noprecipitation experiments further confirm that the two proteins directly associate in vivo (Fig. 3B and Fig. S2A). Notably, this was facilitated by ITK, because it was not detected in Itk−/− thymus (Fig. 3B). We also detected endogenous association of T-bet and GATA-3 in Thp cells treated in culture for 24 hours with Ab to CD3/CD28 and recombinant IL-2 (rIL-2) alone (Fig. S2B) or in the presence of IL-4 and IFN-γ to induce higher expression of T-bet and GATA-3 (Fig. 3C). Again, this was dependent on Y525 phosphorylation by ITK (Fig. 3D). T-bet and GATA-3 were also observed to associate in the human natural killer cell line YT (Fig. S2C). Reconstitution of T-bet−/−, T-bet−/− × IFN-γ−/−, and T-bet−/− × Itk−/− Thy with wild-type or Y525F mutant T-bet confirmed that the T-bet/GATA-3 interaction required Y525 and ITK (Fig. 3D).

We reasoned that the physical interaction between T-bet and GATA-3 might act to sequester GATA-3 away from its binding sites in the Th2 cytokine locus. Indeed, electrophoretic mobility shift assay with nuclear extracts from EL4 cells transfected with wild-type or Y437F T-bet revealed diminished binding of GATA-3 to its target sequence in the IL-5 promoter (Fig. 3E). In contrast, expression of the T-bet Y525F mutant did not affect GATA-3/DNA complex formation (Fig. 3E), nor did it repress GATA-3–dependent IL-5 promoter activity (Fig. 3F) despite all proteins being expressed at equivalent levels in these experiments (Fig. S2, E and F).

Considerable work has implicated ITK and RLK in directing CD4+ Th helper cell differentiation. However, integrating the various studies and model systems into a single unified model has been complex and controversial (11, 14–17). ITK−/− mice display a range of impaired and expanded Th2 phenotypes. Thus, although the exact relation to our studies is not yet resolved, the evidence suggests a pivotal modulation of T helper differentiation by this kinase.

Our studies show that tyrosine phosphorylation of T-bet by this and possibly other kinases is required for the repression of Th2 lineage commitment, gene activation and gene silencing, are physically distinct. Few kinases is required for the repression of Th2 differentiation by this kinase.

The in vivo importance of this observation in the full context of normal immune responses requires further exploration.

References and Notes
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Carotenoid Cation Formation and the Regulation of Photosynthetic Light Harvesting

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Photosynthetic light harvesting in excess light is regulated by a process known as feedback deexcitation. Femtosecond transient absorption measurements on thylakoid membranes show selective excitation of a carotenoid radical cation upon excitation of chlorophyll under conditions of maximum, steady-state feedback deexcitation. Studies on transgenic Arabidopsis thaliana plants confirmed that this carotenoid radical cation formation is correlated with feedback deexcitation and requires the presence of zeaxanthin, the specific carotenoid synthesized during high light exposure. These results indicate that energy transfer from chlorophyll molecules to a chlorophyll-zeaxanthin heterodimer, which then undergoes charge separation, is the mechanism for excess energy dissipation during feedback deexcitation.

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The regulation of photosynthetic light harvesting through a feedback deexcitation quenching mechanism (qE) is one physiologically important strategy used by plants to minimize alternative reaction intermediates (4–6). Elucidation of the biochemical mechanism of this vital regulatory process is fundamental for understanding photosynthesis on a molecular scale. In addition, because qE has been shown to be important for plant fitness (3), it is a requirement for engineering natural and artificial photosynthetic systems to be more robust when exposed to fluctuations in light intensity.

In excess light, a low thylakoid lumen pH (7, 8) has two effects: It activates formation of the carotenoid (Car) zeaxanthin (Zea) from violaxanthin via the xanthophyll cycle (9) (Fig. 1A), and it drives protonation of phyll (Chl) singlet excited states (1Chl*) in photosystem II (PSII) of green plants and algae so as to minimize alternative reaction pathways that generate toxic photo-oxidative intermediates (4–6). The qE involves harmless thermal dissipation of excess energy in the chlorophyll (Chl) singlet excited states (1Chl*) in photosystem II (PSII) of green plants and algae so as to minimize alternative reaction pathways that generate toxic photo-oxidative intermediates (4–6).
PsbS (10, 11), a PSII subunit that is necessary for qE in vivo (12). Determining why Zea is necessary for complete qE induction is central to an understanding of the mechanism(s) of 1Chl* deactivation during qE. Green fluorescence in isolated spinach thylakoid membranes without (black bar above the graph) and with (white bar) continuous high light illumination, $F_m$ is the fluorescence with open reaction centers in the absence of high light; $F_m^-$ and $F_m^+$ denote fluorescence with closed reaction centers (achieved with a saturating pulse, $\sim 2200 \mu \text{mol photons m}^{-2} \text{s}^{-1}$, 1 s) in the absence and presence of continuous high light, respectively. (C) TA data for spinach thylakoids upon excitation at 664 nm and detection at 1000 nm under quenched (circles) and unquenched (triangles) conditions and their corresponding fits (solid lines). (D) Difference between quenched and unquenched TA curves detected at 1000 nm upon excitation at 664 nm (diamonds) and the corresponding fit (solid line). OD, optical density; a.u., arbitrary units.

Additional experiments were performed on thylakoids from two Arabidopsis thaliana mutants in addition to the wild type (WT): (i) $WT + psbS$, which overexpresses PsbS and thus has $\sim 2.5$ times the qE of the wild type (2), and (ii) $npq4-1$, which lacks the $psbS$ gene and is therefore completely qE-deficient (12). Despite the lack of qE capacity, $npq4-1$ carries out light-induced photochemistry at the same rate as the wild type (12). The TA measurements for the three genotypes upon excitation at 664 nm and detection at 1000 nm showed kinetic differences with time constants similar to those observed in spinach only for the wild type and $WT + psbS$, with larger amplitude differences in the latter (Fig. 3). Hence, the kinetic differences we observe are correlated with qE.

TA measurements were also performed on wild-type A. thaliana thylakoids and three mutants with distinct Car composition. The wild-type plant has all of the components necessary to form Zea in high light and has the highest amount of qE of the four plants. The $npq2$ and $npq2lut2$ mutants lack activity of the Zea epoxidase enzyme, resulting in constitutively high levels of Zea in all light conditions (18, 19). The $npq2$ mutant contains the xanthophylls Zea and lutin (Lut), whereas the only xanthophyll pigment in $npq2lut2$ is Zea (19). qE studies on these two mutants have shown that Zea is dominantly responsible for qE in A. thaliana, whereas Lut has a minor role in affecting the rate of qE induction and the net amount of quenching (18). As a result, both $npq2$ and $npq2lut2$ have qE levels comparable to those of the wild type. The $npq1$ mutant lacks Zea in low light and cannot form it in high light, making it severely qE-deficient (6). The TA studies upon excitation at 664 nm and detection at 1000 nm for $npq2$ and $npq2lut2$ (20) showed an additional rise-and-decay component in the quenched case relative to the unquenched case, with time constants similar to those obtained for spinach and the wild-type and $WT + psbS$ A. thaliana plants. The kinetics observed for $npq1$ with and without high light illumination were very similar, displaying only minor differences in the decay component, but neither signal included a rise component (20). The magnitude of the differences varied as follows: wild type $\geq npq2 \geq npq2lut2 \gg npq1$. These findings demonstrate that Zea is necessary to produce the kinetic differences.
On the basis of the similarity in absorption maxima and spectral widths between the kinetic difference spectrum (Fig. 2) and the ground-state absorption spectra of the \( \beta \)-carotene and spheroidene radical cations, we conclude that the changes observed are due to the formation of Car\(^{+} \) selectively under quenched conditions. Identification of the species as a Car\(^{+} \) is consistent with the large amplitude of the differences probed in the near-infrared (near-IR) because the absorption cross section of Car\(^{+} \) is similar to the value for the Car \( S_0 \rightarrow S_2 \) transition (21), which is substantially larger than the excited-state absorption cross section of Chl, the transition that dominates the signal detected at 1000 nm under unquenched conditions (22). Moreover, the lack of additional strongly allowed transitions from other photosynthetic chromophores in the probe region supports the assignment of the species as a Car\(^{+} \). The demonstrated necessity of Zea for the generation of the kinetic changes enables specific assignment of the species observed during qE as a zeaxanthin radical cation (Zea\(^{+} \)).

The observation of Car\(^{+} \) formation upon selective excitation of Chl is in agreement with previous experimental findings in model systems comprising Cars and molecules that are structurally and spectroscopically similar to Chl (23, 24), as well as theoretical studies (25). However, our work shows that Zea\(^{+} \) formation is correlated with qE. The result is in line with calculations that showed Zea to have the lowest ionization potential of the three xanthophyll-cycle Cars (Fig. 1A) (25). A model that is consistent with all our data (13) involves three species: a bulk Chl pool (Chl\(_{\text{bulk}} \)), a Chl-Zea heterodimer that quenches excited Chl\(_{\text{bulk}} \) molecules, and a charge-separated ground state consisting of Chl\(^{+} \) and Zea\(^{+} \) (Fig. 4). The charge-separated state is formed from relaxation of the Chl-Zea excited state, (Chl-Zea\(^{*} \)), which is probably a charge-transfer state (25). In this scheme, the \( \sim 11 \)-ps component corresponds to the net dynamics of the Chl\(_{\text{bulk}} \) molecules that transfer to Chl-Zea. Simulations of the kinetics, including Chl\(_{\text{bulk}} \) annihilation dynamics, show that energy transfer from Chl\(_{\text{bulk}} \) to Chl-Zea occurs in \( \sim 15 \) to 200 ps and the relaxation time scale of (Chl-Zea\(^{*} \)) to form Chl\(^{+} \) and Zea\(^{+} \) is on the order of 0.1 to 1 ps. Such a fast heterodimer relaxation time scale would ensure efficient quenching because it would prevent the energy that is transferred to the heterodimer from returning to Chl\(_{\text{bulk}} \). The Zea\(^{+} \) signal decays on a time scale of \( \sim 150 \) ps, which corresponds to charge recombination between Chl\(^{+} \) and Zea\(^{+} \). Integration of the photophysical quenching pathways uncovered in this work with the currently emerging spatial and dynamical picture of the entire PSII complex (26), including high-resolution crystal structure information on the major light-harvesting protein LHCII (27), is necessary for precise determination of the time scales that generate efficient regulation of photosynthetic light harvesting.

The detection of selective formation of Zea\(^{+} \) under conditions of maximum, steady-state qE identifies the key molecular component involved in energy dissipation in PSII. Our results strongly suggest that the mechanism of nonradiative deactivation of Chl\(^{+} \) during excess light occurs by excitation transfer to a Chl-Zea heterodimer, followed by ultrafast Car\(^{+} \) formation.
Cryo–Electron Tomography Reveals the Cytoskeletal Structure of Spiroplasma melliferum

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Evidence has accumulated recently that not only eukaryotes but also bacteria can have a cytoskeleton. We used cryo–electron tomography to study the three-dimensional structure of Spiroplasma melliferum cells in a close-to-native state at ~4-nanometer resolution. We showed that these cells possess two types of filaments arranged in three parallel ribbons underneath the cell membrane. These two filamentous structures are built of the fibril protein and possibly the actin-like protein MreB. On the basis of our structural data, we could model the motility modes of these cells and explain how helical Mollicutes can propel themselves by means of coordinated length changes of their cytoskeletal ribbons.

One of the key functions of a cytoskeleton is to determine and maintain the shape of cells. In bacteria, the cell wall is generally considered to be the primary determinant of cell shape. However, this has been called into question, most recently by the discovery of the Mollicutes (Mycoplasma, Acholeplasma, and Spiroplasma) (1). Mollicutes are the smallest and simplest free-living and self-replicating cells within prokaryotes, and they are enclosed only by a cholesterol-containing cell membrane. Despite the lack of a cell wall, these cells have distinct morphologies, and their peculiar mode of movement that occurs in the absence of appendages normally implicated in motility (e.g., flagella or secretion organelles) makes the existence of an internal cytoskeleton likely.

Williamson (2) first reported the isolation of a cytoskeleton upon cell lysis and sodium deoxycholate extraction of Spiroplasma isolated from Drosophila. Cytoskeletal elements have also been released from Spiroplasma cells by repeated freezing and thawing (3). Townsend et al. (3) also developed methods for purification of the membrane-associated fibrils from S. citri and suggested that they may be involved in motility. Electron microscopy and SDS gel electrophoresis have shown that S. melliferum possesses a cytoskeletal ribbon comprised of ~4- to 5-nm-wide fibrils with an axial repeat of ~9 nm; these fibrils form pairs within the ribbon and are composed of the 55-kD fibril protein (3, 4). Townsend and Plaskitt (5) further used immunogold staining of thin sections with an antibody to P55 to localize the ribbon built of the fibril protein within S. melliferum cells. More recently, the structure and localization of this cytoskeleton was described as a flat, monolayered, membrane-bound ribbon composed of six or seven pairs of fibrils following the inner, shortest helical path of the cell (6, 7). The fibril protein, which is completely unrelated to any other eukaryotic or prokaryotic protein and has been found exclusively in the genome of Spiroplasma (8), was identified as the only component of this ribbon (7). Because this protein would have a diameter of about 5 nm, assuming a spherical shape, it was proposed that the subunit of the fibrils is a tetramer and that the filaments form pairs to give the 10-nm axial and lateral spacings in the cytoskeletal ribbon (9).

Our tomographic studies (10, 11) of intact vitrified S. melliferum cells, however, reveal a more complex pattern of filamentous structures

![Fig. 1. Superimposed slices from different z heights of a tomogram (A) (z heights indicated by bounding box) and two corresponding 3D visualizations (B and C) of part of a S. melliferum cell showing the arrangement and course of the cytoskeleton. (A) The cytoskeleton is composed of two outer ribbons of thick filaments and a ribbon of thin filaments sandwiched in between. (B) Simplified 3D representation of the filament ribbons (green, purple, and red) that wind in parallel helically around the cell just underneath the cell membrane (blue), showing, in this case, the left-handed course of the cytoskeleton. (C) Idealized visualization of the filaments with smooth transition to the original data (yellow).](image-url)