Paternal Control of Embryonic Patterning in Arabidopsis thaliana

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Paternal Control of Embryonic Patterning in *Arabidopsis thaliana*

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The *YODA* (*YDA*) mitogen-activated protein kinase pathway promotes elongation of the *Arabidopsis* zygote and development of its basal daughter cell into the extra-embryonic suspensor. Here, we show that the interleukin-1 receptor–associated kinase (IRAK)/Pelle-like kinase gene *SHORT SUSPENSOR* (*SSP*) regulates this pathway through a previously unknown parent-of-origin effect. *SSP* transcripts are produced in mature pollen but do not appear to be translated. Instead, they are delivered via the sperm cells to the zygote and the endosperm, where SSP protein transiently accumulates. Ectopic expression of SSP protein in the leaf epidermis is sufficient to activate *YDA*-dependent signaling. We propose that SSP protein produced from paternal transcripts upon fertilization triggers zygotic *YDA* activity, providing an essential temporal cue for the regulation of the asymmetric first division.

Multicellular organisms rely on robust spatial and temporal coordinates to guide embryonic development. Little is known about the cues used by plants to derive such a framework. Growth of plant zygotes is typically polar, and their division asymmetric (*1*). In *Arabidopsis*, the zygote elongates about threefold (Fig. 1A) before dividing into a small apical cell, the founder of the spherical proembryo, and a large basal cell, the founder of a filamentous, largely extra-embryonic support structure called the suspensor (Fig. 1E). This fundamental fate decision is regulated by *YODA* (*YDA*)-dependent signaling. Loss of the mitogen-activated protein kinase kinase (MAPKK) kinase *YDA* (*2*) or the MAP kinases *MPK3* and *MPK6* (*3*) suppress elongation of the zygote (Fig. 1B) and suspensor formation. Hyperactive variants of *YDA* promote extensive elongation of the zygote and exaggerated suspensor growth (*2*). Here, we report that the interleukin-1 receptor–associated kinase (IRAK)/Pelle-like kinase gene *SHORT SUSPENSOR* (*SSP*) links activation of the *YDA* MAP kinase cascade to fertilization through a unique parent-of-origin effect and propose a mechanistic basis for our finding.

Mutations in the *SSP* gene have no discernible impact on adults but closely mimic the effect of *yda* mutations on embryogenesis (*2*). Mutant zygotes fail to elongate and generate basal cells of diminished size (Fig. 1C) (23 ± 5 μm, compared with 61 ± 7 μm for wild type). Reduced growth and aberrant divisions in the basal cell lineage subsequently result in a spectrum of defects, ranging from the absence of a recognizable suspensor (Fig. 1F) to cone-shaped suspensors with multiple cell layers (Fig. 1G) and rudimentary or short suspensors (Fig. 1, H and I). These phenotypes imply that *SSP* promotes extra-embryonic or suspensor fates. In support of this view, expression of a molecular marker for the suspensor (*4*) is not detected in *ssp* mutants (Fig. 1K).

Genetic analysis suggests that *SSP* acts upstream of the *YDA* MAP kinase cascade in a common pathway. Double mutant *ssp-1 yda-1* embryos are anatomically indistinguishable from *yda-1* single mutants (table S1), arguing against an independent function of the two genes. Hyperactive variants of the *YDA* MAPKK kinase reverse the suspensor phenotype caused by *ssp* mutations (table S1), suggesting that *SSP* participates in activating the *YDA* MAP kinase cascade.

Map-based cloning (*5*) revealed that *SSP* corresponds to At2g17090, a member of the RLCK II family of IRAK/Pelle-like kinases [also known as receptor-like kinases (*6*)]. Four mutations, including a premature stop (*ssp-1*) and an RNA null (*ssp-2*), generate essentially indistinguishable mutant phenotypes, which are complemented by the introduction of a 9.8–kilobase pair genomic DNA fragment spanning the locus (316 normal embryos in 580 total) (*7*). Three motifs can be identified in the predicted SSP protein (Fig. 2A): an N-terminal consensus for myristoylation and palmitoylation, a central protein kinase domain of the Pelle/IRAK superfamily, and a C-terminal tetratricopeptide repeat (TPR). Insertion of a yellow fluorescent protein (YFP) moiety on either side of the kinase domain does not impair SSP function (Fig. 2A).

N-myristoylation at Gly^2 and subsequent S-palmitoylation at neighboring cysteines are predicted to mediate stable plasma membrane association (*8*). Consistent with this prediction, cells overexpressing YFP-tagged SSP variants show robust fluorescence at their surface (Fig. 2B). A point mutation abolishing both N-myristoylation and S-palmitoylation [G2→A2 (G2A) (*9*)] renders the mutant protein cytoplasmic (Fig. 2C) and unable to complement *ssp* mutants (n = 479 embryos). Eliminating only palmitoylation [C3→S3 and C4→S4 (C3,4S) (*9*)] has a similar effect (n = 450 embryos). Peptides representing the N terminus of wild-type SSP or the C3,4S mutant are efficiently modified by *Arabidopsis* N-myristoyl transferase NMT1 in vitro (*10*), whereas a peptide representing the G2A mutant is not (Fig. 2D). We conclude that diacylation-mediated membrane association is essential for *SSP* function.

In contrast, protein kinase catalytic activity may be dispensable. Alternative splicing of intron 2 generates a short *SSP* transcript harboring an 18–amino acid in-frame deletion in the kinase domain (Fig. 2A). This cDNA does not complement the phenotype of *ssp* mutants when expressed from the endogenous promoter (n = 311 embryos), whereas the long cDNA species does (218 normal embryos in 449 total), suggesting that gross structural changes in the kinase domain are not tolerated. However, key residues of the active site, most conspicuously the aspartate of the canonical DFG (*9*) motif involved in Mg^{++} binding and phospho-transfer, are not found in SSP. Moreover, a transition-state mutant predicted to reduce catalytic activity to negligible rates while leaving substrate binding unaffected (*11*) fully complements the phenotype of *ssp* mutants (Fig. 2A) [K78R (*9*); 211 normal embryos in 442 total]. Although we cannot rule out that SSP uses a noncanonical reaction mechanism

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(*12*), the primary role of the kinase domain may lie in protein binding. Mammalian IRAK proteins often lack kinase activity but rather contribute to the assembly of receptor complexes (*13*). Similarly, a number of plant IRAK/Pelle kinases does not require catalytic activity for function (*14*).

The importance of protein-protein interactions is further implied by similarity of the SSP C terminus to TPR domains (Fig. 2A) (cluster KOG0548) mediating the association of heat shock protein 70 (HSP70) co-chaperone complexes (*15*). Deletion of the entire TPR motif or only the third, cryptic repeat unit completely inactivates the protein (Fig. 2A) [for mutant Y300Z, $n = 408$ embryos; for W431Z, $n = 433$ embryos (*9*)]. These results argue that SSP acts as an adaptor at the plasma membrane, possibly recruiting a pathway activator.

In the course of our analysis, it became clear that *ssp* mutations result in atypical segregation of normal and mutant phenotypes. Self-fertilized *ssp* plants hemizygous for a functional transgene generate normal and mutant embryos in a ratio of 1:1. The same 1:1 distribution is also seen in the progeny of self-fertilized *ssp-1*/+ or *ssp-2*/+ plants (94 normal embryos in 194 total and 204 in 394, respectively). Our mapping data indicate that transmission of the *ssp-1* allele through the haploid generation is not substantially distorted (*5*), pointing to a parent-of-origin effect as a likely cause for this phenomenon. Reciprocal crosses indeed demonstrate that the phenotype of the embryo is strictly dependent on the genotype of the pollen. When homozygous *ssp* plants were crossed with wild-type pollen, all embryos developed normally (*ssp-1* $n = 104$ and *ssp-2* $n = 185$). Conversely, all embryos resulting from a cross of wild-type plants with pollen from homozygous *ssp* plants developed abnormally (*ssp-1* $n = 121$ and *ssp-2* $n = 185$). We conclude that *SSP* exerts a male gametophytic, or paternal, effect on embryonic patterning (*16*).

Parent-of-origin effects in plants and mammals often arise from imprinting (*17*), in large part mediated by differential DNA methylation (*18*). However, *SSP* function is not sensitive to global changes in DNA methylation (*5*), arguing against epigenetic control. Alternatively, a paternal effect may arise from pollen-specific expression. A reverse transcription polymerase chain reaction (RT-PCR) indicates that *SSP* mRNA accumulates to readily detectable amounts only in mature pollen (fig. S1A). *Arabidopsis* pollen consists of a large vegetative cell that completely encloses two small sperm cells. In situ hybridization of germinated pollen reveals robust signals associated with the sperm (Fig. 3A). In support of this result, microarray profiling of isolated sperm identified *SSP* as 1 of 74 genes preferentially expressed in this cell type (*19*).

Surprisingly, the accumulation of SSP protein, determined by using functional YFP-tagged variants, does not coincide with RNA production. No YFP fluorescence is apparent in germinated pollen (fig. S1, C and D). SSP protein became detectable only upon fertilization, when weak fluorescence of the zygote was observed (Fig. 3D). This signal was transient and no longer seen by the time of the first division. Reciprocal crosses confirmed the absence of a specific signal in zygotes produced by nontransgenic pollen. A fluorescent reporter, consisting of the *SSP* upstream region fused at the translational start site to a nuclear-localized triple YFP, corroborates these results. Again, no YFP fluorescence is apparent in pollen, although *YFP* transcripts are present (fig. S1B). When this construct was crossed via the pollen, a transient fluorescent signal appeared in the nuclei of the zygote and the micropylar endosperm (Fig. 3E).

Our findings suggest a simple mechanistic basis for the paternal effect: *SSP* transcripts are produced but not translated in the sperm cells; rather, they are delivered to the seed, where they become translated and cause transient accumulation of SSP protein in both products of double fertilization, the zygote and central cell. In support of this view, in situ hybridization reveals weak signals in the zygote and the micropylar endosperm (fig. S2). The presence and origin of *SSP* transcripts after fertilization was independently confirmed in RNA samples prepared from about 2000 immature seed dissected 24 hours after pollination of *Ws* plants with *Col* pollen (*5*). RT-PCR from this material detected only RNA transcribed from the paternal *Col* allele (Fig. 3C).

We next sought to clarify whether SSP has a role in the endosperm by examining *fie* seed generated by *fie*; *cdc2a* pollen (*5*). In this genetic combination, a relatively normal seed is formed even though a single sperm fertilizes only

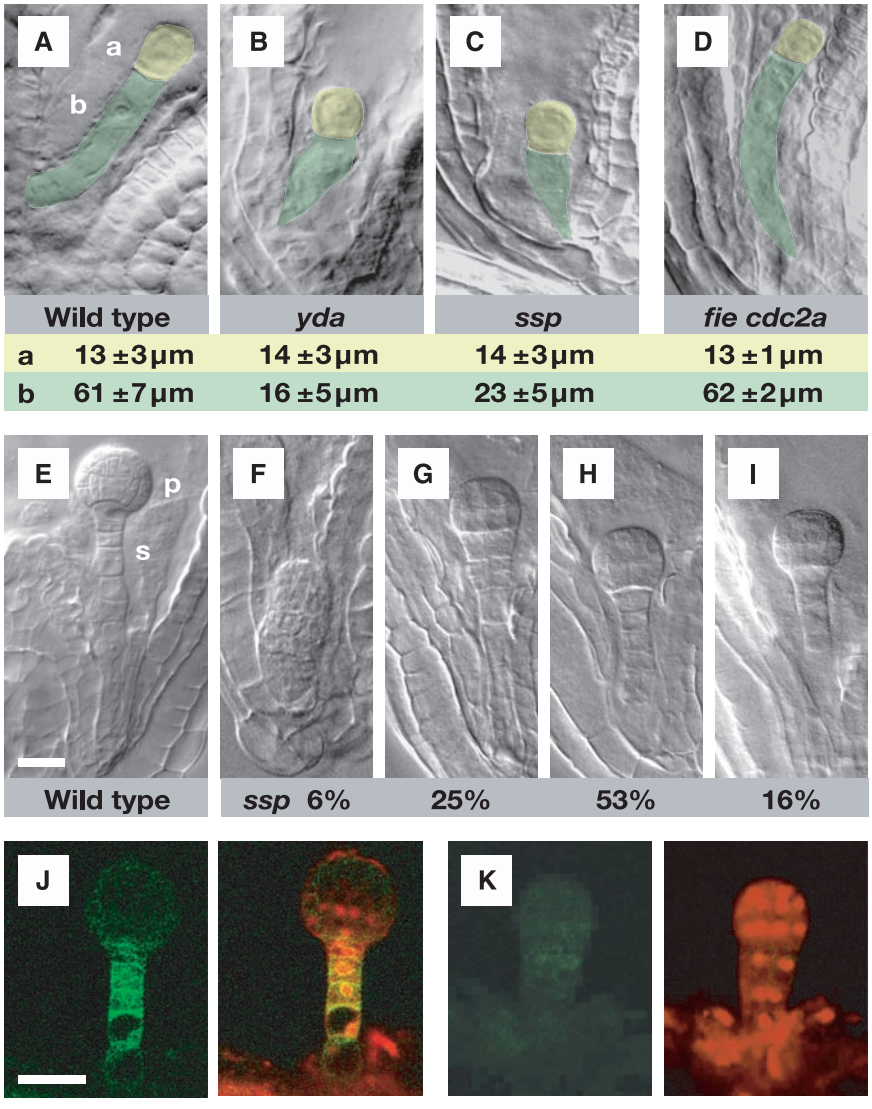
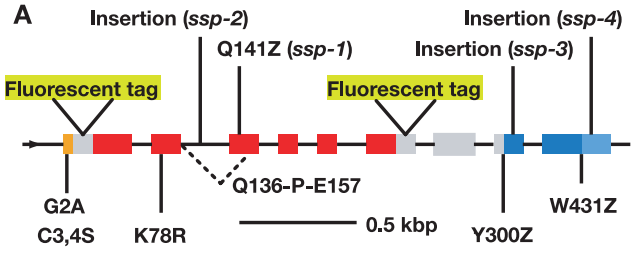


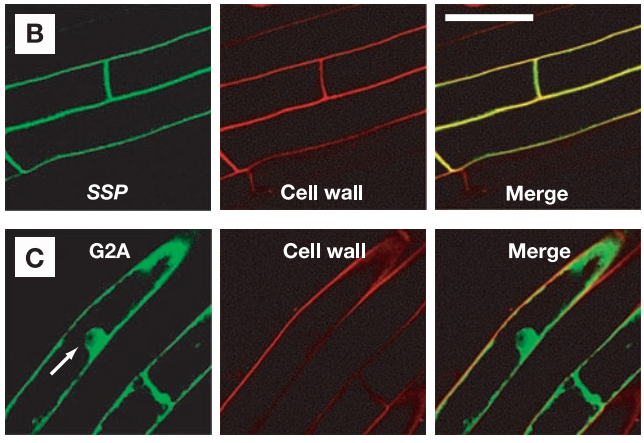
Fig. 1. Phenotype of *ssp* embryos. (**A** to **D**) Size of the apical (a) and basal (b) cell in wild-type (A), *yda* (B), *ssp* (C), and *fie cdc2a* background (*5*) (D); average length and standard deviation ($n > 20$) noted below. (**E** to **I**) Wild-type (E) and *ssp-1* (F to I) globular embryos; frequency of phenotypic classes listed below ($n > 400$); p indicates proembryo; s, suspensor. (**J** and **K**) Confocal microscopy of suspensor-specific marker *SUC3* in wild type (J) and *ssp* (K); green indicates reporter fluorescence; red, propidium iodide counterstain; scale bars, 20 µm.

the egg, leaving the endosperm without paternal contribution (*20*). Despite this, zygote elongation and suspensor development are similar to those in wild type (Fig. 1D), suggesting that *SSP* mRNA is not required in the endosperm.

The tight control of *SSP* expression implies that *YDA*-dependent signaling may be sensitive to the presence of SSP protein. Indeed, seedlings expressing *SSP* from a strong, broadly active promoter (*5*) exhibit a variety of dominant defects that eventually become lethal. Perhaps most strikingly, almost all primary transgenics completely lack stomata on their embryonic leaves (Fig. 4A), faithfully mimicking the phenotype caused by hyperactive *YDA* variants (*21*). This effect is dependent on the presence of a functional *SSP* variant in the construct as well as an intact endogenous *YDA* MAP kinase cascade (Fig. 4, B and C), revealing that SSP protein is sufficient to activate *YDA*-dependent signaling in the leaf epidermis. We propose that, by analogy, SSP protein translated from paternal mRNA triggers activation of the *YDA* MAP kinase cascade in the zygote. According to this model, *SSP* generates a temporal cue linking the onset of *YDA*-dependent signaling to fertilization. Turnover of the sperm-provided mRNA may also limit the duration of this signaling event. It remains to be determined through which mechanism such a temporal signature contributes to regulating the asymmetric first division and thus embryo polarity.

Maternally provided factors are of key importance for animal development because the





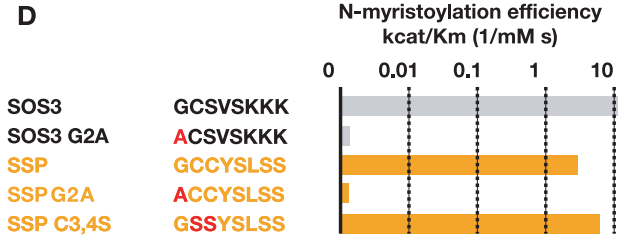
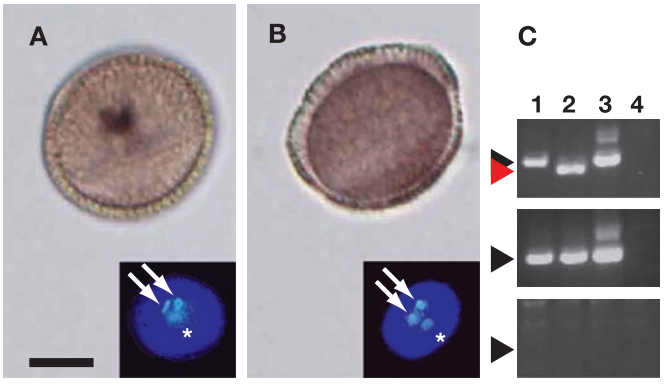


Fig. 2. Functional domains of SSP. (**A**) Gene model showing N-myristoylation motif (orange), kinase domain (red), and two complete (blue) and cryptic (light blue) TRP units; point mutations and insertion of mutant alleles are noted above; alternative splice form and introduced mutations are noted below. (**B** and **C**) Confocal images of root cells overexpressing YFP-tagged functional SSP (B) and a variant with mutated myristoyl-acceptor (C); green, YFP fluorescence; red, propidium iodide counterstain; arrow, cytoplasmic pocket containing nucleus; scale bar, 40 μm. (**D**) In vitro myristoylation of peptides (*9*) representing the N-termini of wild-type and mutant SSP variants by *Arabidopsis* NMT1; SOS3 peptides shown as control (*5*).



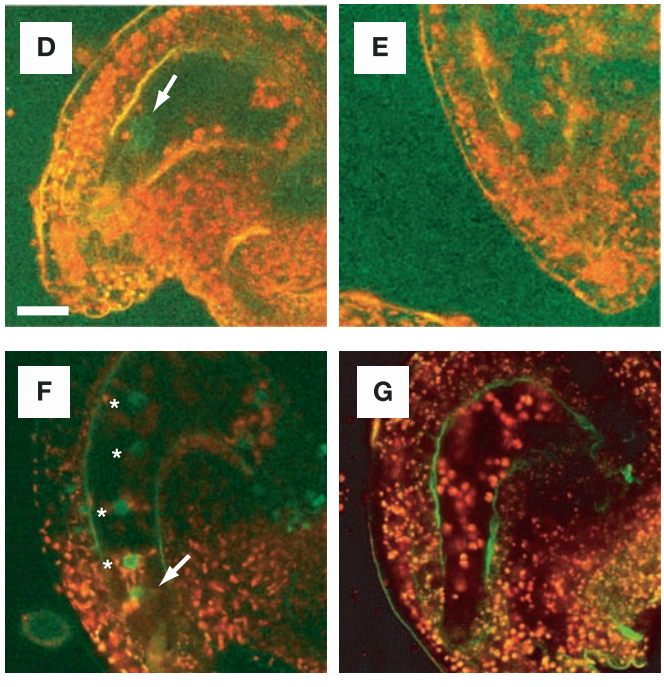


Fig. 3. Expression pattern of *SSP* transcripts and protein. (**A** and **B**) In situ hybridization of germinated wild-type (A) and *ssp*-2 (B) pollen. (Inset) 4′,6′-Diamidino-2-phenylindole (DAPI) staining identifying sperm nuclei (arrows) and the vegetative nucleus (asterisk). (**C**) Detection of paternal *SSP* mRNA in developing seed 24 hours after pollination (*5*); samples of seed from *Ws* pistils crossed with *Col* pollen (lane 1), *Ws* pollen (*2*), *Col* pollen (*3*), and unfertilized *Ws* pistils (*4*). (Top) RT-PCR products restricted with *Bcl*I, cleaving the *Ws* (red arrowhead) but not the *Col* (black arrowheads) allele; (middle) unrestricted products; (bottom) control without reverse transcriptase. (**D** and **E**) Confocal images of zygote-stage seed expressing a YFP-tagged functional SSP variant (D) or no transgene (E); arrow, weak YFP fluorescence of the zygote. (**F** and **G**) Confocal images of seed expressing a nuclear SSP reporter crossed via the pollen (F) or no transgene (G), about 24 hours after pollination; asterisks, endosperm nuclei; arrow, nucleus of zygote; green, YFP fluorescence, red, propidium iodide counterstain; scale bars, 20 μm.

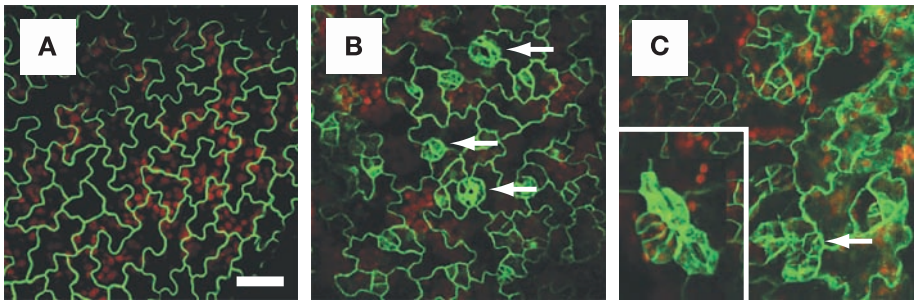


Fig. 4. Ectopic expression of SSP. (**A** to **C**) Confocal images of leaves producing a YFP-tagged functional SSP variant (A), a myristoylation-deficient variant (B), and a functional variant in a *yda* background (C); arrows, stomata; green, YFP fluorescence; red, chlorophyll fluorescence; scale bar, 20 μm. (Inset) Cluster of stomata progenitor cells, as typical for *yda*.

zygotic genome typically does not become active until some time after fertilization [maternal-zygotic transition (*22*)]. Plant embryos apparently develop with more autonomy (*23*), and it is open whether an equivalent to the maternal-zygotic transition of animals exists (*24*). The most prominent parent-of-origin effect in plants targets gene expression in the endosperm and is thought to arise from a parental conflict: Imprinting presumably enforces maternal control over nutrient allocation to the embryo (*25*, *26*). Direct delivery of transcripts to the seed would provide a general mechanism for subverting such epigenetic regulation. Thus, paternal control over growth of the suspensor, the other organ mediating nutrient flux to the embryo, perhaps evolved to antagonize maternal influences in this conflict.

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Preferential Generation of Follicular B Helper T Cells from Foxp3$^+$ T Cells in Gut Peyer's Patches

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Most of the immunoglobulin A (IgA) in the gut is generated by B cells in the germinal centers of Peyer's patches through a process that requires the presence of CD4$^+$ follicular B helper T (T_{FH}) cells. The nature of these T_{FH} cells in Peyer's patches has been elusive. Here, we demonstrate that suppressive Foxp3$^+$CD4$^+$ T cells can differentiate into T_{FH} cells in mouse Peyer's patches. The conversion of Foxp3$^+$ T cells into T_{FH} cells requires the loss of Foxp3 expression and subsequent interaction with B cells. Thus, environmental cues present in gut Peyer's patches promote the selective differentiation of distinct helper T cell subsets, such as T_{FH} cells.

The production and secretion of immunoglobulin A (IgA) by the host is critical for the maintenance of a vast community of commensal bacteria in the intestinal lumen with minimal penetration of the gut epithelium (*1*, *2*). Most of this IgA synthesis requires germinal center (GC) formation in Peyer's patches (PPs), aggregations of lymphoid follicles in the gut. In GCs, activated B cells express activation-induced cytidine deaminase (AID) and switch from making IgM to IgA (*3*–*5*). GC development in PPs requires bacteria in the gut; germ-free mice have small GCs, probably induced by bacterial components in food (*1*). T cells, by providing cytokines and costimulatory molecules to B cells, are also required for GC induction. Mice that lack T cells are devoid of GCs, and GC formation can be rescued by the adoptive transfer of CD4$^+$ T cells (*1*, *6*, *7*).

We investigated the origin of T_{FH} in PPs by examining the contribution of Foxp3$^+$CD4$^+$ T cells (*8*–*10*) obtained from Foxp3^{EGFP} reporter mice (*11*), which express green fluorescent protein (GFP) under the control of the Foxp3 promoter. Total CD4$^+$ T cells, Foxp3/GFP$^+$ CD4$^+$ T cells (hereafter called Foxp3$^+$ T cells) or Foxp3/GFP$^-$ CD4$^+$ (hereafter called Foxp3$^-$ T cells) that were isolated from the spleen and lymph nodes (LNs) of Foxp3^{EGFP} reporter mice were adoptively

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