insulin-resistant and insulin-deficient mice. Second, in obese KKAy mice, a doubling of plasma visfatin levels achieved by an adenovirus vector also led to a reduction of plasma glucose and insulin concentrations. Finally, our biochemical studies indicate that visfatin activates insulin signaling through IR but in a different fashion from insulin.

There are important differences between visfatin and insulin, however. Plasma visfatin levels did not change significantly upon fasting or feeding in mice (fig. S4), whereas plasma insulin levels increased in the fed state and decreased in the fasting state. The plasma concentration of visfatin was 10% that of insulin in the fasting condition and only 3% in the fed condition. These low concentrations of visfatin may account for the modest effect of visfatin on plasma glucose levels relative to that of insulin, as shown by our analysis of visfatin$^{+/-}$ mice. Our biochemical studies showed that at similar concentrations, visfatin and insulin have a comparable ability to activate insulin signaling and glucose uptake and to inhibit glucose release. Moreover, in visfatin$^{+/-}$ mice, a 3 pM reduction in plasma visfatin levels leads to a 10 to 20 mg/dl elevation in plasma glucose concentrations. Taken together, these data suggest that visfatin plays a physiological role in lowering plasma glucose concentrations, but its contribution is small because of its low concentration.

The previously reported function of PBEF/visfatin was enhancement of the effect of interleukin-7 (IL-7) on pre-B cell colony formation (*13*). Insulin and IGFs were also reported to potentiate pre-B cell colony formation (*17*). Thus, this function of PBEF/visfatin may be attributed to another insulin-like effect. Relative to adipose tissue of lean mice, adipose tissue of obese mice contains increased amounts of proinflammatory cytokines such as TNF-α or IL-6. Both of these cytokines increase mRNA levels of PBEF/visfatin (*18*) and thus may be responsible for the increased mRNA levels of PBEF/visfatin in visceral fat.

The discovery of the insulin-mimetic function of visfatin may shed new light on glucose and lipid homeostasis, adipocyte proliferation and differentiation, and other aspects of insulin-related biology. The potential relationship between visfatin and metabolic syndrome also merits further investigation, because plasma visfatin levels increase in proportion to visceral fat accumulation. Finally, our results raise the possibility that visfatin may be a useful target for the development of drug therapies for diabetes.

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T Helper Cell Fate Specified by Kinase-Mediated Interaction of T-bet with GATA-3

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Cell lineage specification depends on both gene activation and gene silencing, and in the differentiation of T helper progenitors to Th1 or Th2 effector cells, this requires the action of two opposing transcription factors, T-bet and GATA-3. T-bet is essential for the development of Th1 cells, and GATA-3 performs an equivalent role in Th2 development. We report that T-bet represses Th2 lineage commitment through tyrosine kinase–mediated interaction between the two transcription factors that interferes with the binding of GATA-3 to its target DNA. These results provide a novel function for tyrosine phosphorylation of a transcription factor in specifying alternate fates of a common progenitor cell.

The immune system T-box protein T-bet controls lineage commitment of the two subsets of cytokine-producing helper T cells, Type 1 (Th1) and Type 2 (Th2) (*1*–*3*) by simultaneously driving Th1 genetic programs and repressing the development of the opposing Th2 subset. T-bet is principally required for expression of the potent inflammatory cytokine interferon-γ (IFN-γ), the hallmark of Type 1 immunity, and simultaneous repression of the signature Th2 cytokines interleukin-4 (IL-4) and IL-5 (*1*, *4*). As a consequence, mice lacking T-bet fail to develop a Th1 compartment but possess an overexpanded Th2 compartment, which leads to a spontaneous asthma-related phenotype (*2*, *5*). Although it is established that T-bet accomplishes Th1 programs partly through direct induction of IFN-γ– and IL-12Rβ2–chain gene transcription (*6*), the mechanism by which it represses Th2 programs is unclear.

To determine whether T cell receptor (TCR) signaling induced alterations in T-bet, a Th1 clone and control Th2 clone were tested after polyclonal TCR engagement. Western blotting revealed a specific phosphorylated species in a Th1 clone that was not present in Th2 cells (fig. S1A). T-bet was rapidly induced in early Th1 differentiation, gradually decreasing at later stages (Fig. 1A). We also observed T-bet tyrosine phosphorylation to occur primarily in Thp cells upon TCR engagement, being most pronounced early in differentiation (day 2), declining by day 4, and being undetectable upon secondary stimulation (Fig. 1A). Tyrosine phosphorylation of T-bet was also enhanced in the presence of the phosphatase inhibitor pervanadate (fig. S1B). Thus, T-bet becomes tyrosine phosphorylated at the earliest stages of Thp dif-

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ferentiation, consistent with its involvement at the initial stages of lineage commitment.

T-bet is constitutively nuclear (*7*), which indicates that its phosphorylation is mediated by one of the few nuclear tyrosine kinases identified in T cells (*8*, *9*). Using the Scansite program (*10*), we identified an ITK phosphorylation motif at the C terminus of T-bet (Y525), as well as three conserved c-Abl sites (Y76, Y107, and Y117). Although phosphorylation of T-bet was restricted to the Tec kinases ITK and RLK (fig. S1C), coexpression studies demonstrated that this was most efficiently performed by ITK (Fig. 1B). In primary CD4 T cells isolated from ITK-, RLK-, or double ITK/RLK-deficient mice (*11*), the greatest diminution of T-bet tyrosine phosphorylation was seen in the absence of ITK (*7*) (Fig. 1C). Furthermore, mutation of T-bet at tyrosine residue 525, but not control tyrosine residue 437, resulted in greatly reduced phosphorylation by ITK (Fig. 1D), revealing that ITK phosphorylates T-bet at residue Y525 after TCR stimulation.

To assess the functional relevance of ITK-induced T-bet phosphorylation, CD4+ T-bet$^{-/-}$ Thp cells were transduced with mutant green fluorescent protein (GFP) retroviruses expressing T-bet wild-type (RV-T-bet) and Y525F (RV-T-bet Y525F), with assessment of cytokine production after secondary stimulation. Both RV-T-bet and Y525F T-bet could restore T-bet function, demonstrated by the repression of IL-2 and the increased induction of IFN-γ (by a factor of >100) (Fig. 1E). However, Y525F T-bet was much less effective in repressing the expression of Th2 cytokines IL-4, IL-5, and IL-13. Similar results were obtained in T-bet$^{-/-}$ × IFN-γ$^{-/-}$ CD4+ Th cells (fig. S1F), revealing that the role of T-bet tyrosine phosphorylation in repressing Th2 cytokines is independent of IFN-γ.

The multidomain protein ITK, an important mediator of TCR signaling in lymphocytes (*9*, *12*, *13*), resides in both nucleus and cytosol, which suggests that it physically associates with T-bet. Selective association of ITK with T-bet in coexpression studies correlated with its ability to phosphorylate T-bet (Fig. 2A). Using a series of ITK mutants, we also revealed that this occurred via the ITK SH1 kinase domain (Fig. 2B). Additionally, this interaction depended largely on the presence of tyrosine 525 within T-bet, as ITK coimmunoprecipitated less well with the Y525F T-bet mutant (Fig. 2C). To look for association under endogenous conditions, T-bet was immunoprecipitated from nuclear extracts of BALB/c wild-type, T-bet$^{-/-}$, and Itk$^{-/-}$ thymocytes followed by Western blotting with antibody (Ab) to ITK. In these experiments, the presence of ITK was apparent in immune complexes from wild-type, but not T-bet$^{-/-}$ or Itk$^{-/-}$, thymocytes (Fig. 2D).

Because T-bet is known to be coregulated with GATA-3, we tested whether this might take place through a physical association. Coexpression of FLAG-tagged GATA-3 or GATA-3 mutants bearing truncations with wild-type or Y525F T-bet did suggest a physical interaction of the two proteins that was diminished by the Y525F mutation (Fig. 3A) and mapped to the GATA-3 N-terminal (amino acids 1–257) domain (fig. S2D). Coimmu-

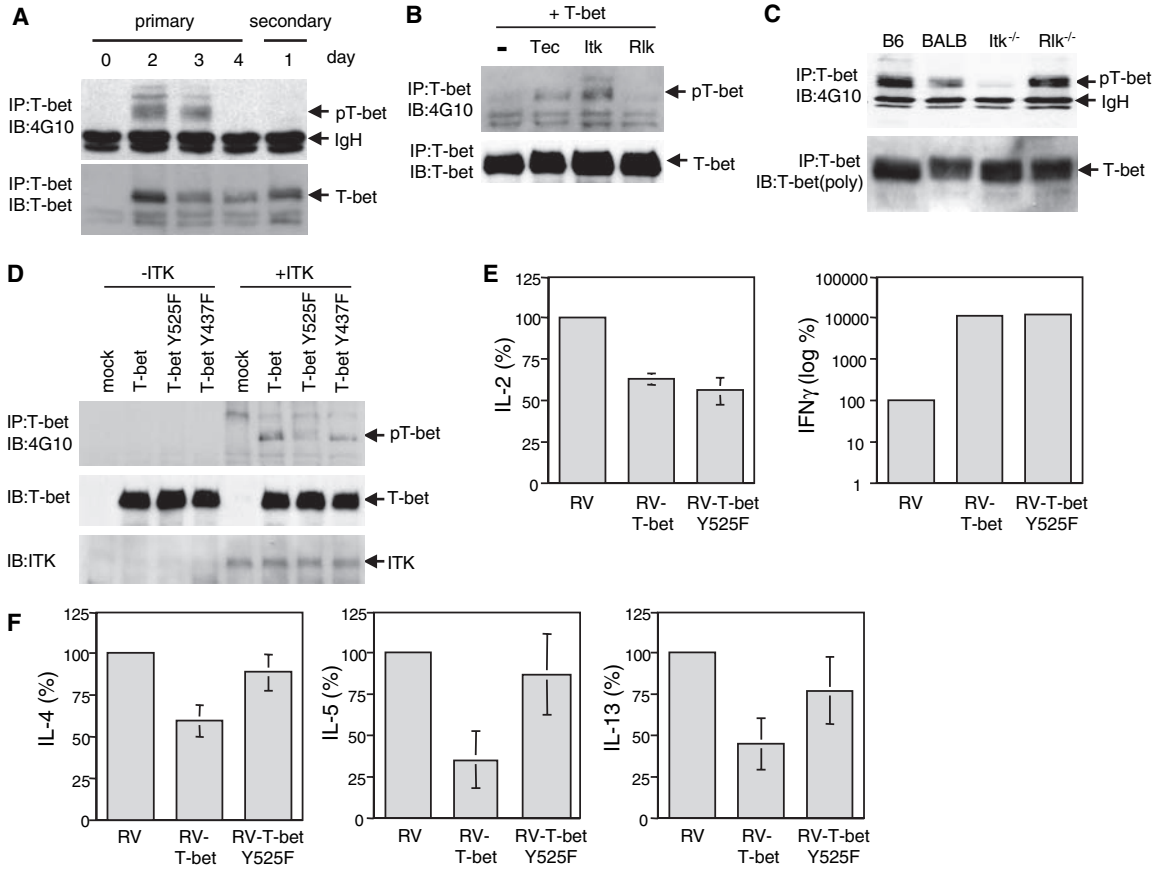

Fig. 1. (**A**) T-bet tyrosine phosphorylation in primary Th1 cells. CD4+ lymph node and spleen Thp cells were stimulated with plate-bound Ab to CD3 (2 μg/ml), Ab to CD28 (1 μg/ml), and IL-2 (100 U/ml) with IL-12 (1 ng/ml) and Ab to IL-4 (10 μg/ml). Total cell extracts were prepared on days 0, 2, 3, and 4 after primary stimulation and on day 1 after secondary stimulation. Pervanadate (100 μM) was added 15 min before cell lysis. (**B**) ITK phosphorylates T-bet. T-bet was cotransfected with Tec kinases TEC, ITK, or RLK in 293T cells, and total cell lysates were prepared after incubation with pervanadate for 15 min. (**C**) Diminished tyrosine phosphorylation of T-bet in Itk$^{-/-}$ CD4+ T cells. CD4+ T cells isolated from the indicated strains were stimulated with plate-bound Ab to CD3 and Ab to CD28 under Th1-skewing condition for 2 days. (**D**) ITK phosphorylates tyrosine 525 residue of T-bet. Wild-type (WT) or tyrosine mutants of T-bet (Y525F and Y437F) were cotransfected with either control vector or ITK into 293T cells. Expression of T-bet or ITK was detected by Western blot using total cell lysates. [(A) to (D)] Tyrosine phosphorylation of T-bet was performed by immunoprecipitation with monoclonal antibody (mAb) to T-bet and by immunoblot with mAb to phosphotyrosine, 4G10, as in fig. S1A. (**E**) WT and Y525F T-bet both restore T-bet function in regulating IL-2 and IFN-γ. Data represent mean ± SD. (**F**) The optimal repression of Th2 cytokines by T-bet requires Y525. *P* value of 0.0002 for IL-4, 0.0074 for IL-5, and 0.002 for IL-13. CD4+ T cells purified from T-bet$^{-/-}$ or T-bet$^{-/-}$ × IFN-γ$^{-/-}$ (fig. S1F) mice were stimulated for 24 hours under Th2-skewing conditions and then transduced with RV, RV-T-bet, or RV-T-bet Y525F. Cells sorted for GFP were expanded for 6 days and restimulated with Ab to CD3 for 24 hours, and cytokines were measured by enzyme-linked immunosorbent assay. Data represent mean ± SD.

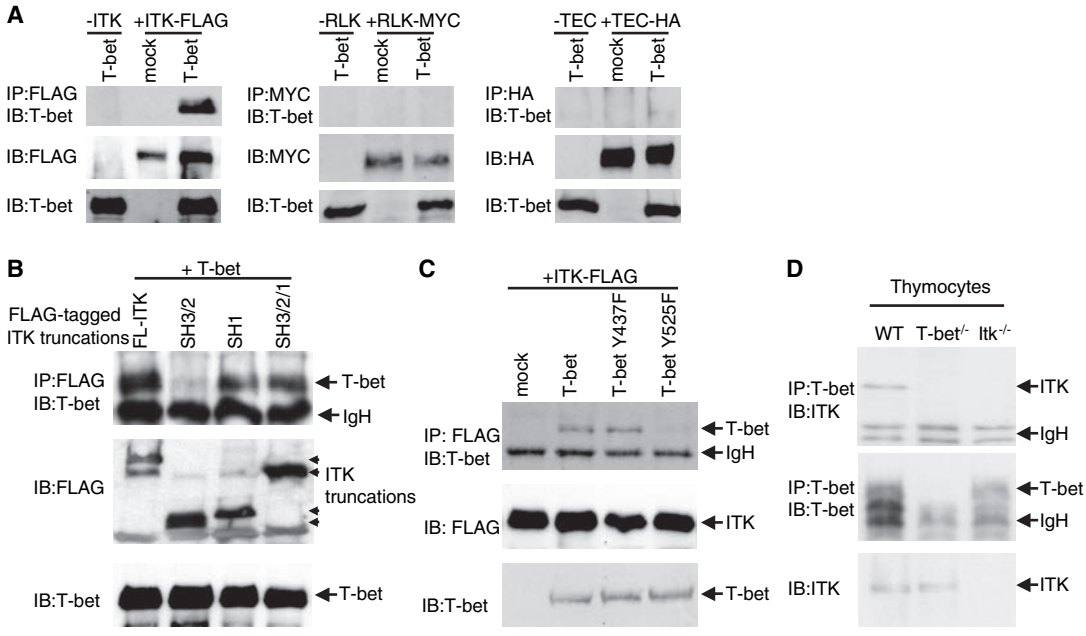

Fig. 2. T-bet physically interacts with ITK. (**A**) T-bet was cotransfected into 293T cells with peptide-tagged TEC kinases ITK-FLAG, RLK-MYC, or TEC-hemagglutinin (TEC-HA). Total cell extracts were incubated with Abs to FLAG, MYC, or HA, and immunocomplexes were resolved by 7.5% Tris-Glycine (Tris-HCl Gel, BIO-RAD, Hercules, CA) gel and probed with Ab to T-bet. Expression of T-bet or TEC kinases was assayed by Western blot using total cell lysates. (**B**) ITK interacts with T-bet through its SH1 domain. The FLAG-tagged ITK truncations were cotransfected with T-bet into 293T cells and immunoprecipitated with Ab to FLAG followed by Western blot with Ab to T-bet. Expression of ITK truncations and T-bet was detected with Abs to FLAG T-bet. (**C**) ITK interacts with the tyrosine Y525 residue of T-bet. FLAG-tagged ITK was coexpressed with WT or tyrosine mutants (Y525F or Y437F) of T-bet, immunoprecipitated with FLAG-M2 agarose, and the protein blot was probed with Ab to T-bet. Similar expression levels of T-bet proteins and ITK were detected in 30 μg of total cell lysates. (**D**) T-bet interacts with ITK in vivo in thymus. Nuclear extracts were prepared from BALB/c WT, T-bet$^{-/-}$, and Itk$^{-/-}$ thymi with NE-PER (Pierce, Rockford, IL). Two mg of nuclear extracts were incubated with 4B10 in 150 mM NaCl, and immune complexes were resolved and probed with mAb to ITK (2F12) and then sequentially with 4B10 after stripping. ITK expression was assayed in 30 μg of nuclear extracts.

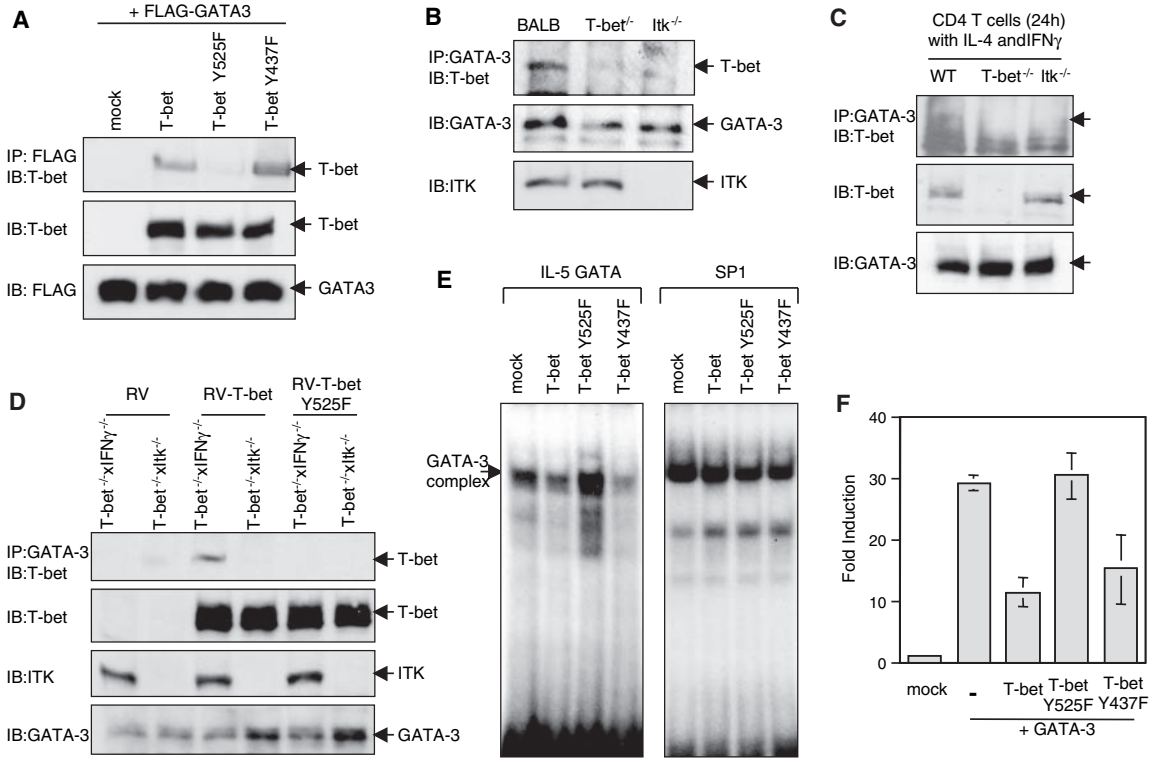

Fig. 3. T-bet interacts with GATA-3 and sequesters it away from binding to target DNA. (**A**) FLAG-tagged GATA-3 was cotransfected into 293T cells with WT, Y525F, or Y437F T-bet and immunoprecipitated with FLAG-M2 agarose, and protein blots were probed with 4B10. Expression of T-bet and GATA-3 was analyzed by Western blot of total cell lysates. (**B**) T-bet interacts with GATA-3 in thymocytes in an ITK-dependent manner. Thymocyte nuclear extracts (3 mg) isolated in Fig. 2D were used for immunoprecipitation and immunoblot analyses as described in fig. S2A. (**C**) Naive Thp from BALB/c WT, T-bet$^{-/-}$, and Itk$^{-/-}$ mice were cultured for 24 hours with Ab to CD3 plus Ab to CD28 and IL-2 in the presence of rIL-4 and rIFN-γ, nuclear extracts were prepared, and immunoprecipitation and immunoblot analyses were performed as in fig. S2A. (**D**) T-bet interacts with GATA-3 in vivo, in an ITK-dependent manner. CD4+ T cells isolated from T-bet$^{-/-}$ × IFN-γ$^{-/-}$ or T-bet$^{-/-}$ × Itk$^{-/-}$ mice were activated and transduced with retroviruses as described (Fig. 1, E and F). Nuclear extracts were prepared for immunoprecipitation with Ab to GATA-3 and for immunoblot analyses of T-bet, GATA-3, and ITK. We observed comparable expression of GATA-3 in Th cells transduced with control, WT, or Y525F T-bet (fig. S2E). (**E**) DNA binding activity of GATA-3 is blocked by T-bet but not by T-bet Y525F. EL4 cells were transfected with WT, Y525F, or Y437F T-bet and nuclear extracts were incubated with radiolabeled GATA-3 binding sites from the IL-5 promoter, or with SP1 binding sites, resolved in native 6% polyacrylamide gel, and subjected to autoradiography. (**F**) WT but not Y525F T-bet inhibits GATA-3 driven IL-5 promoter activation. EL4 cells were cotransfected with an IL-5 promoter reporter gene with GATA-3 and T-bet cDNAs and a β-gal reporter gene. Relative luciferase activity was assayed, standardized with β-galactosidase activity, and shown as fold induction. There was comparable expression of GATA-3 and T-bet (fig. S2F). Data represent mean ± SD.

noprecipitation experiments further confirmed 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$^{-/-}$ Thp with wild-type or Y525F mutant T-bet confirmed that the T-bet/GATA-2 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+ T 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 genetic programs rather than the activation of Th1 genetic programs, which reveals that the two major functions of T-bet in directing Th lineage commitment, gene activation and gene silencing, are physically distinct. Few transcription factors are known to be tyrosine phosphoproteins (*18–21*), and although there is precedent for transcription factors that cross-regulate one another in cell lineage commitment, no structural basis for such repression has been established (*22*). Our studies reveal one such mechanism, whereby tyrosine phosphorylation activity of a nuclear kinase controls the interaction of two opposing transcription factors, T-bet and GATA-3, in the repression of Th2 lineage development. The in vivo importance of this observation in the full context of normal immune responses requires further exploration.

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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 formation 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.

The regulation of photosynthetic light harvesting through a feedback deexcitation quenching mechanism (qE) is one physiologically important strategy used by plants to minimize the deleterious effects of short-term high light exposure (*1–3*). qE involves harmless thermal dissipation of excess energy in the chlorophyll (Chl) singlet excited states (^{1}Chl*) in photosystem II (PSII) of green plants and algae so as to minimize alternative reaction pathways that generate toxic photo-oxidative intermediates (*4–6*). Elucidation of the biophysical 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

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