LETTER TO THE EDITOR

REPLY: The revised model for chloroplast protein import

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Sometimes, data shown in the classical literature cannot be reproduced or turn out to be inconsistent with data obtained by improved modern techniques. In such cases, there is a need for careful examination of all the data and, if necessary, to revise or extend models which have long been accepted in a research community. In their Letter to the Editor concerning our recent article (Kikuchi et al. 2018; highlighted by Herrmann, 2018), Li et al. (2019) remind us of the classical view of the molecular mechanism of chloroplast protein import. My aim in this reply is not to dismiss the previous findings cited by Li et al. (2019) or their associated claims, but rather, to clarify what I believe to be the most important core of the issue, which has remained latent behind the long-accepted model for chloroplast protein import (Nakai, 2015a; Nakai 2015b; Nakai 2018).

The Chaperone Model

The long-accepted model for chloroplast protein import, as also described by Li et al. (2019), has been that Tic110 and Tic40 form a general translocon in the inner chloroplast membrane (TIC), which recruits stromal chaperones Hsp93, cpHsp70, and Hsp90C that function as import motors. I myself learned a lot from the classical literature on this topic, including most of the important works cited by Li et al. (2019). However, I often came away with different interpretations of these previous findings (for details, see Nakai 2015a; Nakai 2015b; Nakai 2018). In all cases, one simple question always remained: why is there a lack of conclusive evidence demonstrating any functional or physical cooperativity between Tic110/Tic40—two long-believed TIC proteins—and Tic20, the most widely-accepted central TIC channel component (Richardson et al. 2018)?
Identification of a Novel TIC and Direct Evidence for its Involvement in Chloroplast Protein Import

To answer this question, we first demonstrated that Tic20 forms a rigid one-megadalton complex at the chloroplast inner envelope membrane with three completely novel constituents named Tic56, Tic100, and Tic214(Ycf1) (Kikuchi et al. 2013). Quite surprisingly, we found almost no association of Tic110 or Tic40 with the newly identified Tic20-containing complex. Next, we asked whether the complex actually functioned as a general TIC. What should be the most reliable way to demonstrate its direct involvement in chloroplast protein import? We were convinced that the most straightforward way would be isolation of preprotein translocation intermediates followed by determination of the associating proteins/complexes. For this sake, we utilized a “gold standard” method by adding a well-established affinity tag: Protein A tag (a part of TAP tag), FLAG tag, or HA tag, at the C-terminus of often-used model preproteins, such as Rubisco small subunit preprotein or ferredoxin preprotein. Using these model preproteins to arrest a translocation intermediate under conditions of low ATP concentration in in vitro import experiments with isolated chloroplasts, we succeeded in purifying the intermediate complexes (summarized in Figure 1A). With all model preproteins tested, subsequent immunoblot analyses of affinity-purified fractions allowed the identification of all the novel TIC constituents, namely Tic20, Tic56, Tic100, and Tic214/Ycf1 as major protein partners (revealed by silver staining), together with well-known TOC constituents Toc159, Toc75, and Toc33/34. Importantly, we could not detect Tic110 nor Tic40, nor stromal chaperones such as Hsp93 or cpHsp70.

Li et al. (2019) state that “it is also important to note that this group did not investigate the roles of Tic110, Hsp93, cpHsp70, and Hsp90C in their previous publications”. This statement is incorrect. In our papers (Kikuchi et al. 2013, 2018), we investigated the association of Tic110, Hsp93, and cpHsp70 proteins to preproteins using specific antibodies and found almost no association. Our purification method may be considered as “unbiased” to identify the import machinery “in action”, since it depends on an established affinity tag attached to a cargo protein, rather than the potentially variable (and in some cases, problematically low) specificities of antisera raised against each component, often used for co-immunoprecipitation studies. Hence, even if parallel import pathways exist, components of both pathways should be co-purified together by our improved method. The observed absolute absence of Tic110 or Tic40 (see also Figure 1B) in the purified translocation intermediates with various model preproteins argues against the often-described possibility that Tic110/Tic40 may function in parallel with the one megadalton TIC consisting of Tic20, Tic56, Tic100, and Tic214 (Ycf214). Our work suggests that
only the latter TIC functions in chloroplast protein import, and this requires timely revision of the long-believed chaperone model (Nakai, 2015a; Nakai 2015b; Nakai 2018).

Identification of Novel Import Motor(s) Physically Associated with the Novel TIC

The successful identification of a novel TIC further pushed us to determine its associated ATP-driven import motor (Kikuchi et al. 2018). What criteria should be fulfilled to be such an import motor? We settled on two: i) it should interact with preproteins at the very early stage of ATP-dependent translocation across the inner envelope membrane, and ii) it should physically interact with TIC for their mechanical cooperation. In the process of searching for such a candidate, we identified a completely novel two-megadalton inner envelope-bound heteromeric AAA ATPase, named the Ycf2/FtsHi complex, which consists of Ycf2, FtsHi1, FtsHi2, FtsHi4, FtsHi5, FtsH12, and NAD-MDH. We further demonstrated its physical interaction with our novel TIC. Thanks to the above-mentioned method, all these components were confirmed to bind specifically to various translocation intermediates arrested at the early stage of import together with TOC and TIC constituents by immunoblot analysis (summarized in Figure 1A).

We then questioned to what degree translocation intermediate-associating proteins contribute to the import process by analyzing the entire purified translocation intermediate fractions by highly sensitive LC-MS/MS. This was done because the immunoblot analyses can tell us their specific association but nothing about their quantities directly. As summarized in Figure 1B, TOC, our novel TIC, and Ycf2/FtsHi complex components were reasonably identified with comparable levels by LC-MS/MS, strongly indicating their cooperative contribution to the formation of an import pathway. Consistent with the immunoblot data, this method failed to detect Tic110, Tic40, or Tic236, a recently proposed link between TOC and TIC (Chen et al. 2018). With additional biochemical and genetic evidence (described in Kikuchi et al. 2018), we proposed a revised model for chloroplast protein import: TOC and our novel TIC cooperate in preprotein translocation across the outer and inner envelope membranes with the aid of the Ycf2/FtsHi complex, which provides pulling force as the ATP-driven motor. Recent high-quality LC-MS/MS data reported by other laboratories appear to be highly consistent with our model (Zufferey et al. 2017; Schreier et al. 2018).

Some may argue that association of the Ycf2/FtsHi complex with the translocating preproteins might be due to the use of high concentrations of purified preproteins in in vitro import experiments which might jam at the import site and thus could trigger a sort of cleaning or degradation mechanism involving this complex. However, we concluded that this is not the case.
because, as demonstrated in Kikuchi et al. (2018; Figure 5), even using unpurified preproteins synthesized in in vitro cell-free translation systems, similar sets of Ycf2/FtsHi complex components were found in the translocation intermediates together with TOC and TIC components. It should be noted that purified preproteins synthesized in \textit{E. coli} cells have long been utilized in this research field not only by our own but also by others (Schnell et al. 1993; Kessler and Blobel 1996; Richardson et al. 2018).

We were unable to detect Hsp90C by LC-MS/MS in the translocation intermediates but could detect Hsp93 as well as cpHsp70, albeit as a minor contributor (Figure 1B), which might reflect on their relative role in the import process as also described by Li et al. (2019). However, as we demonstrated in our article (Kikuchi et al., 2018), these chaperones most likely contribute at a later stage, after import, for folding/assembly or degradation in the stroma. Thus, it is critically important to distinguish their direct participation in import from their chaperoning functions or from indirect secondary actions. So far, we have observed no direct physical interaction between these stromal chaperones and the novel TIC or Ycf2/FtsHi complex. Nevertheless, as discussed by Li et al. (2019), it is true that there are distinct interpretations of data presented in the literature for their functions. Thus, it will be important to reexamine the roles of these stromal chaperones in chloroplast protein import, as highlighted previously (Herrmann, 2018). Similarly, the stereotypical view of Tic110/Tic40 as central to chloroplast import has tended to preclude reconsideration of their direct roles in chloroplast biogenesis, rather than in protein import. Indeed, while Tic110 was previously shown to be crosslinked with a translocating preprotein (Inaba et al. 2003), this finding was not reproducible even by the same research group (Richardson et al. 2018). Thus, we conclude that it is now time to reevaluate what might be the genuine functions of Tic110/Tic140 (Nakai 2015a; Nakai 2015b; Nakai 2018).

\textbf{Why are the Models Seemingly Mutually Exclusive?}

The core of the issue is of the lack of reproducibility of the previous observations by which Tic110 and Tic40 were proposed to be central translocon components. Although the two proteins have long been believed to be key translocon proteins, in fact there is a limited number of papers published from only a few research groups, and the evidence supporting their direct involvement seems not that dependable as described above (for details, see Nakai 2015a). Some may argue that these discrepancies may be due to different experimental conditions. However, I believe this does not adequately explain the complete absence of Tic110 or Tic40 in our observations. Rather, I have considered that initial misidentification of these two proteins as TIC components might be
the reason. In the mid-1990s, Schnell et al. (1994) found a 100 kDa protein seemingly associated with translocation intermediates. However, it later was discovered that they had cloned the cDNA for a well-known high-abundant 100kDa envelope protein, and not for the 100 kDa protein recovered with the intermediates; the former is a protein now known as Tic110 (Kessler and Blobel 1996). It seems there is no absolute guarantee that the original 100 kDa protein associated with the translocation intermediates was Tic110. Because, Tic100 (not Tic110) of our TIC complex possesses very similar electrophoretic mobility with that of Tic110, it seems possible that the 100 kDa translocation intermediate-interacting protein described by Schnell et al. (1994) is Tic100, not Tic110.

A similar case of mis-identification could have happened when the Tic40 cDNA was cloned because it was associated with considerable confusion (see Nakai 2015a). A Canadian group initially found a 44 kDa protein that seemed to be associated with translocation intermediates after chemical cross-linking (Wu et al. 1994). Later this group cloned a partial Brassica cDNA which they believed to be a full-length cDNA for the 44 kDa protein, although the cDNA encoded a 36 kDa protein which was localized to the outer envelope and thus was named as Toc36 (Pang et al. 1999). Independently, Hsou-min Li had screened Arabidopsis pale mutants and selected one such mutant because the mutation was located in a gene corresponding to that reported in Pang et al. (1999), and this protein is now known as Tic40 (Chou et al. 2003). However, again, there remains uncertainty whether the 44 kDa protein initially reported by Wu et al. (1994) was the same Tic40 characterized by Chou et al. (2003). In our analyses (Kikuchi et al. 2018), another 44 kDa protein, which we called Tgd4-like protein, seems loosely associated with translocation intermediates. While, at present, the function of this nonessential protein in chloroplast protein import remains unclear, this protein could be a genuine candidate for the 44 kDa protein initially identified by Wu et al. (1994). Thus, we might need to consider whether initial mis-identification of Tic110 or Tic40 could be a plausible reason for the discrepancies between the two models.

**Green Lineages, Including Most Monocots, Retain the TIC and Ycf2/FtsHi Motor**

Contrary to the claim of Li et al. (2019), the novel TIC and Ycf2/FtsHi complex components are well conserved across the green lineage including the vast majority of monocots, with one minor exception of the grasses (Nakai, 2018). This is of course very surprising and it would be extremely interesting to clarify the identities of the TIC and motor components that function in the grasses. Our current model is that grasses—somehow—have evolved to utilize solely a “non-photosynthetic-type” alternative TIC and a single remaining FtsHi protein, all of which are well-
conserved across the green lineage (Nakai, 2018); such a “non-photosynthetic-type” TIC also involves Tic20 as a core (Kikuchi et al. 2018). This idea is not unprecedented, because a similar “non-photosynthetic-type” TOC is well known and involves Toc75 as a core, but contains a set of peripheral receptor components that is distinct from that of the “photosynthetic-type” TOC (Nakai 2015a; Nakai 2015b; Nakai 2018). Alternatively, it might be possible that, during evolution, grasses somehow gained an energetically more efficient protein import system involving mechanically-coupled Hsp70-type molecular chaperones just like an extant mitochondrial protein import system (Herrmann, 2018); it remains as an intriguing open question.

Bright Outlook

Our recent studies with successful purification of translocation intermediates (Figure 1) should pave the way to elucidating the detailed molecular structures and underlying mechanisms of the TOC-TIC-Ycf2/FtsHi motor complexes in this cryo-EM era, which will surely clarify how this supramolecular organization pulls the preprotein from the cytosol to the chloroplast interior. I remain optimistic about the prospects of achieving this goal.

Acknowledgements

I thank all the past and current members of the Nakai Laboratory as well as collaborators. Our work was supported by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, Japan [17H05668, 17H05725, 19H03183] (MN).

Author Contributions

The author is solely responsible for the content of this article.

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Figure 1. (A) Improved method to purify and identify translocation machineries. Translocation intermediates are arrested under low [ATP] in in vitro experiments. Proteins identified by immunoblot analyses in our studies (Kikuchi et al. 2013; Kikuchi et al. 2018) are shown. (B) LC-MS/MS determination of purified translocation intermediate-associating components (adapted from Kikuchi et al. 2018; Table 1). Areas of circles are depicted in proportion to the observed MS spectral counts (shown under protein names) except for those not detected. For TOC, TIC, and Ycf2/FtsHi complexes, total spectral counts of respective constituents are combined (shown under circles). *Tic20 has been known to be an extremely difficult protein to be detected by MS but was clearly detected by immunoblot analysis as well as by silver staining (Kikuchi et al. 2013) as indicated in (A).
