Uncovering the Protein Translocon at the Chloroplast Inner Envelope Membrane

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Chloroplasts require protein translocons at the outer and inner envelope membranes, termed TOC and TIC, respectively, to import thousands of cytoplasmically synthesized preproteins. However, the molecular identity of the TIC translocon remains controversial. Tic20 forms a 1-megadalton complex at the inner membrane and directly interacts with translocating preproteins. We purified the 1-megadalton complex from *Arabidopsis*, comprising Tic20 and three other essential components, one of which is encoded by the enigmatic open reading frame *ycf1* in the chloroplast genome. All four components, together with well-known TOC components, were found stoichiometrically associated with different translocating preproteins. When reconstituted into planar lipid bilayers, the purified complex formed a preprotein-sensitive channel. Thus, this complex constitutes a general TIC translocon.

Translocation of nuclear-encoded preproteins across the double envelope membranes of chloroplasts is mediated by two protein translocons, the TOC and TIC complexes (*1*–*5*). Tic20, an integral inner membrane protein with four predicted transmembrane helices, has been proposed to form part of a general protein-conducting channel (*6*, *7*). Recombinant Tic20 has further been reported to have the capacity to form a channel (*8*). A 1-megadalton (MD) translocation intermediate complex has been recently identified at the inner membrane, in which Tic20 is in close contact with a translocating preprotein (*9*). Whereas Tic20 forms a stable 1-MD complex in the inner membrane, Tic21 interacts only weakly with the complex, and other known Tic proteins such as Tic110 and Tic40 are not involved in the complex (*9*). Thus, detailed organization of this 1-MD complex has remained elusive.

Here, we generated transgenic *Arabidopsis* plants expressing a protein A–tagged Tic20-I (the major Tic20 isoform in *Arabidopsis*), and the tagged Tic20-I–containing complexes were purified (fig. S1). Three proteins with molecular masses of 214, 100, and 56 kD were specifically copurified with Tic20-I, which were confirmed to form a stable 1-MD complex together with Tic20-I (Fig. 1A). Subsequent mass spectrometric analysis revealed three previously uncharacterized *Arabidopsis* proteins (fig. S2A).

Surprisingly, the 214-kD protein, AtCg01130, is encoded by the previously enigmatic chloroplast gene *ycf1* (hypothetical chloroplast open reading frame 1) (*10*–*12*). The deduced protein, here renamed Tic214, is 1786 amino acids in length with a calculated relative molecular mass M_r of 213,742 and is predicted to contain six N-terminal transmembrane domains (figs. S2B and S3). The 100-kD protein, At5g22640, is a nuclear-encoded protein of 871 amino acids with a calculated M_r of 99,954 (fig. S4). This protein likely has no cleavable transit peptide (fig. S2A) and thus was named Tic100. The 56-kD protein, At5g01590, is also a nuclear-encoded protein of 527 amino acids with a calculated M_r of 61,625 (fig. S5). This protein is most likely synthesized as a preprotein and processed to form a mature protein upon import (fig. S6A) and thus was designated Tic56. Tic100 and Tic56 are highly conserved among most land plants, but show no remarkable overall sequence similarity to any proteins of known function and have no predicted transmembrane segment.

In wild-type chloroplasts, Tic214, Tic100, Tic56, and Tic20-I appeared to form stable 1-MD TIC complexes (Fig. 1, B and C, and fig. S6, B and C), which were shown to associate with preproteins (fig. S7). If we assume that the stoichiometry of the components in the 1-MD complex is 1:1:1:1 (fig. S8), we would expect that the complex represents a trimeric assembly of Tic214, Tic100, Tic56, and Tic20-I (1170 kD). The stoichiometry of Tic20-I:Toc75 (the channel protein of the TOC complex) in the envelope membranes was roughly estimated to be 1:2.5 (fig. S8). Tic20-I and Tic214 likely constitute the membrane-integral part of the 1-MD TIC complex; Tic20-I is entirely buried in the core of the complex, whereas Tic214

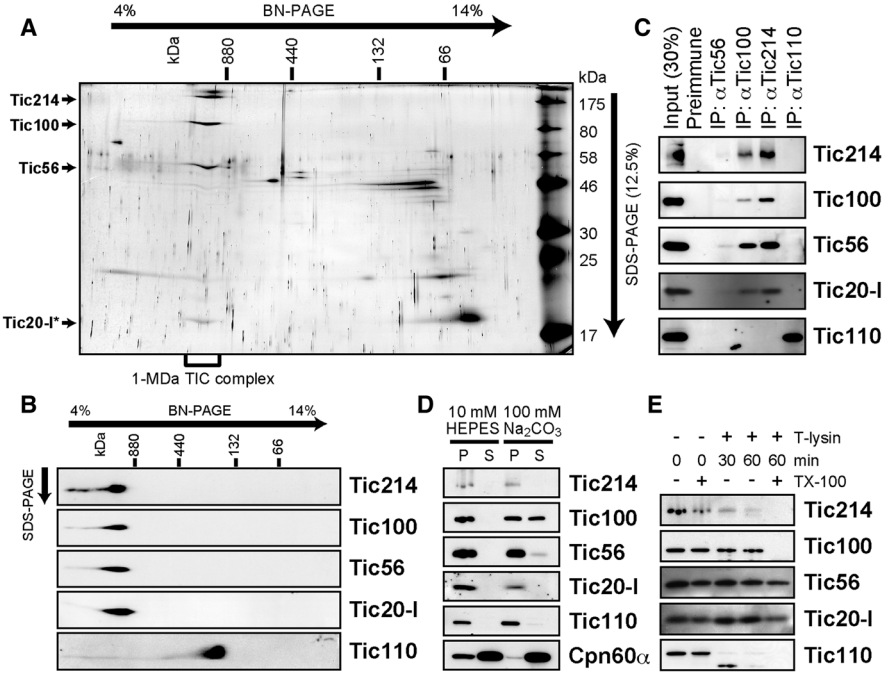

Fig. 1. The 1-MD complex is composed of Tic20-I and three other Tic proteins. (**A**) Tic214, Tic100, and Tic56 were copurified with protein A–tagged Tic20-I (PA2-Tic20-I). The purified fraction was separated by two-dimensional blue native (2D-BN) SDS-PAGE followed by silver staining. Tic20-I*, tobacco etch virus (TEV) protease–cleaved PA2-Tic20-I. (**B**) Wild-type *Arabidopsis* chloroplasts were solubilized with 1% digitonin in the presence of 1 M NaCl and then separated by 2D-BN SDS-PAGE followed by immunoblotting. (**C**) Co-immunoprecipitation of the 1-MD TIC complex components. (**D** and **E**) The localization and topology of the different TIC complex components were analyzed by alkaline (D) and protease (E) treatment. (D) Chloroplasts were treated with either 10 mM HEPES-KOH, pH 7.5 (hypotonic lysis), or 100 mM Na_2CO_3 on ice for 30 min, followed by centrifugation to obtain supernatant (S) and membrane pellet (P) fractions. (E) Inverted inner envelope membrane vesicles (*26*) were treated with or without thermolysin (T-lysin; 100 μg/ml) and with or without Triton X-100 [TX-100; 0.1% (w/v)] on ice for the indicated time periods.

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is exposed to both sides of the inner membrane (Fig. 1, D and E, and fig. S6D). Tic100 is associated peripherally in the complex at the intermembrane space side, whereas Tic56 is mostly embedded in the complex.

In a search for evidence of direct involvement of the 1-MD TIC complex as a general protein translocation machinery at the inner membrane, we used purified model preproteins in import experiments in vitro to isolate translocation intermediate–associated proteins from *Arabidopsis* chloroplasts (Fig. 2 and fig. S9) (*13*–*15*). Almost all major isolated proteins could be assigned to either the well-known TOC components (including Toc159, Toc75, and Toc33) or the 1-MD TIC complex components (including Tic214, Tic100, Tic56, and Tic20-I) (Fig. 2). Association of these TOC and TIC components with preproteins absolutely required adenosine triphosphate (ATP) in the import reactions that could drive preprotein translocation across the envelope membranes (fig. S9B). Tic214, Tic100, Tic56, and Tic20-I were purified at levels comparable to those of TOC components and preproteins (Fig. 2 and fig. S9B). Two different model preproteins, pSTEV and pFdTEV, resulted in essentially similar profiles of associated proteins (Fig. 2), which supports the direct involvement of the 1-MD TIC complex in protein translocation as a general import machinery in concert with the well-established TOC complex.

By contrast, we observed hardly any specific association of Tic110 or Tic40 with the translocation intermediate complexes (Fig. 2 and fig. S9B). This is surprising because Tic110, an abundant inner membrane protein, was initially identified by a similar method (*15*) and has long been considered to be a central player (*5*), together with Tic40, in preprotein translocation across the inner membrane (*16*). The migration of Tic110 upon SDS–polyacrylamide gel electrophoresis (PAGE) was almost the same as that of Tic100. However,

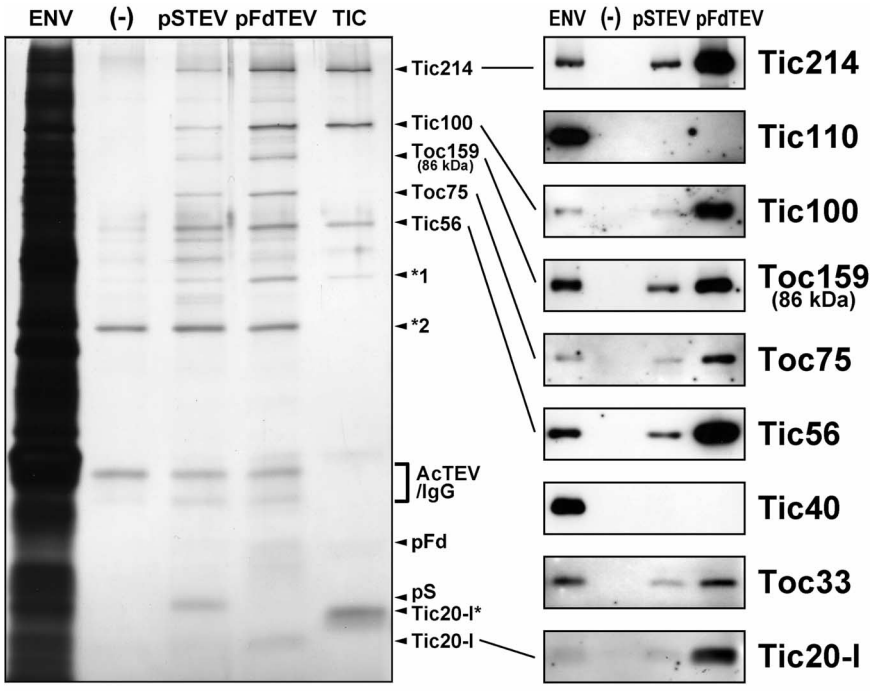

Fig. 2. Association of the 1-MD TIC complex components with two distinct model preproteins. *Arabidopsis* chloroplasts were incubated with 100 nM urea-denatured protein A–tagged preproteins pSTEV or pFdTEV in the presence of 0.5 mM ATP for 10 min at 25°C. As a control, incubation without preprotein (–) was also performed. Reisolated chloroplasts were directly solubilized with 1% digitonin, and insoluble material was removed by ultracentrifugation. The resulting supernatant was incubated with immunoglobulin G–sepharose for 2 hours at 4°C. After the beads were washed thoroughly, bound protein complexes were eluted under nondenaturing conditions by TEV cleavage. The denatured samples were separated by 12.5% SDS-PAGE followed by silver staining (left) or immunoblotting (right). ENV, *Arabidopsis* mixed envelope fraction; TIC, the 1-MD TIC complex purified from PA2-Tic20-I plants; *1, unidentified protein currently under investigation; *2, unknown contaminating protein; AcTEV, TEV protease; pFd and pS, TEV-cleaved preproteins; Tic20-I*, TEV-cleaved PA2-Tic20-I.

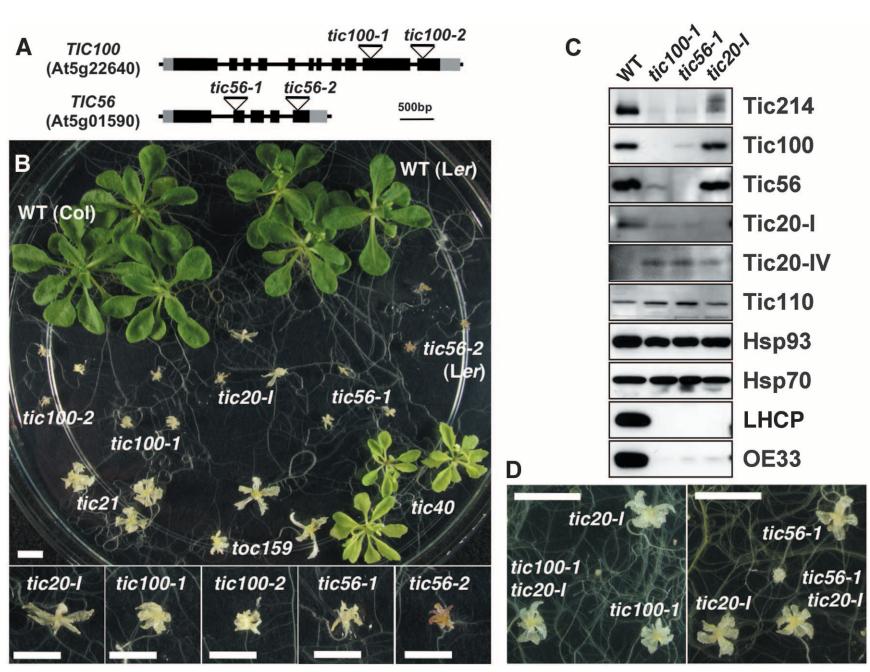

Fig. 3. Characterization of *tic100* and *tic56 Arabidopsis* knockout mutants. (**A**) Schematic representation of the genes encoding Tic100 and Tic56. Triangles, T-DNA insertions; solid boxes, exons; thin lines, introns; shaded boxes, untranslated regions. (**B**) Visible phenotypes of various *tic* and *toc* mutants (*16*, *19*–*22*, *27*) grown on sucrose-containing media for 3 weeks. Scale bars, 5 mm. (**C**) Total proteins were extracted from 3-week-old wild-type *Arabidopsis* and from homozygous albino *tic100-1*, *tic56-1*, and *tic20-I* mutants; 24 μg of total protein was subjected to SDS-PAGE and immunoblotting. Hsp93 and Hsp70, stromal molecular chaperones; LHCP, light-harvesting chlorophyll-binding protein; OE33, 33-kD protein of the oxygen-evolving complex of photosystem II. (**D**) Double and single knockouts were identified in the progeny derived from a *+/tic20-I +/tic100-1* heterozygous plant (left) and from a *+/tic20-I +/tic56-1* heterozygous plant (right). Scale bars, 1 cm.

they are completely different proteins, and Tic110 exists as a very distinct 200- to 300-kD entity in the inner membrane (Fig. 1B) (*9*) most probably without any stably associated proteins (fig. S10). Physical interaction between Tic110 and any component of the 1-MD TIC complex was hardly observed even after chemical cross-linking (Fig. 1C and fig. S11). Thus, although Tic110 might act as a scaffold for stromal molecular chaperones at a later stage during import (*17*, *18*), direct participation of these Tic proteins in preprotein translocation is unclear.

The null mutant of *Arabidopsis TIC20-I* displays developmental defects during embryogenesis and results in an albino, seedling-lethal phenotype due to a strong defect in protein import into chloroplasts (*19*–*21*). Null mutants of *TIC100* (*22*) and *TIC56* showed very similar phenotypes (Fig. 3, A and B, and figs. S12 and S13). Absence of either Tic100 or Tic56 led to marked reductions of the remaining 1-MD TIC complex components, indicating that they are important for assembly of the complex (Fig. 3C and fig. S12E). Furthermore, chloroplasts isolated from a viable *tic56-3* pale green mutant had a notable protein import defect (figs. S14 and S15).

Like the *tic20-I* mutant (*20*), no photosynthetic proteins accumulated in the albino seedlings of the *tic100-1* and *tic56-1* mutants, although some housekeeping proteins did accumulate (Fig. 3C). This residual import ability in the *tic20-I* mutant can be attributed to partial compensation by the elevated expression of the minor, partially redundant homolog Tic20-IV (Fig. 3C) (*20*), because a *tic20-I tic20-IV* double-knockout mutant exhibits more severe embryo lethality (*20*, *21*). The *tic20-I tic56-1* and *tic20-I tic100-1* double-knockout mutants showed phenotypes similar to those of the single-knockout mutants, *tic20-I, tic56-1, or tic100-1*, indicating that neither Tic56 nor Tic100 contributes to the compensation provided by Tic20-IV (Fig. 3D and fig. S12). By contrast, neither a *tic20-IV tic56-1* nor a *tic20-IV tic100-1* double-knockout mutant could be identified, which suggests that such double-knockout mutants are also embryo-lethal. Hence, there seems to be a residual import pathway for some housekeeping proteins in which Tic20-IV but neither Tic20-I, Tic56, nor Tic100 is involved (Fig. 3C), and impairment of both pathways causes embryo lethality (*20*).

The chloroplast gene initially called *ycf1* was reported to be an essential gene in tobacco (*11*) and in *Chlamydomonas* (*12*). Thus, one can anticipate that Tic214 is an essential *Arabidopsis* protein. Overall, like Tic20-I, both Tic100 and Tic56 (and most likely Tic214) are indispensable components of the 1-MD TIC complex required for photosynthetic protein import, and are therefore essential for plant viability.

When reconstituted into planar lipid bilayers, the purified 1-MD TIC complexes showed ion channel activity (Fig. 4 and fig. S16). In most cases, three identical channels appeared to be simultaneously incorporated into the bilayer as a unit (fig. S16C). This also suggests a trimeric assembly of Tic214, Tic100, Tic56, and Tic20-I to form the 1-MD complex. The single-channel current-voltage (*I-V*) curve (Fig. 4B) indicates weak rectification, because current was slightly larger at negative voltages than at positive voltages. The average slope conductance at 0 mV was 266 ± 18 pS ($N = 10$), a value comparable to those obtained with other protein translocons (*23*–*25*). Channel gating had a weak dependence on the membrane voltage, as spike-like short closures were frequently observed at negative high voltages (Fig. 4A). Because they always showed rectifying and voltage-dependent gating properties, we concluded that TIC channels incorporated into planar bilayers had the same orientation (fig. S16B).

In the presence of 0.1 μM preprotein (pS-protA) in the physiological intermembrane space side of the membrane (fig. S16B), TIC channels were blocked at positive voltage but open at negative voltage (Fig. 4C). At high positive voltages, the open probability decreased with an increase in preprotein concentration (fig. S16D). In contrast, TIC currents were only slightly affected, even by a high concentration (1 μM) of mature protein (mS-protA) lacking a transit peptide (Fig. 4D and fig. S16D). When preproteins were added to the opposite (stromal) side of the membrane, TIC channels were not blocked either at positive or negative voltage (fig. S16E). Thus, the TIC complex forms membrane channels, where preproteins specifically interact with and plug the channel pore.

Although homologs of Tic20 have been identified in cyanobacteria and are well conserved among virtually all plastid-containing lineages (*21*), phylogenetic analysis (fig. S17) revealed

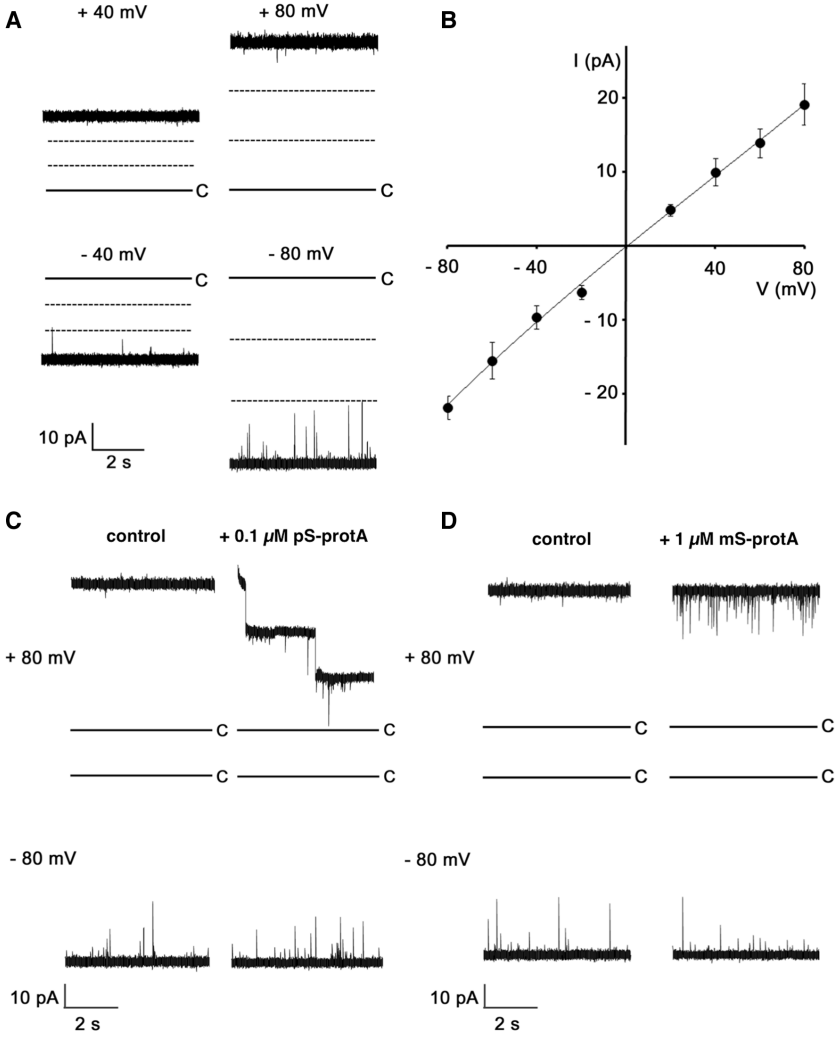

Fig. 4. Reconstituted 1-MD TIC complexes form preprotein-sensitive channels in planar bilayer membranes. (**A**) Representative current fluctuations in reconstituted 1-MD TIC complexes at different membrane voltages. Solid line labeled C indicates zero current. The distance between dashed lines corresponds to a unitary step. (**B**) The single-channel current-voltage (*I-V*) relationship. Data are means ± SD ($N = 8$ to 19). (**C**) Representative current recordings in the absence (control) and presence of 0.1 μM pS-protA in the intermembrane space side at the indicated membrane voltage. (**D**) Representative current recordings in the absence (control) and presence of 1 μM mS-protA in the intermembrane space side at the indicated membrane voltage.

no direct homologs for Tic214, Tic100, or Tic56 in extant cyanobacteria, Glaucophyta, or Rhodophyta, indicating that this TIC transport system evolved largely after the initial endosymbiotic event. Thus, the chloroplast inner membrane protein translocon that we describe here has changed markedly during evolution through modifications of both nuclear and chloroplast genomes.

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Acknowledgments: We thank K. Iwasaki, N. Miyazaki, S. Thompson, T. Takao, C. Awada, H. Mori, and J. Mima for helpful suggestions and valuable discussions; P. Jarvis, D. Schnell, and I. Nishimura for vectors; the Arabidopsis Biological Resource Center, Institut National de la Recherche Agronomique, and Cold Spring Harbor Laboratory for the T-DNA lines; and RIKEN Bioresource Center for the full-length cDNAs. Supported by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (20059022, 22020024, 24117511, and 24657073, M.N.; 21107518 and 22370059, T.I.; 23770190 and 23113519, M.H.) and by a Japan Society for the Promotion of Science postdoctoral fellowship for foreign researchers (J.B.). S.K., J.B., Y.H., M.O., M.I., M.T., and M.N. performed biochemical and genetic analyses; M.H. and T.I. performed electrophysiological analyses; M.N. conceived and supervised the whole project and wrote the paper.

Supplementary Materials

www.sciencemag.org/cgi/content/full/339/6119/571/DC1
Materials and Methods
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24 August 2012; accepted 30 November 2012
10.1126/science.1229262

Emergent Sensing of Complex Environments by Mobile Animal Groups

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The capacity for groups to exhibit collective intelligence is an often-cited advantage of group living. Previous studies have shown that social organisms frequently benefit from pooling imperfect individual estimates. However, in principle, collective intelligence may also emerge from interactions between individuals, rather than from the enhancement of personal estimates. Here, we reveal that this emergent problem solving is the predominant mechanism by which a mobile animal group responds to complex environmental gradients. Robust collective sensing arises at the group level from individuals modulating their speed in response to local, scalar, measurements of light and through social interaction with others. This distributed sensing requires only rudimentary cognition and thus could be widespread across biological taxa, in addition to being appropriate and cost-effective for robotic agents.

Research on collective intelligence has demonstrated how organisms, including humans, can improve decision-making accuracy by appropriately pooling individual estimates (*1*–*8*). In the earliest study, in 1907, Sir Francis Galton made a near-perfect estimate of the weight of an ox by using 787 guesses made by others (*9*). Pooling of information is not exclusive to the human domain; for example, it has also been suggested that aggregating imperfect estimates may help organisms, or cells, navigate weak or noisy environmental gradients (*2*–*5*). If each individual makes an error-prone estimate of the local gradient, it may benefit by also basing its movement decisions on the direction of motion of others, termed the "many wrongs" hypothesis (*3*).

Despite the importance of collective sensing to the ecology of many social species (*10*, *11*) and the value to bio-inspired technological applications [such as particle swarm optimization (*12*), or swarm robotics (*13*)], with the exception of the social insects (*8*), we do not know how grouping enhances sensing capabilities in animal groups. Here, we use an integrated experimental and theoretical approach to address this deficit. We use schooling fish (golden shiners, *Notemigonus crysoleucas*) as our model experimental system and take advantage of their natural preference for a shaded (darker) habitat (*14*). Thus, our experiments do not require training and are not susceptible to confounding factors relating to competition for, or consumption of, a preferred resource. Shiners school naturally in shallow water (*15*) and remain highly cohesive (*14*), which allowed us to explore the role of group size during a gradient detection task.

Our experiments were conducted with juvenile fish (body length 4.9 ± 0.5 cm) in a shallow tank (213 cm by 122 cm, 8-cm water depth). Dynamic light fields were projected onto the tank. These fields consisted of a circular patch that was darkest at its center and transitioned smoothly (as an exponential) to the brightest light levels. Noise was added to this gradient to generate local variability in space and time. Furthermore, the circular patch itself moved at a constant speed between a series of randomly selected locations within the tank. See (*16*) for further details. The task for the fish was to track the preferred, darker regions of this dynamic environment.

We investigated the performance of single fish and groups of 2, 4, 8, 16, 32, 64, 128, and 256 individuals. Three levels of environmental noise were employed. For the lowest value, the light field was dominated by the simple circular patch; for the highest level of noise, the field largely consisted of ephemeral, local peaks. Measured light levels at the surface of the tank ranged from 4.2 lux (approximately twilight) to 150 lux (overcast day), corresponding to their natural environment in the morning or evening. We stress, however, that light is used as a proxy for any important environmental cue (such as temperature or salinity), with the circular patch representing large-scale features and the noise recreating fine-scale structure.

Because golden shiners are highly cryptic when in dark regions, the tank was lit with infrared light. The level of the projected light field, with respect to their positions, was then used to calculate a

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Editor's Summary

Chloroplast Translocon Revealed

Protein translocation across biological membranes requires supramolecular complexes, called translocons. Chloroplasts require translocons in their double-envelope membranes to import thousands of nucleus-encoded proteins synthesized in the cytosol. However, the identity of the translocon at the inner envelope of the chloroplast (TIC) has long been a matter of debate; two proteins, Tic20 and Tic110, have been proposed to be central to protein translocation across the inner envelope membrane. Using transgenic *Arabidopsis* plants expressing a tagged form of Tic20, **Kikuchi *et al.*** (p. 571) report the isolation of a 1-megadalton complex composed of Tic56, Tic100, and Tic214 involved in protein translocation across the inner envelope. Thorough in vitro biochemical and in vivo genetic experimentation suggest that the isolated translocon contains both nuclear- and organellar-encoded components. Tic110 was not part of the isolated translocon.