidopsis root cells, imaged with VA-TIRFM. The image is a section of the first frame of a stack of images with the background subtracted. (Scale bar: 1 μm.) (C-E) Representative time courses of EGFP emission of AMT1;3-EGFP spots in fixed Arabidopsis root cells after background correction: one-step bleaching (C), two-step bleaching (D), and three-step bleaching (E). Movies of 150 frames were captured with a 200-ms frame interval.

Fig. 3. Dynamic analysis of AMT1;3-EGFP internalization induced by high-ammonium stress in Arabidopsis root cells. (A and B) Average size (A) and fluorescence intensity (B) of AMT1;3-EGFP fluorescent spots in wild type under N-sufficient conditions and high-ammonium treatment, and in gln1;2 mutant background under N-sufficient conditions (n = 200), showing that external high-ammonium stress and internal ammonium accumulation induced the massing of AMT1;3-EGFP oligomers into clusters. The data came from three separate replicates. Values given are means ± SD. (C) Representative time course of EGFP emission of AMT1;3-EGFP spots under high-ammonium stress in fixed Arabidopsis root cells after background correction, showing the approximately exponential distribution of the bleaching steps. (D–G) The raw frames of a representative area of Arabidopsis root cells expressing AMT1;3-EGFP, imaged live using VA-TIRFM at the indicated times. (D) In wild-type background under N-sufficient condition. (E) In wild-type background under high-ammonium stress. (F) In gln1;2 mutant background under N-sufficient conditions. (G) In gln1;2 mutant background under high-ammonium stress. (Scale bar: 1 μm.) (H) Corresponding average surface fluorescence measurements in D–G. In wild type, no distinct change of surface fluorescence was found over a 30-min period under N-sufficient conditions. After high-ammonium treatment, only 31.6 ± 1.6% of AMT1;3-EGFP remained on the membrane surface. In the gln1;2 mutant, 46 ± 2.7% AMT1;3-EGFP remained under N-sufficient conditions, but only 25.1 ± 1.2% of AMT1;3-EGFP remained on the membrane surface after high-ammonium treatment. Approximately five to eight cells in at least five different seedlings were measured. The analysis was based on three independent repetitions. Values given are means ± SD. (I) Analysis of AMT1;3-EGFP endocytic rate (n = 200). The fast-spot internalization required only about 0.86 s, whereas slow-spot internalization required about 6.8 s. The analysis was based on three separate replicates. Values given are means ± SD.
uptake rate of roots after treatments

\[ I \mu \] is one of the genes encoding N-suf, J and A, and Arabidopsis H. Under high-ammonium is knocked out, the internal ammonium level is increased (22). Using SIET and \[^3^N\] analysis, we confirmed that NH\(_4^+\) uptake in glh1;2 mutants was indeed lower than in the wild type under N-limiting, N-sufficient, or high external-ammonium treatment, demonstrating that an abnormality of ammonium assimilation can affect the external NH\(_4^+\) uptake (Fig. S10 C and D). Furthermore, we analyzed the dynamic behavior of AMT1;3-EGFP spots in the glh1;2 mutant background using VA-TIRFM. We found that, under N-sufficient conditions, the individual spots amassed into clusters with bigger size and higher fluorescence intensity in the mutant, compared with spots in wild type (\( P < 0.05 \); Fig. 3 A and B). However, there was a significant reduction in the overall fluorescence intensity of the proteins on the plasma membrane (Fig. 3 F and H), suggesting the internal ammonium accumulation can promote internalization of AMT1;3-EGFP. When glh1;2 mutants were treated with high ammonium for 30 min, heavier clustering of the individual spots occurred (Fig. 3 A and B) compared with those in wild type, and only 25.1 ± 1.2% of overall fluorescence remained on the plasma membrane (Fig. 3 G and H). Western blot analysis further confirmed that, in glh1;2 mutants, the AMT1;3-EGFP protein underwent some degrees of degradation under high-ammonium stress (Fig. S9). Collectively, our data suggest that the ammonium-dependent regulation of AMT1;3 clustering and internalization provides a fast and efficient way of controlling AMT1;3 activity to avoid cellular ammonium toxicity.

Although the number of transporter molecules in the plasma membrane, which is closely linked to transport capacity, can be regulates by endocytosis and recycling (23), the molecular mechanisms that control the endocytic trafficking of AMTs are not defined. Previous studies indicate that internalization of molecules can occur not only through the classical clathrin-mediated pathway but also through clathrin-independent routes (24). In our study, the internalization time of spots differed significantly, with fast-spot internalization requiring only about 0.66 s, and slow-spot internalization requiring about 6.8 s (Fig. 3f), with a 10-fold increment in protein endocytic rate. This large discrepancy in the time of AMT1;3 internalization suggested that AMT1;3 internalization may occur through more than one endocytic pathways.

To test this hypothesis, we examined AMT1;3 internalization in the chc2 mutants, which are defective in bulk endocytosis, as well as in internalization of prominent plasma membrane proteins (25). In chc2, we found that the internalization of AMT1;3-EGFP spots in N-sufficient conditions was inhibited and the spots amassed into clusters with larger spot size and higher fluorescence intensity (Fig. 4, A, I, and J and Movie S4), compared with that in wild type (\( P < 0.05 \)). Under high-ammonium treatment, the internalization of AMT1;3-EGFP was nearly halted, and the spot size and fluorescence intensity were significantly increased compared with that in wild type (\( P < 0.01 \); Fig. 4 B, I, and J). Western blot analysis confirmed that AMT1;3-EGFP was relatively stable under high ammonium in the chc2 mutant (Fig. S9). Furthermore, our phenotyping experiment indicated that chc2 mutant Arabidopsis seedlings were more susceptible to ammonium toxicity (Fig. S11 A and B). In addition, we found that treatment with tyrophostin (tyr) A23, a specific inhibitor of clathrin-dependent endocytosis (26), can reduce the internalization of AMT1;3 spots and resulted in AMT1;3-EGFP coalescing into larger particles with an increased spot size and fluorescence intensity both under N-sufficient conditions (Fig. 4 C, I, and J and Movie S4) and high-ammonium treatment (Fig. 4 D, I, and J and Movie S5), similar to the results in the chc2
A

B

C

Fig. 5. FCS measurement of AMT1;3-EGFP density in plasma membrane. (A) Typical image showing our selection of the FCS measurement region. (Scale bar: 10 μm.) (B) Representative graph of fluorescence intensity fluctuation (or counts) of AMT1;3-EGFP during the detection time under N-sufficient conditions. (C) Distribution of AMT1;3 transporter density in Arabidopsis root cells under different conditions. In N-deprived seedlings, the density of AMT1;3 transporters was about 38 molecules per square micron. Under N-sufficient conditions and high-ammonium stress, the density decreased to about 31 molecules and about 13 molecules per square micron. In the chc2 mutant background or by tyrA23 treatment under high-ammonium stress, the density increased to about 25 molecules or 26 molecules per square micron. In the Flo1 amiRNA15-5 line or by mCD treatment under high-ammonium stress, the density increased to 18 molecules or 19 molecules per square micron. The data came from three separate replicates. Am, ammonium.

Fig. 6. AMT1;3-EGFP and mCherry-CLC/mCherry-Flot1 spots colocalize at the plasma membrane. (A and B) Expanding root epidermal cells expressing AMT1;3-EGFP (A) and mCherry-CLC (B) imaged with VA-TIRFM. (C) Merged image of A and B; the yellow dots represent colocalization spots, indicating that impairment of the AMT1;3-EGFP spots colocalize with mCherry-CLC particles. (D) An example of real-time dynamic observation of AMT1;3-EGFP and mCherry-CLC spot. The time series shows AMT1;3-EGFP (Top), mCherry-CLC (Middle), and a merged image (Bottom). (E) Three-dimensional luminescence plots of the corresponding spots in D. (F and G) Expanding root epidermal cells expressing AMT1;3-EGFP (F) and mCherry-Flot1 (G) imaged with VA-TIRFM. (H) Merged image of A and B; the yellow dots represent colocalization spots, indicating that only a small fraction of AMT1;3-EGFP spots colocalize with mCherry-Flot1 particles. (I) An example of real-time dynamic observation of AMT1;3-EGFP and mCherry-Flot1 spot. The time series shows AMT1;3-EGFP (Top), mCherry-Flot1 (Middle), and a merged image (Bottom). (J) Three-dimensional luminescence plots of the corresponding spots in D. (K) A typical 3D plot of AMT1;3-EGFP and mCherry-CLC cross-correlation vs. pixel shift. (L) Mean PPI values showing high (PPi, 0.61 ± 0.03) and low (PPi, 0.32 ± 0.18) colocalization degree of AMT1;3-EGFP and mCherry-CLC/Flot1. Approximately 10–15 cells in at least five different seedlings were measured. (Scale bars: A–C and F–H, 1 μm; D and I, 0.25 μm.)

Besides clathrin-dependent endocytosis, clathrin-independent entry pathways have been reported in plant cells, including the membrane microdomain-associated endocytic pathway (29). Previous proteomics studies revealed a tendency of AMT1 transporters to partition in membrane microdomains (30). However, whether they could be internalized through the membrane microdomain-associated endocytic pathway remained to be determined. In the present experiment, we used a Flo1 (a membrane microdomain marker) amiRNA15-5 line (31) to analyze whether membrane microdomains were involved in AMT1;3-EGFP internalization. We found that, in the Flo1 amiRNA15-5 line, the internalization of AMT1;3-EGFP spots was reduced, but the spot size and fluorescence intensity remained almost the same as controls under N-sufficient condition (Fig. 4 G, I, and J) and high-ammonium treatment (Fig. 4 H, I, and J). All of these results suggested that the membrane microdomain-associated endocytic pathway may be also involved in AMT1;3 internalization.

To further confirm the role of clathrin or membrane microdomains in regulating AMT1;3 endocytic trafficking, we provide three lines of evidence to demonstrate that clathrin and membrane microdomains contributed differently to the internalization of AMT1;3. First, FCS measurements showed that, when the clathrin-dependent endocytic pathways were disrupted in chc2 mutants or by tyrA23 treatment, the density of AMT1;3 in the plasma membrane was 92% or 100% higher than with just high-ammonium treatment. However, when the microdomain-associated endocytic pathway was impaired in Flo1 amiRNA15-5 line or by mCD treatment, the membrane density of AMT1;3 was only 46% or 50% higher than that in high ammonium without inhibitor (Fig. 5 A–C), indicating that impairment of the

mutant. Indeed, a similar phenomenon was reported previously, showing that PIN protein clustering was linked to reduced dynamics of PIN proteins in the plasma membrane (27). However, the inactive analog of tyrA23, tyrA51 (28), had no effects on the behavior and fluorescence intensity of AMT1;3 (Fig. S12), indicating the effect of tyrA23 is specific. These results suggested that AMT1;3 may internalize through a clathrin-dependent endocytic pathway.

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clathrin-dependent endocytic pathway played a dominant role in AMT1;3 internalization, but the involvement of membrane microdomain-associated endocytic pathway in AMT1;3 endocytosis cannot be excluded.

Materials and Methods

Plant materials, plant culture, drug treatments, image analysis, and FCS and FCCS measurement and analysis are described in SI Materials and Methods.

Taking these results together, we concluded that the clathrin-dependent endocytic pathway plays a dominant role in AMT1;3 internalization, but the involvement of membrane microdomain-associated endocytic pathway in AMT1;3 endocytosis cannot be excluded.

Materials and Methods

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Single-particle fluorescence imaging was performed on an inverted microscope (IX71; Olympus) equipped with a total internal reflective-fluorescence illuminator and a 100x oil-immersion objective ( Olympus; NA 1.45) as described (12). The gain of our EM CCD camera was set at 300 throughout all single-particle imaging experiments, and the setting was in the linear dynamic range of the EM CCD camera. Movies of 100–300 frames were captured in a single 200-ms frame.

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amined the colocalization of AMT1;3-EGFP/mCherry-Flot1 (Fig. S14 A–C), indicating the interaction between AMT1;3-EGFP and mCherry-Flot1 was stronger than that between AMT1;3-EGFP and mCherry-Flot1. This conclusion is further supported by the FCCS analysis, which yielded detailed information about the transient and dynamic interaction of AMT1;3 with clathrin or membrane microdomains. We found that the relative cross-correlation amplitude was 0.57 ± 0.03 for AMT1;3-EGFP/mCherry-CLC, but the value was 0.36 ± 0.02 for AMT1;3-EGFP/mCherry-Flot1 (Fig. S14 A–C), indicating the interaction between AMT1;3-EGFP and mCherry-Clc was also shown by immunofluorescence (Fig. S13 A–J).
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