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Zfrp8, the *Drosophila* ortholog of *PDCD2*, functions in lymph gland development and controls cell proliferation

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We have identified a new gene, *Zfrp8*, as being essential for hematopoiesis in *Drosophila*. Zfrp8 (Zinc finger protein RP-8) is the *Drosophila* ortholog of the PDCD2 (programmed cell death 2) protein of unknown function, and is highly conserved in all eukaryotes. *Zfrp8* mutants present a developmental delay, lethality during larval and pupal stages and hyperplasia of the hematopoietic organ, the lymph gland. This overgrowth results from an increase in proliferation of undifferentiated hemocytes throughout development and is accompanied by abnormal differentiation of hemocytes. Furthermore, the subcellular distribution of γ-Tubulin and Cyclin B is affected. Consistent with this, the phenotype of the lymph gland of *Zfpr8* heterozygous mutants is dominantly enhanced by the *l(1)dd4* gene encoding Dgrip91, which is involved in anchoring γ-Tubulin to the centrosome. The overgrowth phenotype is also enhanced by a mutation in *Cdc27*, which encodes a component of the anaphase-promoting complex (APC) that regulates the degradation of cyclins. No evidence for an apoptotic function of *Zfrp8* was found. Based on the phenotype, genetic interactions and subcellular localization of Zfrp8, we propose that the protein is involved in the regulation of cell proliferation from embryonic stages onward, through the function of the centrosome, and regulates the level and localization of cell-cycle components. The overproliferation of cells in the lymph gland results in abnormal hemocyte differentiation.

KEY WORDS: *Drosophila* hematopoiesis, Lymph gland hyperplasia, Centrosome

INTRODUCTION

The developmental mechanisms of human and *Drosophila* blood systems show remarkable parallels. In humans, several blood cell types with specific functions develop from the same pluripotent stem cells. In *Drosophila*, only a few specialized cell types exist, with functions similar to human cells. These are thought to originate from a common set of hematopoietic precursors. The development and specification of blood cells in humans and flies are controlled by conserved signaling pathways. Because of its relative simplicity, hematopoiesis in *Drosophila* is frequently used as a model to investigate the genetic control of hematopoiesis in flies and humans (reviewed by Evans et al., 2003; Hartenstein, 2006; Mandal et al., 2004; Meister, 2004; Orkin, 2000).

In *Drosophila*, mature hemocytes arise from two distinct sources: the mature larval circulating hemocytes derive from the embryonic head mesoderm, whereas the lymph gland hemocytes are normally released into circulation at the onset of metamorphosis and perdure into the adult stage (Holz et al., 2003; Jung et al., 2005; Lanot et al., 2001). As in vertebrate blood and vascular systems, the *Drosophila* lymph gland hemocytes and heart cells derive from a common progenitor, called the hemangioblast or cardiogenic mesoderm, which further splits into the lymph gland and cardiogenic progenitors (Mandal et al., 2004).

Among the earliest requirements for the specification of blood progenitors in mammals and *Drosophila* are the highly conserved, GATA zinc-finger transcription factors (Fujiwara et al., 2004; Mandal et al., 2004; Rehorn et al., 1996). The *Drosophila* GATA-factor Pannier (Pnr) is required for early specification of the hemangioblast/cardiogenic mesoderm. Another GATA-factor, Serpent (Srp), plays a central role in committing mesodermal precursors to the hemocyte fate (Mandal et al., 2004; Rehorn et al., 1996).

By the end of embryogenesis, the lymph gland is fully formed and contains mostly pro-hemocytes. The third instar larval lymph gland contains a pair of primary and several secondary lobes. Each primary lobe is subdivided into (1) the medullary zone, populated by slowly proliferating pro-hemocytes; (2) the cortical zone, containing differentiated hemocytes; and (3) the posterior signaling center (PSC), first defined as a small group of cells expressing the Notch ligand Serrate (Ser). Under the control of the EBF-homolog (early B-cell factor) *collier* (*col*; *knot* – Flybase), PSCs function as a hematopoietic niche to maintain a population of blood cell precursors (Krzemien et al., 2007; Lebestky et al., 2003; Mandal et al., 2007). The blood cell precursors differentiate into three groups of hemocytes: plasmatocytes, crystal cells and lamellocytes. All three are released into the open circulating hemolymph during the onset of metamorphosis or as a part of an immune reaction (Holz et al., 2003; Jung et al., 2005). Differentiated plasmatocytes and crystal cells are found in both the cortical zone of the lymph gland and the larval hemolymph, but lamellocytes are rare.

Plasmatocytes, the predominant form of hemocytes in larvae, perform phagocytic functions and secrete extracellular matrix components and immune peptides similar to human white blood cells. Crystal cells are non-adhesive hemocytes responsible for melanization during wound healing and encapsulation of parasites. Crystal cell differentiation requires the cell-autonomous expression of the transcription factor Lozenge (Lz), homologous to the mammalian acute myeloid leukemia 1 protein (Aml1 or Runx1) (Daga et al., 1996; Okuda et al., 2001).

Lamellocytes function in encapsulation. Their number is significantly increased at the onset of metamorphosis and in response to infection (Evans et al., 2003; Lavine and Strand, 2002; Meister, 2004; Rizki and Rizki, 1992). Differentiation of lamellocytes is connected to two major pathways – the *Drosophila* Toll/NF-κB and the JAK/STAT – that regulate blood cells

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proliferation and activation during immune response. Constitutive activation of either pathway leads to overproliferation of circulating and lymph gland hemocytes, an increase in lamellocytes and activation of the cellular immune response (Luo et al., 2002; Minakhina and Steward, 2006; Qiu et al., 1998).

We identified a new gene, *Zfrp8*, essential for lymph gland growth and for the normal development of *Drosophila* larvae. Mutant larvae show hyperplasia of the hematopoietic organs. This phenotype is not linked to apoptosis but rather to an increase in cell proliferation. Mutant lymph glands also show a drastic increase in the number of lamellocytes.

These phenotypes are suppressed by mutations in the GATA factor gene *pnr*. Mutations in the two cell-cycle genes *Cdc27* and *l(1)dd4* [*lethal (1) discs degenerate 4*], have the opposite effect as they enhance the lymph gland overgrowth phenotype of *Zfrp8*/+. *Cdc27* encodes a subunit of the APC complex, responsible for the turnover of cyclins, and *l(1)dd4* encodes Dgrip91, a component of the centrosome involved in γ-Tubulin anchoring. In the *Zfrp8* mutant lymph gland cells, both Cyclin B (CycB) and γ-Tubulin exhibit abnormal subcellular distribution, suggesting that Zfrp8 plays an important role in their regulation.

MATERIALS AND METHODS

Drosophila strains and collection of larvae

Fly stocks bearing the mutations $dronc^{2}$, $dronc^{51}$, $Su(var)205^{04}$ were kindly provided by Drs A. Bergmann (University of Texas, Houston, TX), J. Abrams (UT Southwestern Medical Center, Dallas, TX) and L. Wallrath (University of Iowa, Iowa City, IA) respectively. Flies carrying $UAS\text{-}Ras1^{V12}$, *hs-rpr*, *GMR-rpr*, *GMR-grim* and *GMR-hid* (*hid* is also known as *Wrinkled* – Flybase) were kindly provided by Drs E. Hafen (University of Zurich, Switzerland), H. Steller (The Rockefeller University, New York, NY) and K. White (Massachusetts General Hospital, Charlestown, MA). Other mutants used for genetic interaction experiments, and drivers were obtained from the Bloomington Stock Center (Bloomington, IN).

T(2;3)B3, CyO; TM6B, Tb^{1} and GFP-marked balancers were used to identify mutant larvae. Homozygous and hemizygous mutant larvae were collected on egg-laying plates and incubated with additional yeast on standard cornmeal/molasses food at 23°C until they reached the third instar larval stage. Because the *Zfrp8* larvae have a severe growth delay – they reach pupariaton 2-7 days after wild type – we collected the mutant larvae after their second molt and incubated for an additional 70 hours. The lymph glands of mutant animals were most similar to those of wild-type larvae grown 60 hours after the molt.

The C-terminally 3×HA-tagged (*UAS-Zfrp8-HA*) construct was made by PCR amplification of the *Zfrp8* coding region and subsequent cloning into the Gateway vector pDONR4 (Life Technologies). The gene was transferred into pPWH (Gateway, Carnegie Institution) and transgenic flies were created following standard protocols. This transgene expressed under the control of the Hsp70-GAL4 driver was able to rescue *Df(2R)SM206*.

Zfrp8 genetic interactions

Gain-of-function mutations (*M*) in the hematopoietic/immunity genes *Toll*, *hop* and *Ras1* (*Ras85D* – Flybase) were crossed into a heterozygous *Zfrp8* loss-of-function background (*Zfrp8/+;M/+*). The gain-of-function phenotypes including enlargement of the lymph gland, melanotic tumor formation and lethality were monitored (Asha et al., 2003; Govind, 1996; Luo et al., 2002; Minakhina and Steward, 2006; Qiu et al., 1998). Between 50 and 100 animals were examined for each genotype. Removal of one copy of *Zfrp8* did not affect any of the dominant phenotypes (see Table S1 in the supplementary material).

The second approach was based on the mild haplo-insufficient phenotype of *Zfrp8*/+ larvae. The lymph gland of *Df(2R)SM206*/+ larvae is on average twice (2.2±0.5) as large as that of wild type (Fig. 3A,C). We found that the smallest gland of 50 *Df(2R)SM206*/+ lymph glands analyzed was larger than the largest wild-type gland. To test genes for their ability to modify the lymph gland phenotype, loss-of-function mutations in genes reported to function in fly hematopoiesis, cell-cycle genes and genes with an established function in programmed cell death, were crossed with *Df(2R)SM206*/+. From each cross, 30 transheterozygous larvae at wandering stage were analyzed; the area of 2D images of 6-12 lymph glands (one pair of primary and two pairs of secondary lobes) were measured, using Adobe PhotoShop and normalized to the size of the wild-type gland (see Table S1 in the supplementary material).

Dominant suppressor of *Df(2R)SM206*/+ lymph gland overgrowth, pnr^{MD237}, was also tested for its ability to suppress the phenotype of *Df(2R)SM206* homozygous larvae. We found that pnr^{MD237} not only dominantly suppresses the lymph gland phenotype, but also improves the survival of mutants through the larval stages (Fig. 3B,F and see Table S1 in the supplementary material).

Because the apical caspase Dronc (also known as Nedd2-like caspase) is known to function in the induced apoptosis of blood cells (Chew et al., 2004), homozygous and hemizygous combinations of *dronc* and *Zfrp8* ($Zfrp8^{M\text{-}1\text{-}1}/Df(2R)206$; $dronc^{2}$/+ and $Zfrp8^{M\text{-}1\text{-}1}$/+; $dronc^{2}/dronc^{51}$) were tested. No modifications of the $Zfrp8^{M\text{-}1\text{-}1}/Df(2R)206$ $dronc^{2}/dronc^{51}$ phenotype were observed.

Immunostaining

For antibody staining, third instar larvae were dissected in phosphate-buffered saline (PBS) and lymph glands with adjacent pericardial cells, brain and discs were immediately transferred to a glass slide. Excess liquid was removed with tissue paper and the samples air dried for 30 seconds and then fixed at 80°C for 1 minute. The slides were kept at –70°C for several days until staining. The samples were additionally fixed for 40 minutes in 4% paraformaldehyde in PBST (PBS, 0.1% Tween 20) and washed several times in PBST. Antibody staining was performed essentially as described (Jung et al., 2005; Minakhina and Steward, 2006; Oegema et al., 1999). Between 20 and 40 larvae of each genotype were stained with each antibody.

Rat anti-Ser antibodies were obtained from Dr C. Irvine (Rutgers University, Piscataway, NJ). Anti-Hemese monoclonal antibody (H2), antibodies specific for lamellocytes (L1) and for plasmatocytes (P1) were obtained from Dr I. Ando (Biological Research Center, Szeged, Hungary) and used at 1:500 dilution. Rabbit polyclonal anti-H3P antibody (Upstate, Lake Placid, NY, 1:1000), rabbit anti-HA (Sigma, St Louis, MO, 1:1000), mouse anti-HA (Roche, 1:100) and mouse anti-γ-Tubulin (Sigma, St Louis, MO, 1:100) were used. Mouse monoclonal anti-Cyclin B (F2F4) and anti-Cyclin A (A12) antibodies developed by Drs C. Lehner (University of Bayreuth, Bayreuth, Germany) and P. O'Farrell (University of California, San Francisco, CA) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA) and used 1:120. Rabbit anti-Dgrip84 and anti-Dgrip91 were kindly provided by Dr M. Moritz (UCSF, San Francisco, CA), and anti-CP309 by Drs D. Ducat and Y. Zheng (Carnegie Institution of Washington/HHMI, Baltimore, MD) and used 1:1000. Rabbit anti-active Caspase 3 (BD Biosciences, San Jose, CA) and anti-Srp were obtained from Dr K. Hoshizaki (University of Nevada, Las Vegas, NV) and used at 1:1500.

Secondary goat anti-mouse Cy3, goat anti-rat Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) and Alexa Fluor 488 donkey anti-rabbit antibodies (Molecular Probes, Eugene, OR) were used at 1:500. DNA was stained using Hoechst 33258 (Molecular Probes, Eugene, OR). Samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and examined with a Zeiss Axioplan-2 microscope. Hematopoietic nodule images were captured using a Leica DM IRBE laser scanning confocal microscope. The images were analyzed with Image Pro Plus and Leica Microsystems software and further processed using Adobe PhotoShop.

Analysis of cell cycle in lymph glands

To determine the S-phase index, BrdU incorporation was performed as previously described (Jung et al., 2005). Immediately upon fixation, lymph glands were stained with mouse monoclonal anti-BrdU antibody (Becton Dickinson, Franklin Lakes, NJ, 1:100 dilution). The number of positive nuclei per field was counted, and the ratio of BrdU-positive nuclei to the total number of nuclei was calculated. This ratio was averaged over at least ten fields from different lobes of lymph glands of five larvae (more than 2000 cells counted for each genotype).

To count the number of centrosomes, we stained cells with anti-CP309, anti-Dgrip91 and anti-Dgrip84 antibodies (Kawaguchi and Zheng, 2004; Oegema et al., 1999). We captured a series of confocal sections through one layer of lymph gland cells (about 5 μm) at ×63 magnification, and counted the number of cells with one or two centrosomes using 3D projection. Cells in at least five independent fields were counted for each marker. All three markers gave similar results.

RESULTS

PDCD2/Zfrp8 is a highly conserved protein

We isolated *Zfrp8* in an ethylmethansulfonate (EMS) screen for alleles of NF-κB pathway genes and pursued it because of its hematopoietic phenotype. *Zfrp8* encodes a conserved protein, called PDCD2 in mammals, that has not been functionally characterized so far. Like the mammalian gene, *Zfrp8* is transcribed at low levels and is ubiquitously expressed in embryos and the majority of tissues (Mihola et al., 2007; Rebhan et al., 1997) (data not shown).

PDCD2/Zfrp8 is present in eukaryotes from yeast to humans. The mammalian proteins are more than 80% identical, and the identity between the fly and the human orthologs is ~40%, distributed along the entire protein (see Fig. S1 in the supplementary material). The C-terminal half of PDCD2/Zfrp8 proteins is called the PDCD2(C) domain (Chen et al., 2005). It is present only in PDCD2/Zfrp8 orthologs and in one predicted protein in vertebrates and flies. It has not been functionally characterized and might not represent a bone fide domain.

The zinc-finger domain, named MYND because of the conservation of its general structure in the myeloid tumor gene 8 (also known as MTG8, ETO and RUNX1T1), the *Drosophila* proteins Nervy and in Deaf1 (Gross and McGinnis, 1996), is also present in a number of other transcriptional regulators including MTGR1, NUDR and BS69 (also known as CBFA2T2, DEAF1 and ZMYND11, respectively). In these proteins, MYND fosters protein-protein interaction and recruits co-repressors (Ansieau and Leutz, 2002; Ibanez et al., 2004; Jensik et al., 2004; Lutterbach et al., 1998). The sequence adjacent to the MYND domain (see Fig. S1 in the supplementary material, underlined) has been shown to interact with Host cell factor 1 (also known as HCF-1 and HCFC1), an important regulator of cell cycle (Mahajan and Wilson, 2000; Scarr and Sharp, 2002). We find that *Drosophila* Hcf and Zfrp8 also interact in vitro in a manner similar to the human and worm orthologs (results not shown).

Zfrp8 is essential for fly hematopoiesis

To investigate the in vivo function of *Zfrp8*, we used two previously described deficiencies, *Df(2R)SM206* and *Df(2R)SM183* (Minakhina et al., 2003) and the new EMS allele, $Zfrp8^{M\text{-}1\text{-}1}$. The deficiencies were originally obtained by mobilizing a P-element located at the 5′ end of the *Zfrp8* protein-coding region. Whereas the larger deletion, *Df(2R)SM183*, abolishes expression of the two genes *tamo* and *Zfrp8*, the smaller deletion, *Df(2R)SM206*, does not affect the expression of the neighboring *tamo* gene (Minakhina et al., 2003). The EMS allele, $Zfrp8^{M\text{-}1\text{-}1}$, was isolated as a lethal over both deficiencies. More than 90% of $Zfrp8^{M\text{-}1\text{-}1}$ hemizygous and homozygous late third instar larvae produce melanotic masses that are seen as black spots outlining the shape of the lymph glands in pupae (see Fig. S2B,D in the supplementary material). In $Zfrp8^{M\text{-}1\text{-}1}$, the absolutely conserved glutamic acid 296 is changed to a lysine (E296K; see Fig. S1 in the supplementary material).

Homozygotes for the *Zfrp8*-null allele, *Df(2R)SM206*, show a severe growth and developmental delay and most animals die in larval stages. Few animals survive until pupariation and are smaller than their heterozygous siblings. About 2% of homozygous *Df(2R)SM206* escapers survive until adulthood, but have poor viability and female fertility. $Zfrp8^{M\text{-}1\text{-}1}$ homozygous and hemizygous larvae show ~5% adult survival.

All *Zfrp8* larvae have an enormous overgrowth of the hematopoietic organs, the lymph glands. The mutant lymph glands are at least 25 times the size of the organ of wild-type third instar

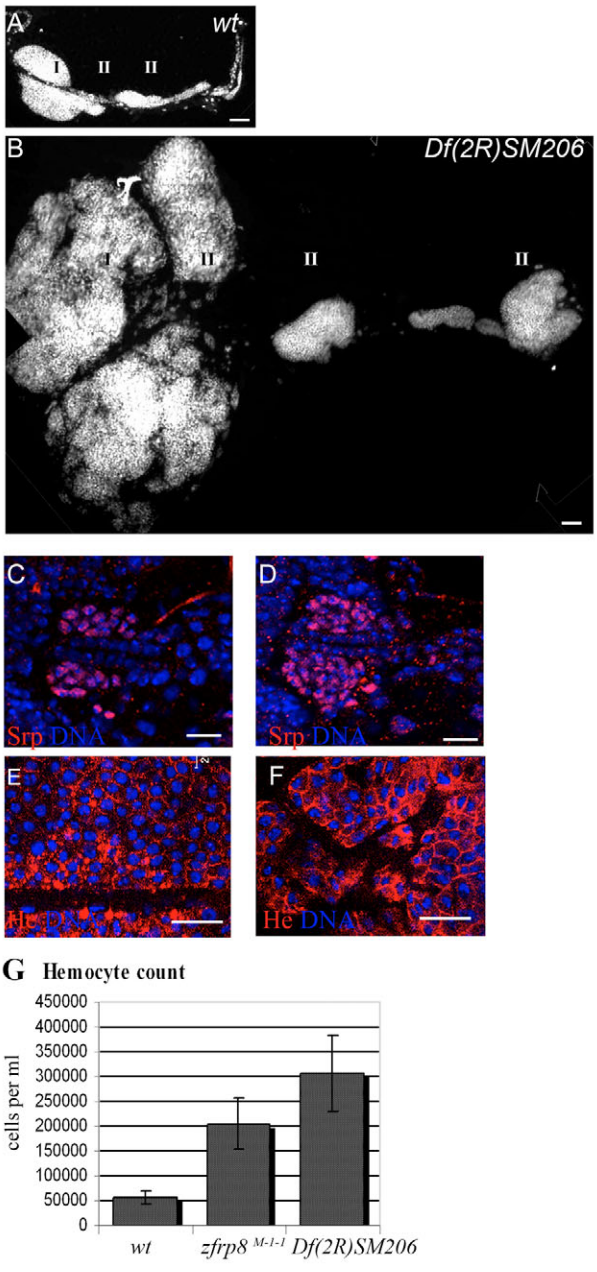

Fig. 1. The *Zfrp8* lymph gland phenotype. (**A**,**B**) Wild-type (A) and *Df(2R)SM206*/ *Df(2R)SM206* (B) *Drosophila* lymph glands. Primary lobes are marked with I, secondary lobes with II. (**C-F**) Confocal crossections of 16- to 17-stage wild-type (C) and a *Zfrp8*-null (D) embryos stained with anti-Srp antibody (Srp) show the early increase in size of mutant lymph glands. Wild-type (E) and *Zfrp8*-null (F) larval lymph glands stained with anti-Hemese antibody (He). (**G**) Hemocyte counts in wild-type and in *Zfrp8* and *Df(2R)SM206* mutant larvae. Hemocytes were counted from at least ten third instar larvae of each genotype. Scale bars: 70 μm in A,B; 15 μm in C,F.

larvae. In developmentally delayed *Zfrp8* animals, it could grow to up to 70 times the normal size (Fig. 1A,B, and see Fig. S2A,B in the supplementary material). Other organs were often reduced in size proportional to the size of the larvae. *Zfrp8* was also found to have a mild haplo-insufficient phenotype; the lymph glands of *Df(2R)SM206/+* larvae were about twice (2.2±0.5) the size of the average wild-type gland (Fig. 3, and see Table S1 in the supplementary material).

Lethality, growth delay and the lymph gland overgrowth phenotypes of *Df(2R)SM206*, $Zfrp8^{M\text{-}1\text{-}1}$/*Df(2R)SM206* and $Zfrp8^{M\text{-}1\text{-}1}$/*Df(2R)SM183* are efficiently rescued by a wild-type UAS-*Zfrp8* transgene expressed under the control of the ubiquitous promoter, *arm-GAL4* (Minakhina et al., 2003). Up to 90% of mutant animals expressing the transgene survived and were fertile. The size of their lymph glands was about two to three times the size of that of wild type (2.8±1.0). It is possible that the lymph gland phenotype would be rescued to wild-type size if the transgene were expressed at higher levels specifically in the gland. Alternatively, the growth of the gland could be controlled extrinsically.

We found that the organization and location of the *Zfpr8* mutant lymph glands were similar to those of wild-type larvae. The relative proportions of primary and secondary lobes (marked I and II in the figures) were preserved and all lobes were overgrown (Fig. 1A,B, Fig. 2 and see Fig. S2A,B in the supplementary material). Anti-Hemese (He) antibody (Kurucz et al., 2003) recognized the plasma membrane-associated protein specific for pro-hemocytes and hemocytes in both wild-type and mutant organs (Fig. 1E,F). Anti-Srp antibody (Sam et al., 1996) staining showed that *Zfpr8* lymph glands are already twice as large as wild-type glands by stage 16 to 17. The number of Srp-positive cells in wild-type organs varied from 19 to 25, whereas *Df(2R)SM206* homozygous organs contained 35-50 cells (Fig. 1C,D). During the 5 days of larval growth and development, the size difference between wild-type and mutant lymph glands increased exponentially, and in third instar mutant larvae the glands virtually filled the mid-anterior part of the larva.

The *Zfrp8* phenotype is completely different from that observed in mutants of genes functioning in the immune response. In mutants with constitutively induced immunity, such as in *Toll* or *JAK/STAT* (*hop/Stat92E*) gain-of-function mutants, the number of circulating hemocytes is markedly increased (Luo et al., 2002; Minakhina and Steward, 2006; Qiu et al., 1998). By contrast, in *Zfrp8* larvae, the number of cells in the lymph glands was dramatically increased, but the number of circulating hemocytes was only about six times higher than in the wild type (Fig. 1A,B,G).

Hemocyte differentiation in *Zfrp8* mutants

We further defined the role of *Zfrp8* in hemocyte differentiation by staining mutant and wild-type lymph glands with antibodies recognizing specific markers. These antibodies were for plasmatocytes (P1); lamellocytes (L1); crystal cells (anti-Lozenge); and specific for the PSC (anti-Ser) (Lebestky et al., 2003). Despite the increase in the number of the plasmatocytes, their proportion in the mutant lymph glands was similar to that in wild type (Fig. 2C,D). Crystal cells, positive for anti-Lozenge staining (Jung et al., 2005), were also increased proportionally to overgrowth and were distributed similar to those in wild type (data not shown). By contrast, the number of L1-positive pro-lamellocytes or lamellocytes was drastically increased in the mutant (Fig. 2F). In wild-type lymph glands and hemolymph, lamellocytes are rare (<0.5% of hemocytes, Fig. 2E) (Holz et al., 2003; Jung et al., 2005; Sorrentino et al., 2002), but in mutant glands the percentage of L1-positive cells varied from 5% to 60% and they were not restricted to the cortical zone of the primary lymph gland lobes, but were found in the presumptive medullary zone and in secondary lobes. The number of circulating lamellocytes varied from very few to several hundred per larva (>10% hemocytes). The increase in the number of lamellocytes

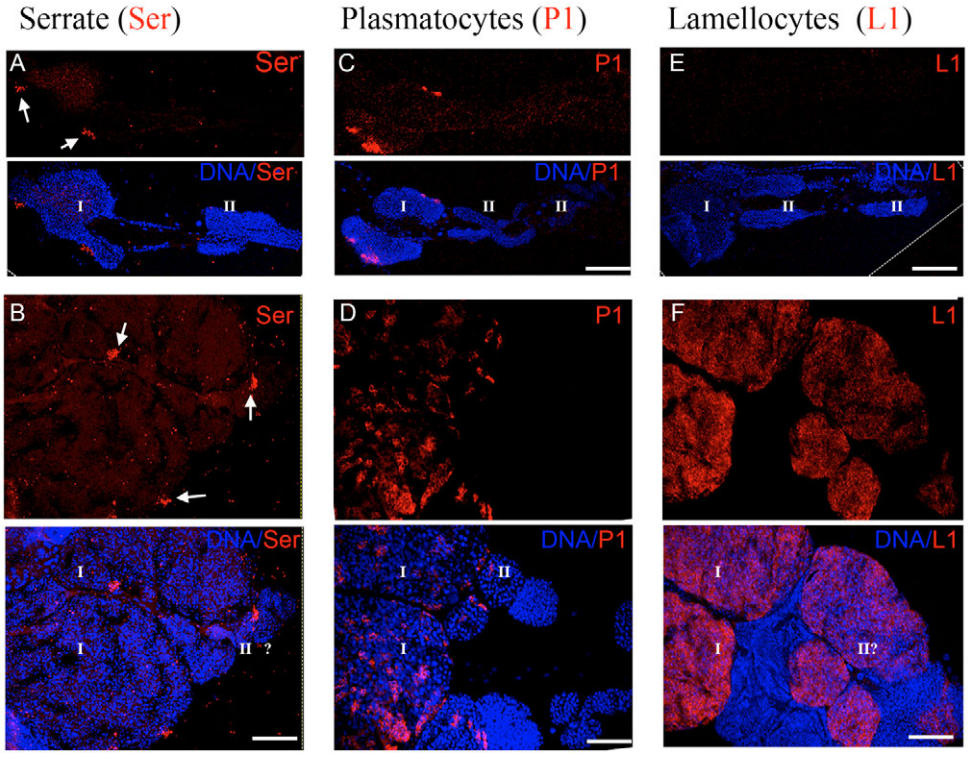

Fig. 2. The mutant lymph gland contains differentiated hemocytes. Confocal images of wild-type (**A**,**C**,**E**) and *Df(2R)SM206* (**B**,**D**,**F**) *Drosophila* lymph glands stained with (A,B) anti-Ser to locate PSCs, (C,D) P1 antibodies as a plasmatocyte marker, and (E,F) L1 antibodies to mark lamellocytes. Scale bars: 150 μm.

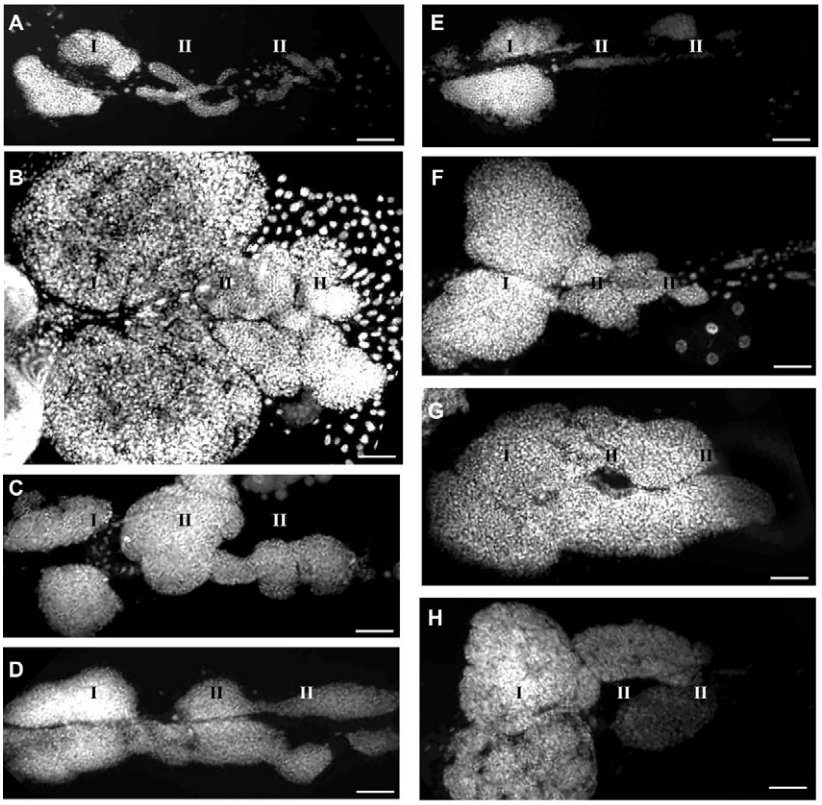

Fig. 3. *Drosophila* lymph gland size is dependent on the dose of Zfrp8. Lymph glands from (**A**) wild-type, (**B**) *Df(2R)SM206*, (**C**) *Df(2R)SM206*/+, (**D**) *Df(2R)SM206/Ark*^{k11502}, (**E**) *Df(2R)SM206*/+, *pnr*^{MD237}/+, (**F**) *Df(2R)SM206, pnr*^{MD237}/+, (**G**) *Df(2R)SM206*/+, *Cdc27*^{L7123}/+ and (**H**) *l(1)dd4*^{2}/+, *Df(2R)SM206*/+ third instar wandering larvae stained with Hoechst 33258 for DNA. Scale bars: 70 μm.

reflects reprogramming of pro-hemocytes, similar to what is observed in response to parasitization or during metamorphosis (Krzemien et al., 2007).

Despite the increase in lamellocyte differentiation, the overgrown *Zfrp8* lymph glands always contained a pool of undifferentiated hemocytes. Some of these undifferentiated cells were found in primary lobes. In the secondary lobes, the number of undifferentiated cells increased with the posterior localization of the lobes.

The size and appearance of the PSC was similar in *Zfrp8* and wild-type late third instar larvae. In the wild-type, small islands of Ser-positive hemocytes were located at the posterior of each primary lobe (Fig. 2A,B) (Jung et al., 2005). More than half of the mutant organs analyzed had three to five Ser-positive islands (Fig. 2B), but the number of positive cells was not massively increased. The number of Ser-positive islands were proportional to the size of the gland, suggesting that the increase in hemocytes results in the formation of the additional PSCs, possibly by splitting the islands during growth.

Zfrp8 genetically interacts with *pannier*, which encodes a GATA factor essential for lymph gland development

To investigate connections between *Zfrp8* and genes known to function in *Drosophila* hematopoiesis, we took advantage of the dominant lymph gland phenotype observed in *Df(2R)SM206*/+ larvae, and looked for dominant modification of this phenotype in transheterozygous larvae (see Material and methods; Fig. 3A,C). We also investigated whether loss of one copy of *Zfrp8* modified the phenotypes caused by specific gain-of-function mutations. Mutations in 14 genes were tested (for full results see Table S1 in the supplementary material). Although most mutations did not modify the *Df(2R)SM206*/+ lymph gland phenotype, two alleles of *pnr*, *pnr*^{VX6} and *pnr*^{MD237}, dominantly suppressed the lymph gland overgrowth of *Df(2R)SM206*/+ (Fig. 3E). Moreover, in *Df(2R)SM206/Df(2R)SM206; pnr*^{MD237}/+ larvae, the lymph glands were significantly smaller than in *Df(2R)SM206/Df(2R)SM206;* +/+ larvae (Fig. 3B,F).

Pnr functions in the specification of the hemangioblasts, mesodermal cells giving rise to both cardiac and lymph gland cells (Mandal et al., 2004). Zfrp8 might function with Pnr, a transcription factor, in controlling the early establishment of the lymph gland. The interaction of *Zfrp8* with *pnr* is not restricted to embryonic development because the dose reduction of *pnr* not only suppresses the lymph gland phenotype, but also increases the larval survival rate of *Zfrp8* mutants, suggesting that the two proteins also function together at other stages of development and in other tissues.

These genetic studies, in which we failed to detect an interaction of *Zfrp8* with genes functioning in hemocyte differentiation, suggest that *Zfrp8* regulates proliferation of blood cells prior to differentiation.

Role of *Zfrp8* in the regulation of cell proliferation

The overgrowth phenotype suggests that *Zfrp8* is essential for controlling cell death of hematopoietic precursor cells or, alternatively, the number of cell divisions. To investigate these possibilities we performed genetic interaction assays with mutants in 14 genes with an established function in programmed cell death or involved in the cell cycle (see Table S1 in the supplementary material). Mutations in two genes involved in cell-cycle regulation, *Cdc27* (*Cdc27*^{L7123}) and *l(1)dd4* (*l(1)dd4*^{1} and *l(1)dd4*^{2}), showed dominant enhancement of the *Df(2R)SM206*/+ lymph gland phenotype, at least doubling the size of the gland. The resultant (*Df(2R)SM206*/+; *Cdc27*^{L7123}/+ and

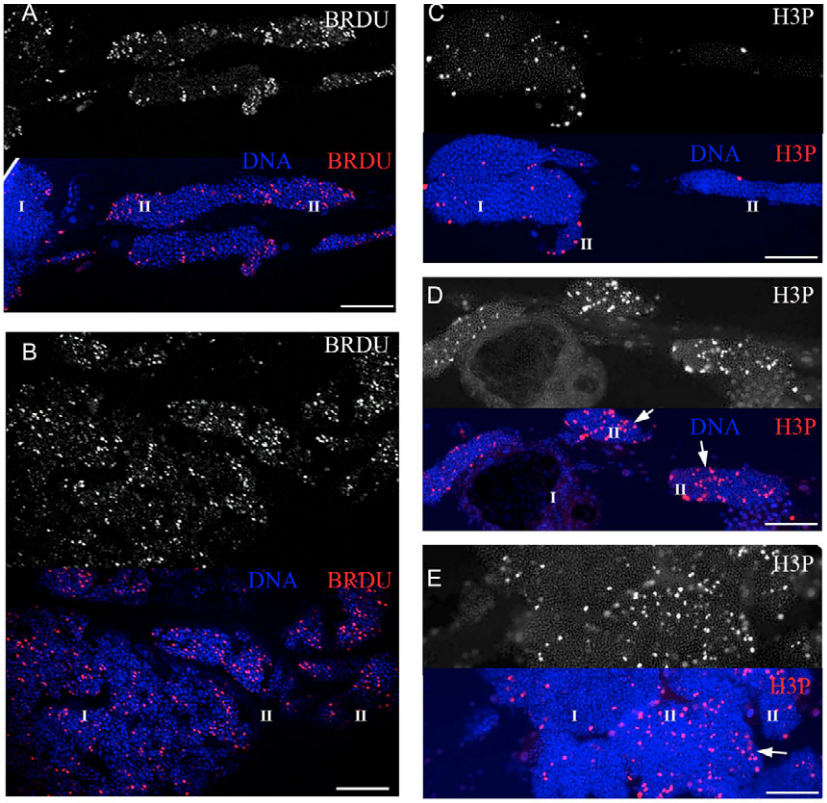

Fig. 4. Cell proliferation in wild-type and mutant *Drosophila* lymph glands. (**A**,**B**) Lymph glands from wild-type (A) and *Df(2R)SM206* (B) mid-third instar larvae stained for cells in S phase. Confocal section from the middle of the organ shows BrdU incorporation in cells of primary (I) and secondary (II) lobes. (**C-E**) Mitotic cells in the lymph glands from wild-type (C), *Zfrp8*$^{M\text{-}1\text{-}1}$ (D) and *Df(2R)SM206* (E) mid-third instar larvae. Projection through 10 μm section of the organ shows cells with condensed DNA, stained with anti-H3P antibodies. Arrows point to areas with high mitotic activity. Scale bars: 70 μm.

l(1)dd4^{2}*/+;Df(2R)SM206/+*) lymph glands were five times the size of wild-type lymph glands (Fig. 3G,H, and see Table S1 in the supplementary material). This demonstrates that the *Df(2R)SM206/+* lymph gland size is sensitive to the dosage of genes involved in the cell cycle and links *Zfrp8* itself to the cell cycle.

We monitored the progression through the cell cycle in *Zfrp8* lymph glands. We surveyed entry into S phase by measuring BrdU incorporation (Fig. 4A,B). In wild-type lymph glands, S-phase cells were distributed throughout the lymph gland until late third larval instar (Fig. 4A), when proliferating cells become largely restricted to the cortical zone of the primary lobes (Jung et al., 2005). In mutant glands, the proportion of cells in S phase was about 1.3 (25±2%) times greater than in wild type (20±2%). As in wild type, BrdU-positive cells were found in all lobes.

We next evaluated the mitotic index (mitotic cells/total cells) in wild-type and mutant glands by staining of histone H3 phosphorylated at serine 10 (H3P), which is specific to mitosis (Fig. 4C-E). The average mitotic index in the entire lymph glands increased from 6±1% in wild type to an average of 8.5±1% in *Df(2R)SM206/Df(2R)SM206* and *Zfrp8* $^{M\text{-}1\text{-}1}$*/Zfrp8* $^{M\text{-}1\text{-}1}$. Overall, the mutant tissue showed a mild increase in cell proliferation, but the continuous growth increase over a period of several days converted the slightly increased gland observed in embryos into the massively overgrown lymph gland observed in third instar larvae.

In *Zfrp8* mutants, the increase in mitotic cells was concentrated in secondary lymph gland lobes (II) that usually contain less differentiated hemocytes. Such areas had greater than 10% of cells in mitosis, indicating actively growing regions (Fig. 4D,E arrows). Primary lobes (I) usually had fewer mitotic cells.

In *Drosophila*, accumulation of Cyclins A and B (CycA and CycB) is detected in cells from late S/G2 to prophase and anaphase, respectively (Huang and Raff, 1999; Parry and O'Farrell, 2001). Our staining experiments showed that CycA and CycB are abundant in wild-type brain and discs (Fig. 5). CycA was also detected in the lymph glands, whereas CycB was virtually absent from the glands (Fig. 5A,C,D,G,H). In mutant lymph glands, CycB was dramatically increased. The protein was detected in the cytoplasm of more than 50% of cells and was particularly abundant in ~25% (Fig. 5B,E,F). However, in mutant brains, the CycB level was comparable to that in wild type. The CycA level and distribution were similar in mutant and wild-type glands and brains (Fig. 5G-J).

The apparent high levels of CycB in many cells of mutant glands raised the question of whether it reflects an increase in the number of cells in G2. We visualized centrosomes using antibodies to γ-Tubulin and two other components of the γ-Tubulin ring complex (γ-TuRC), *Drosophila* gamma-ring proteins (GRIPs) Dgrip84 (Grip84 – Flybase) and Dgrip91 (Fig. 6 and see Fig. S3 in the supplementary material) (Colombie et al., 2006; Oegema et al., 1999). We also stained the centrosome protein CP309, which is required for microtubule nucleation (Kawaguchi and Zheng, 2004). The number of centrosomes is indicative of G1 (one centrosome) and G2 (two). We counted cells containing one and two foci of Dgrip84, Dgrip91 or CP309 in lymph gland lobes and found that the ratio of cells before and after chromosome duplication was about 1:1 in wild-type and mutant glands, suggesting that the ratio of G1/G2 cells in mutants and wild type is the same.

The number of centrosomes and appearance of Dgrip91, Dgrip84 and CP309 were similar in mutant and wild-type lymph gland cells, but the key γ-TuRC component, γ-Tubulin, showed abnormal distribution in *Zfrp8* mutants (Fig. 6A-D, and see Fig. S3 in the supplementary material). Multiple γ-Tubulin foci that did not overlap with other centrosomal markers were observed. The phenotype suggests that Zfrp8 is involved in γ-Tubulin recruitment to centrosomes (Fig. 6C,D, and see Fig. S3 in the supplementary

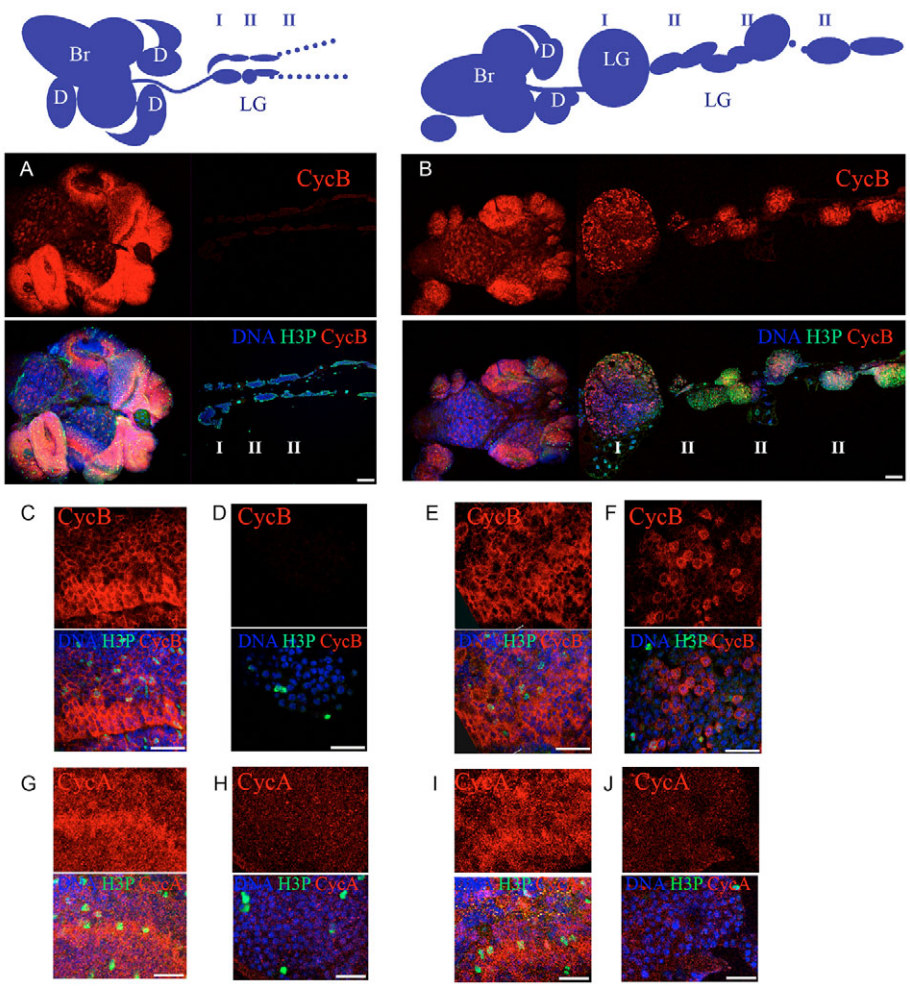

Fig. 5. Distribuiton of CycA and CycB in *Zfrp8* mutants. Comparative analysis of CycA and CycB distribution in (**A**,**C**,**D**,**G**,**H**) wild-type and (**B**,**E**,**F**,**I**,**J**) *Df(2R)SM206 Drosophila* lymph glands and brains. The combined confocal fields (A,B) show larval brain with adjacent discs and lymph glands (partial) stained with anti-CycB, anti-H3P and Hoechst 33258. The schematics depict the tissues shown in the panels beneath. Br, brain; D, imaginal discs; I,II, primary and secondary lobes, respectively, of lymph glands (LG). High magnifications of brain (C,G,E,I) and lymph gland secondary lobes (D,H,F,J) show differences in CycB (C-F) and CycA (G-J) distribution. (*Df(2R)SM206* and *Zfrp8*$^{M\text{-}1\text{-}1}$ give similar results.) Scale bars: 70 μm in A,B; 18 μm in C,J.

material). This phenotype is consistent with our genetic finding that the lymph gland overgrowth of *Zfpr8* is dominantly enhanced by the mutation in *l(1)dd4*, which encodes Dgrip91 involved in γ-Tubulin centrosome anchoring.

Zfrp8 and centrosomes

To determine the subcellular localization of Zfrp8, we expressed an HA-tagged protein in larval tissue under the control of CG(collagen)-Gal4 and hsp70-GAL4 drivers. Both drivers gave similar results. Zfrp8 was mostly found in the cytoplasm. In discs and lymph glands, the Zfrp8 foci were adjacent to γ-Tubulin and Dgrip84 foci at centrosomes (Fig. 6E,F). We also observed similar localization of human PDCD2 in ML-1 cultured cells (data not shown). These results suggest that *Zfrp8* might function with the centrosomes and participate in anchoring proteins to this organelle.

DISCUSSION

Zfrp8 as a developmental regulator of hematopoiesis

In the literature, the Zfrp8 vertebrate ortholog, PDCD2, is routinely referred to as an apoptotic gene solely because it was upregulated during steroid-induced programmed cell death in rat thymocytes (Owens et al., 1991). Subsequent studies, using different cells and assay conditions, found no connection between PDCD2 expression and programmed cell death (Chen et al., 2005; Fan et al., 2004; Kawakami et al., 1995; Steinemann et al., 2003).

It is unlikely that a reduction in cell death is the cause of the lymph gland overgrowth observed in *Zfrp8* mutant larvae. Very few or no apoptotic cells are detected in wild-type larval lymph glands (Chiu and Govind, 2002; Jung et al., 2005). We found a statistically insignificant increase in the number of apoptotic cells in *Zfrp8* mutants (see Fig. S4 in the supplementary material). No other evidence of change in programmed cell death in *Zfrp8* mutant animals, no increase in apoptotic gene expression, no change in caspase cleavage (data not shown) and no genetic interaction of *Zfrp8* with known apoptotic genes (and see Table S1 in the supplementary material) were found.

Our results are consistent with an increase in cell division in *Zfrp8* mutants throughout development. This conclusion is supported by the observation that *Zfrp8* lymph glands are already twice the size of their normal counterparts in late-stage embryos, and that the number of cells in mitosis is about 30% higher in the mutant glands than in wild type.

Detailed analysis of *Zfrp8* lymph glands shows that its phenotype is different from that of *Drosophila* hematopoietic/immunity mutants. Unlike hematopoietic/immunity mutants, the increase in lymph gland cell numbers is much larger than the increase in circulating hemocytes. Furthermore, the blood cell overproliferation in *Zfrp8*-null mutants is not accompanied by constitutive activation of immunity. *Zfrp8* larvae show normal induction of immune peptide genes in response to bacterial challenge (see Fig. S2E in the supplementary material) and normal wound clogging and wound

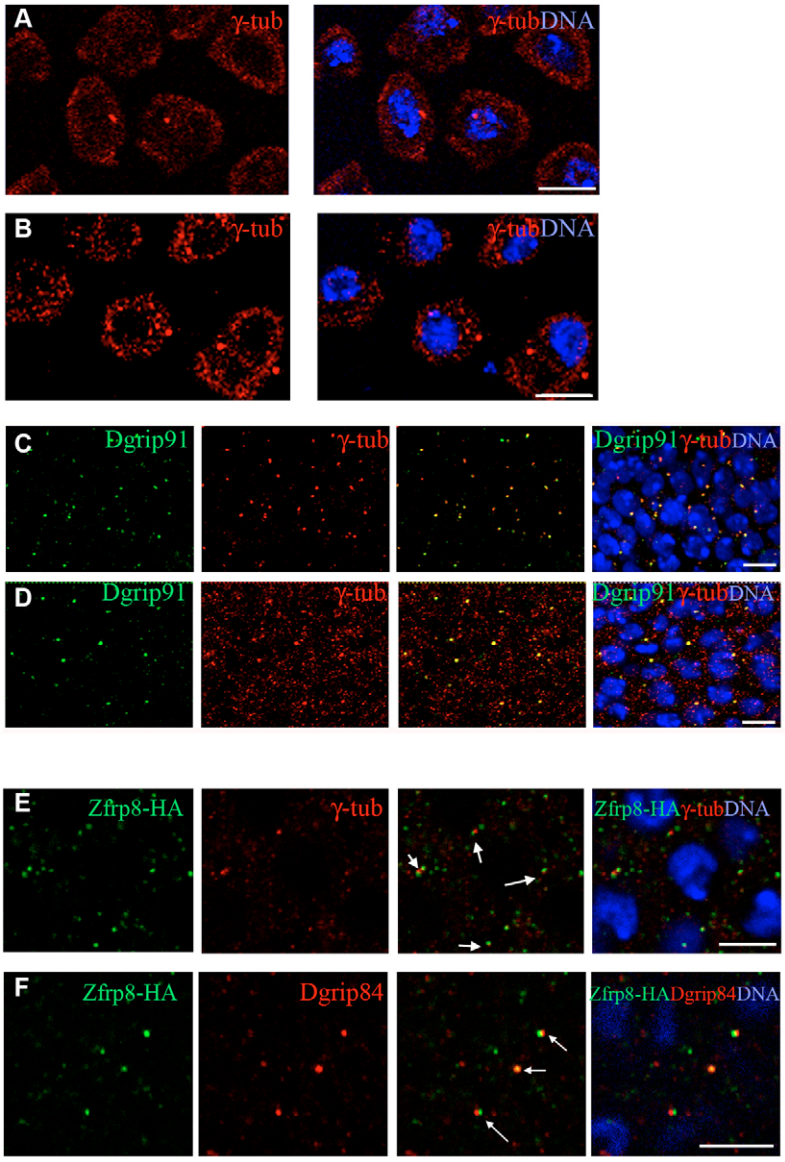

Fig. 6. *Zfrp8* and centrosome organization. Confocal projection through one layer of lymph gland cells from squashed (**A**,**B**) and intact (**C-F**) lymph glands of wild-type (A,C,E) and *Zfrp8* mutant (B,D,F) *Drosophila*. (*Df(2R)SM206* and *Zfrp8*$^{M-1-1}$ give similar results.) Cells were stained with anti-γ-Tubulin (A-E), anti-Dgrip91 (C,D) and anti-Dgrip84 (F) to visualize centrosomes. Confocal sections of lymph glands (E,F) show Zfrp8-HA adjacent to centrosmes (arrows). DNA is stained with Hoechst 33258. Scale bar: 5 μm.

melanization (not shown). That the requirements are different for *Zfrp8* and known hematopoiesis and immunity genes is underlined by the absence of their genetic interaction (and see Table S1 in the supplementary material).

In normal lymph glands, plasmatocytes are found mostly in the cortical region and very few lamellocytes are detected. The PSC is formed at the base of each primary lobe. The presence of additional PSCs in mutant lymph glands might indicate that additional primary lobes are formed by the large number of cells.

Two recent papers report that the PSCs are essential for maintaining the undifferentiated hemocyte population in the medullary zone and that they control lamellocyte differentiation during parasitic infection (Krzemien et al., 2007; Mandal et al., 2007). Lack of the transcription factor *collier*, essential for PSC maintenance, leads to a decrease in the pro-hemocyte population and abolishes lamellocyte differentiation. Loss of *Zfrp8* leads to the opposite phenotype – an increase in pro-hemocyte proliferation, beginning during embryogenesis, and an increased number of cells acquiring the lamellocyte fate. Expansion of the PSCs alone does not account for this phenotype. Ectopic expression of the homeotic gene *Antennapedia* results in expansion of the PSCs, and a concomitant increase of the medullar zone, but not the gland overgrowth (Mandal et al., 2007). Therefore, it is unlikely that *Zfrp8* is directly involved in the establishment of PSCs.

Zfrp8 and the cell cycle

Our results link the *Zfrp8* overgrowth phenotype to a defect in normal cell proliferation. In mutant lymph glands, the cell-cycle markers γ-Tubulin and CycB are misregulated. *Zfrp8* genetically interacts with at least two genes functioning in the cell cycle, *Cdc27* encoding a subunit of the anaphase-promoting complex (APC), and *l(1)dd4* encoding the *Drosophila* gamma-ring protein Dgrip91 (Barbosa et al., 2000; Oegema et al., 1999).

Dgrip91 and γ-Tubulin are components of the γ-TuRC microtubule-nucleating complex anchored to centrosomes. Beyond the conventional role in microtubule organization, centrosomes also serve as a scaffold for anchoring a number of cell-cycle regulators. For instance, centrosome-association of Cdc27 and CycB proteins plays an important role in CycB activation, degradation and entrance into mitosis (Debec and Montmory, 1992; Jackman et al., 2003; Kramer et al., 2004; Wakefield et al., 2000).

The link between the phenotypes described above and *Zfrp8* function became clear when we discovered that a proportion of Zfrp8 protein localizes adjacent to the centrosome in wild-type tissue. This subcellular localization is consistent with a function of Zfrp8 in centrosome organization and in the anchoring of proteins such as γ-Tubulin and CycB to this organelle.

Zfrp8 might also affect the expression of bona fide cell-cycle regulators. The protein contains a zinc-finger domain, MYND, present in a number of transcriptional regulators, that fosters protein-protein interactions and recruits co-repressors (Ansieau and Leutz, 2002; Ibanez et al., 2004; Jensik et al., 2004; Lutterbach et al., 1998). PDCD2/Zfrp8 is known to interact with the HCF-1 transcriptional regulator, which suggests that PDCD2/Zfrp8 might be involved in regulating the cell cycle at the transcriptional level.

Zfrp8 might have a dual function, through its association with the centrosome and as a transcriptional regulator of the cell cycle. Several transcriptional regulators have been found to localize to the centrosome, but their centrosomal function has not been documented (Andersen et al., 2003; Doxsey et al., 2005; Hsu and White, 1998).

Zfrp8 function is essential for the control of cell proliferation already in the embryo. With this being the case, it functions upstream from most of the conserved signaling pathways involved in fly hematopoiesis and immunity. Because of the similarity of the protein in flies and vertebrates, it is possible that PDCD2 has a similar function in vertebrate hematopoiesis (Hartenstein, 2006; Evans et al., 2003).

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/13/2387/DC1

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Table S1. *Zfpr8* genetic interactions

Pathway	Control genotype	Tested genotype	Monitored phenotype	Result	Lymph gland size
Ras	*UAS-Ras1^{v12} /+; hsp70-GAL4/+*	*Df(2R)SM206/UAS-Ras1^{v12}; hsp70-GAL4/+*	Lethality, melanotic phenotype	–	
	UAS-Ras1^{v12} /+; hsp70-GAL4/+	*Zfrp8 $^{M-1-1}$/UAS-Ras1^{v12}; hsp70-GAL4/+*	Lethality, melanotic phenotype	–	
	UAS-Ras1^{v12} /+; arm-GAL4/+	*Df(2R)SM1183/UAS-Ras1^{v12}; arm-GAL4/+*	Lethality	–	
	Df(2R)SM206/+	*Df(2R)SM206/UAS-Ras1.v12; hsp70-GAL4/+*	Lymph gland size increase	–	2.1 (0.2)
Toll	*Toll^{10B}*	*Df(2R)SM206/+; Toll^{10B}*	Melanotic masses, lymph gland overgrowth	–	Same as *Toll^{10B}*
	Toll^{10B}	*Zfrp $^{M-1-1}$/+; Toll^{10B}*	Melanotic masses, lymph gland overgrowth	–	Same as *Toll^{10B}*
	Toll^{3}	*Df(2R)SM206/+; Toll^{3}*	Melanotic masses, lymph gland overgrowth	–	Same as *Toll^{3}*
	Df(2R)SM206/+	*Df(2R)SM206/cactus^{A2}*	Lymph gland size increase	–	2.5 (0.7)
	Df(2R)SM206/+	*Df(2R)SM206/cactus^{1}*	Lymph gland size increase	–	
JAK/STAT	*Hop^{Tum}*	*Df(2R)SM206/+; Hop^{Tum}*	Melanotic masses	–	Same as *Hop^{Tum}*
	Hop^{Tum}	*Zfrp8^{M-1-1}/+; Hop^{Tum}*	Melanotic masses	–	Same as *Hop^{Tum}*
	Hop^{Tum}	*Df(2R)SM206/+; Hop^{Tum}*	Lymph gland phenotype	–	Same as *Hop^{Tum}*
GATA	*Df(2R)SM206/+*	*Df(2R)SM206/+; srp ^{3}/+*	Lymph gland size increase	–	2.0 (0.4)
	Df(2R)SM206/+	*Df(2R)SM206/+: srp^{01549}/+*	Lymph gland size increase	–	2.2 (0.5)
	Df(2R)SM206/+	*Df(2R)SM206/+; pnr^{MD237}/+*	Lymph gland size increase	Su	1.2 (0.3)
	Df(2R)SM206/+	*Df(2R)SM206/+; pnr^{VX6}/+*	Lymph gland size increase	Su	0.9 (0.3)
	Df(2R)SM206	*Df(2R)SM206; pnr ^{MD237}/+*	Lymph gland over-growth	Su	3.3 (1.6)
Notch	*Df(2R)SM206/+*	*Df(2R)SM206/+; Ser^{1}/+*	Lymph gland size increase	–	2.1 (0.6)
Other genes causing blood-related phenotypes	*Df(2R)SM206/+*	*Df(2R)SM206/+; Su(var)205 ^{04}/+*	Lymph gland size increase	–	2.0 (0.3)
	Df(2R)SM206/+	*Df(2R)SM206/dom^{k08108}*	Lymph gland size increase	–	2.2 (0.4)
	Df(2R)SM206/+	*Df(2R)SM206/ytr^{k13108}*	Lymph gland size increase	–	2.3 (0.6)
	l(3)mbn/+	*Df(2R)SM206/+; l(3)mbn/+*	Melanotic masses	–	Same as *l(3)mbn/+*
	Df(2R)SM206/+	*Df(2R)SM206/+; l(3)mbn/+*	Lymph gland size increase	–	Same as *l(3)mbn/+*
	Df(2R)SM206/+	*skpA^{G0389}/+; Df(2R)SM206/+*	Lymph gland size increase	–	2.0 (0.7)
	Df(2R)SM206/+	*PrSet7^{20}/+; Df(2R)SM206/+*	Lymph gland size increase	–	1.8 (0.1)
	Df(2R)SM206	*Bc^{1}/Df(2R)SM206*	Lymph gland size increase	–	2.1 (0.7)
	Bc^{1}/+	*Bc^{1}/Df(2R)SM206*	'Black' crystal cells phenotype	–	
Cell cycle	*Df(2R)SM206/+*	*Df(2R)SM206/+; cdc27$^{[L7123]}$/+*	Lymph gland size increase	E	5.1 (1.3)
	Df(2R)SM206/+	*Df(2R)SM206/cdc2^{E1-23}*	Lymph gland size increase	–	2.3 (0.8)
	Df(2R)SM206/+	*Df(2R)SM206/cycB^{KG08886}*	Lymph gland size increase	–	1.9 (0.5)
	Df(2R)SM206/+	*Df(2R)SM206/+; polo^{01673}/+*	Lymph gland size increase	–	2.1 (0.5)
	Df(2R)SM206/+	*Df(2R)SM206/+; aur^{1}/+*	Lymph gland size increase	–	2.2 (0.5)
	Df(2R)SM206/+	*Df(2R)SM206/cnn^{HK2}*	Lymph gland size increase	–	2.0 (0.5)
	Df(2R)SM206/+	*l(1)dd4^{2}/+; Df(2R)SM206/+*	Lymph gland size increase	E	4.7 (1.3)
Programmed cell death	*Df(2R)SM206/+*	*Df(2R)SM206/Ark^{k11502}*	Lymph gland size increase	–	2.6 (0.3)
	Df(2R)SM206/+	*Df(2R)SM206/Ark$^{Df(2R)P803-Delta15}$*	Lymph gland size increase	–	2.4 (0.5)
	Df(2R)SM206/+	*Df(2R)SM206/dcp-1^{k05606}*	Lymph gland size increase	–	2.1 (0.5)
	Df(2R)SM206/+	*Df(2R)SM206/+; th^{4}/+*	Lymph gland size increase	–	2.1 (0.7)
	Df(2R)SM206/+	*Df(2R)SM206/+; dronc^{2}/+*	Lymph gland size increase	–	2.3 (0.7)
	Df(2R)SM206/+	*Df(2R)SM206/+; dronc^{51}/ +*	Lymph gland size increase	–	2.3 (0.4)
	Df(2R)SM206/+	*Df(2R)SM206/+; dronc^{51}/ dronc^{2}*	Lymph gland size increase	–	Same as *dronc*
	dronc^{51}/dronc^{2}	*Df(2R)SM206/+; dronc^{51}/ dronc^{2}*	Lethality, lymph gland increase	–	Same as *dronc*
	Zfrp8^{M-1-1}/Df(2R)SM206	*Zfrp8^{M-1-1}/Df(2R)SM206; dronc^{2}/+*	Lymph gland overgrowth	–	2.2 (0.7)
	GMR-grim/+	*Df(2R)SM206/GMR-grim*	Small eye	–	
	GMR-grim/+	*Zfrp8^{M-1-1}/GMR-grim*	Small eye	–	
	GMR-hid/+	*Df(2R)SM206/GMR-hid*	Small eye	–	
	GMR-hid/+	*Zfrp8^{M-1-1}/GMR-hid*	Small eye	–	
	GMR-rpr/+	*Df(2R)SM206/+; GMR-rpr/+*	Small eye	–	
	GMR-rpr/+	*Zfrp8^{M-1-1}/+; GMR-rpr/+*	Small eye	–	
	GMR-rpr/+	*ey-FLP; FRT G13 Zfrp8^{M-1-1}; GMR-rpr/+*	Small eye	–	
	hs-rpr/+	*Df(2R)SM206/+; hs-rpr/+*	Temperature induced lethality	–	

Lymph gland size the area of 2D lymph gland images were measured (in pixels) and normalized to the area of wild-type lymph gland. The average lymph gland size for wild-type was 1 (s.d. 0.3) and for *Df(2R)SM206/+* was 2.2 (s.d. 0.5).
–, no change in phenotype.
Su, phenotype suppression.
E, phenotype enhanced.