Yantar, a conserved arginine-rich protein is involved in Drosophila hemocyte development

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Abstract

To identify novel factors involved in Drosophila hematopoiesis, we screened a collection of lethal recessive mutations that also affected normal hemocyte composition in larvae. We present the characterization of the gene yantar(ytr) for which we isolated null and hypomorphic mutations that were associated with severe defects in hemocyte differentiation and proliferation; ytr is predominantly expressed in the hematopoietic tissue during larval development and encodes an evolutionarily conserved protein which is predominantly localized in the nucleus. The hematopoietic phenotype in ytr mutants is consistent with a defect or block in differentiation of precursor hemocytes: mutant larvae have enlarged lymph glands (LGs) and have an excess of circulating hemocytes. In addition, many cells exhibit both lamellocyte and crystal cell markers. Ytr function has been preserved in evolution as hematopoietic specific expression of the Drosophila or mouse Ytr proteins rescue the differentiation defects in mutant hemocytes.

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Introduction

Studies in the last two decades have revealed a strong evolutionary conservation in genes and molecular pathways involved in vertebrate and invertebrate development (Evans et al., 2003; Potter et al., 2000; Reiter et al., 2001). Despite differences in the composition of cell types, this conservation has held true for mammalian and Drosophila genes involved in hematopoiesis (Evans et al., 2003). The Drosophila hematopoietic system is primitive and largely confined to cells analogous to those of the vertebrate myeloid lineage. At the larval stage, Drosophila hemocytes belong to three major classes: monocytelike plasmatocytes, involved in host defense and phagocytosis; crystal cells which carry out melanization reactions; and lamellocytes that are involved in encapsulation of objects that are too big to be phagocytosed (Rizki and Rizki, 1983). The conserved transcription factors—Serpent (Srp) a GATA protein, Glial cell missing (Gcm), Ushaped (Ush), the Friend of GATA (FOG)-ortholog and Lozenge (Lz), a Runx protein with homology to mammalian AML1—have been implicated in lineage specification of hemocytes. Srp, Gcm, and Ush transcription factors are required for the maintenance and specification of early hemocyte precursor cells to the plasmatocyte lineage, while Lz plays a similar role for the crystal cell lineage (Alfonso and Jones, 2002; Fossett et al., 2001; Lebestky et al., 2000). The fact that the corresponding mammalian transcription factors (GATA-1 and -2, FOG and AML1) also play pivotal roles in vertebrate hematopoiesis validates the notion that the basic mechanisms involved in cell fate specification, survival, proliferation, and differentiation of hemocytes are conserved between flies and vertebrates. Furthermore, signal transduction pathways implicated in mammalian hematopoiesis (e.g.,
when placed in trans (Wharton et al., 1999). The bgcn^{Q52} allele was kindly provided by B. Ohlstein (University of Texas). All other stocks, including enhancer trap lines: P[w^{+mC} = UAS – mC D8::GFP Pl];LY4 y^{+} P[w^{+mW.hs} = GawB]iz–Gal4 and w^{+} P[w^{+mW.hs} = GawB]izs015, were obtained from the Bloomingston Stock Center. Oregon-R, w^{118} or ytr heterozygous flies were used as control strains. Flies were maintained on standard Drosophila cornmeal/sucrose/yeast medium at 20°C, 25°C, or 29°C, as necessary.

Genetic mapping of the 5E24 mutation

Meiotic recombination analysis mapped the 5E24 mutation between the px and sp recessive markers. A (al, dp^{w1118}, b, pr, c, px, 5E24/Cyo, GFP) stock was established and subsequently used in deficiency mapping. Seven deletions (Df(2R)b23, Df(2R)106, Df(2R)bwDra, Df(2R)reg12, Df(2R)chi, Df(2R)or-Br, Df(2R)bwS4) that uncovered the 59–61 genetic interval on chromosome II failed to complement 5E24. Their distal breakpoints as well as that of two deficiencies (Df(2R)OVi1 and Df(2R)x32) that complemented our mutation were estimated using complementation with mapped lethal P insertions (P[w^{+mC} = lacW]iz [5.13214], P[w^{+mC} = lacW]iz [5.13214], P[y^{+7.2} = PZ]ken02970, and P[hse] [3.3]neo11). Taking into account the molecular mapping of Df(2R)reg12 provided in (Wharton et al., 1999), we placed the mutation between the distal breakpoints of Df(2R)OVi1 and Df(2R)reg12.

Molecular analysis and excision of P[w + mC = lacW]iz

The insertion site of P[w + mC = lacW]iz transposon, a ytr allele, was determined after plasmid rescue by inverse PCR [http://www.fruitfly.org/about/methods/inverse.pcr.html]. The forward primer, P[GalW] GCGTCGGTTTGAAGCAGCGAGCTT, and reverse primer, Plac1 CACCCAAAGCT-TGCTCCCACAAT, were used to amplify an approximately 850-bp fragment. Additional ytr alleles (AP22, AP2) were isolated by imprecise excision of P[w + mC = lacW]iz using a stock carrying the Δ2–3 transposase. Deletions in these ytr alleles were confirmed with PCR mapping and complementation with gbb and elf6 mutations.

Molecular biology

DNA from homozygous mutant or wild-type larvae was isolated and the overlapping fragments of ytr genomic locus were amplified using eight sets of primers. Potential changes due to allelic variation were controlled for by sequencing the ytr locus in pkdr^{5K29} mutant which shares the same genetic background as ytr^{5E24}. Sequencing reactions were performed at the core facility at the Dana-Farber Cancer Institute. Protein domain predictions were made with BLAST [http://www.ncbi.nlm.nih.gov/BLAST/]. KpnI

Materials and methods

Strains and genetics

The 5E24, 3A8, and 5K29 mutant alleles were generated in a large-scale mutagenesis using ethyl methanesulfonate (N. Perrimon, unpublished). An additional EMS-induced ytr allele, Ae-3, was originally isolated on the basis of its lethality when placed in trans with the deficiency Df(2R)bwS46.
and BglII linkers were added to the coding region of ytr cDNA (EST LD12325) as well as to the mouse ytr (mytr) cDNA (BF682621, IncyteGenomics) at the 5′ and 3′ ends, respectively, to facilitate cloning into the pUASP vector. Transgenic lines were generated as previously described (Callus and Mathey-Prevot, 2002). To facilitate expression of HA-mYtr in mammalian cells, 5′ EcoRI and 3′ XhoI restriction sites were added to the mYtr coding region and cloned into the pcDNA3.1-KozakHAHA vector, kindly provided by Dr. H. Widlund (Huber et al., 2003).

**Northern blot analysis**

*Drosophila* polyA+ RNA was prepared after Trizol reagent (Gibco-BRL) extraction and purification with PolyATract® mRNA Isolation System IV (Promega). Northern blot hybridization was performed as described (Callus and Mathey-Prevot, 2002).

**Immunocytochemistry and hemocyte counts**

*ytr* Mutant homozygous larvae were transferred to new vials containing fresh food for 4–7 days before collecting hemocytes. To determine total hemocyte numbers in different mutants, the hemocytes from five larvae of each genotype were collected in 50 μl of Ringer solution. The resuspended cells were counted using a hemocytometer, and counts were used to determine the number of cells per single larva. To count L1-positive (L1+), and Lz-positive (Lz+) cells, hemocytes from 40 larvae of each genotype were collected into 400 μl of Ringer solution. A 40-μl aliquot of the hemocyte suspension (corresponding to 4 larvae) was placed on a single well of a 10-well glass slide (PGC Sciences). Cells were let to adhere for 40 min before fixation with acetone (8 min) or 2% formaldehyde solution (20 min). Fixed cells were treated with specific antibodies, as indicated in the appropriate figure legends: anti-L1 and -H2 antibodies directed against specific hemocyte markers (previously described in Asha et al., 2003; Kurucz et al., 2003; anti-Lz antibody (kindly provided by Dr. U. Banerjee); antiphosphohistone H3-specific antibodies (Upstate). Request for L1 and H2 hemocyte-specific antibodies should be addressed to Dr. I. Ando. Biotinylated antimouse antibodies with the “ABC” system (DAKO) or FITC-labeled secondary antibodies (DAKO) were used according to the manufacturer’s instructions. An equivalent area of the slide was scanned to count positive and negative cells. Multiple independent areas from the same slide were analyzed, and the analysis was repeated multiple times to ensure reproducibility. Numbers of Lz- or L1-positive cells relative to the total number of hemocytes were calculated in each case. These numbers were used to calculate back the numbers of positive cells circulating in larvae and are shown with their standard deviations. For rescue experiments, hemocytes from a single larva of each genotype were collected in 50 μl of Ringer solution in a single well of a 10-well glass slide.

Homozygous ytr mutant larvae were selected based on the absence of the balancer CyO chromosome (marked with GFP) and the presence of small, mutant imaginal discs. Cells were let to adhere before fixation with a 2% formaldehyde solution (Sigma). DNA was counterstained with 4,6-diamidino-2-phenylindole (DAPI), and final preparations were mounted in 80% glycerol in PBS. Multiple (10) independent samples for each genotype were analyzed to ensure reproducibility. Cell images were taken under different magnifications with a light (Nomarski optics) and fluorescent microscope (Zeiss Optical).

**RNA in situ hybridization**

A ytr digoxigenin (DIG)-labeled riboprobe was prepared according the kit manufacturer’s instruction (Roche). In situ hybridization of staged embryos was carried out according to Protocol 12.5 of Sullivan et al. (2000). For in situ hybridization of larval tissues, third instar larvae were dissected directly in 4% paraformaldehyde in PBS for 20 min. Hemocytes were washed and prehybridized before the probe was added. Hybridization was performed at 55°C in a hermetically closed humid chamber (4 h to overnight). Detection was performed with anti-DIG antibody conjugated to alkaline phosphatase (Roche) (Sullivan et al., 2000).

**Cell culture and transfection**

NIH 3T3 and COS7 cells were cultured in RPMI supplemented with 10% FCS. Transfections were carried out with the FUGENE-6 transfection reagent (Roche) according to the manufacturer’s instructions. Briefly, subconfluent cultures of cells grown on glass slides (Lab-Tek II Chamber slide system, Nalge Nunc, Int.) were transfected with 2.5 μl of the FUGENE-6 reagent containing either 0.5–1 μg of HA-mYtr-, HA-MITF-, or HA-Tsp68C-pcDNA3.1 plasmids in 200 μl serum-free RPMI. RPMI containing 10% FCS medium was added 1 h later. Twenty-four hours after transfection, cells were fixed with 3% formaldehyde in PBS for 20 min and incubated in 0.1% Tween 20, 0.3% BSA in PBS. Fluorescein-labeled HA-specific monoclonal antibodies (3F10, Roche) was added to the fixed and permeabilized cells for 2 h, and DNA was counterstained with DAPI. Cells were then examined by fluorescence microscopy (Zeiss Optical).

**Results**

**Screen for Drosophila hematopoietic mutants**

Our screen was based on the fact that distinct black masses (also known as melanotic tumors) often arise in the
larval body cavity of mutants with aberrant hematopoiesis (Alfonso and Jones, 2002; Badenhorst et al., 2002; Dearolf, 1998; Gateff, 1978). We identified 2000 mutant stocks were screened in this fashion, and we identified 18 EMS-induced lethal mutations defining 18 complementation groups. Three of these mutations (SE24, 5K29, and 3.48) were selected because of their reproducible and fairly penetrant melanotic tumor phenotype, and we named their corresponding genes yantar (ytr), polka dots (pkdt), and splotchy (splo). Hemoocytes from these mutants were visualized after immunostaining using two hemoocyte-specific monoclonal antibodies, MAb H2 and L1. MAb H2 recognizes the hemese antigen present in all Drosophila hemocytes, whereas MAb L1 is specific for lamellocytes (Asha et al., 2003; Kurucz et al., 2003). We observed a marked elevation in circulating hemoocytes in ytrSE24 and splo3.48 homozygous larvae (4- and 1.8-fold increase, respectively) compared to counts in wild-type or heterozygous larvae (Fig. 1 and data not shown). In contrast, the pkdt5K29 mutant showed a 3.2-fold reduction in circulating hemoocytes. In agreement with previous studies (Lanot et al., 2001; Russo et al., 2001), only 0.3% of peripheral hemocytes circulating in wild-type hemolymph were L1-positive (L1+) cells (lamellocytes) (Fig. 1). In contrast, the percentage of L1+ cells was dramatically increased in all three mutants they represented, 25% and 27% of circulating hemoocytes in ytrSE24 and pkdt5K29 mutants, respectively, and 8% in the splo3.48 mutant. Interestingly, L1+ cells from these mutants often had morphology distinct from typical lamellocytes (smaller and more round) but were usually larger than wild-type plasmatocytes. Crystal cells in wild-type and mutant larvae were readily identified based on their round morphology and the presence of para-crystalline inclusions in the cytoplasm. We did not observe dramatic differences in the relative number or morphology of crystal cells between any of the mutants and wild-type samples, suggesting that crystal cell differentiation in the mutants was largely unaffected (data not shown). As the ytrSE24 mutation was associated with the most pleiotropic phenotype showing a significant overaccumulation of hemoocytes and an uncharacteristically high proportion of morphologically diverse L1+ cells, we chose to characterize the molecular lesion in this mutant.

The gene CG18426 is affected in ytrSE24 mutant

Initial deficiency mapping placed the site of the ytrSE24 mutation between the subdivisions 59 and 60 of the right arm of chromosome II. To further narrow down the interval, we selected four deficiencies (Df(2R)b23, Df(2R)egl2, Df(2R)x32, and Df(2R)OV1) which had been characterized and used to identify other mutations in this region (Munn and Steward, 2000; Ohlstein et al., 2000; Wharton et al., 1999). Complementation studies between the ytrSE24 mutation and these deficiencies indicated that the mutation lied in the interval between the distal breakpoints of Df(2R)OV1 and Df(2R)egl2. This interval was confirmed using Df(2R)Chi which overlaps with the distal region of Df(2R)egl2 and extends further toward the telomere (Fig. 2A). Several genes mapped within or close to that interval (Fig. 2B) (Adams et al., 2000; Lukacsovich et al., 1999). We quickly ruled out gbb and bgcn by complementation. To help us identify which of the remaining genes was mutated in ytrSE24, we searched for lethal P-element transposon insertions and additional EMS-induced zygotic lethal mutations in the region. We identified two P-element insertions, P[w+mC = lacW]k13108b (P(lacW)k13108b) and P[w+mC] = lacW]/(2)k13214 (eIF6 k13214). Complementation studies indicated that the former was allelic to ytrSE24, whereas the latter which disrupts the coding sequence of the elF6 gene (Spradling et al., 1999) complemented the ytrSE24 mutation. Plasmid rescue on DNA isolated from P(lacW)k13108b homozygous larvae revealed that the P-element was inserted in the second intron of the annotated gene CG18426 (Fig. 2B). To verify that the lethality associated with P(lacW)k13108b was linked to the transposon, we mobilized the P-element associated with P(lacW)k13108b and isolated flies which had lost the P-element. Stocks derived from precise excisions were homozygous viable and fertile, and showed no hematopoietic defects. Along with the precise excisions of the P-element ytrk13108b, we isolated several imprecise excision lines. As expected, two of them (AP2, AP22) failed to complement ytrSE24 and ytrk13108b, but complemented eIF6k13214, gbb, and bgcn mutations. In addition, four EMS-induced mutations belonging to the F complementation group reported in Wharton et al. (1999) were tested by complementation. Three of them, including ytrk13108b, failed to complement ytrSE24 and ytrk13108b but complemented mutants in eIF6 and gbb. These data indicate that CG18426 is the affected gene in ytrSE24 mutants. The appearance of mutant pupae with melanotic masses reminded us of amber with inclusions, and we named the gene yantar (amber in Russian).

Characterization of molecular lesions associated with the ytr alleles

ytr Resides in a locus densely packed with genes (Lukacsovich et al., 1999). It lies next to gbb in a head-to-head orientation, 453 bp from the initiating ATG codon of gbb. The single exon gene eIF6 is located in the third intron of ytr, in the same orientation as ytr (Fig. 2B). Sequences from a ytr cloned expressed sequence tag (EST) (LD12035, AY061143) and a ytr cDNA isolated from a Drosophila embryonic cDNA library (cDNA11, AY171243) revealed that the two clones differed in their use of S' untranslated exons but encoded the same open reading frame (ORF) consisting 194 amino acids (aa) (Fig. 2C). Northern blot analysis of poly(A-) RNA isolated from embryos and third instar larvae confirmed that the cDNAs correspond to full-length transcripts as we can detect ytr mRNA bands ranging in size from about 2.0 to 2.2 kb (Fig. 3A).
Fig. 1. Aberrant composition of hemocytes in Drosophila mutant larvae. (A) Hemolymph from a similar number of third instar wild-type or mutant larvae was collected in the same final volume of buffer and processed for immunostaining using the pan-hemocyte monoclonal antibody H2 and the lamellocyte-specific monoclonal antibody L1 (see Materials and methods for details). (B) Cell counts per mutant or wild-type larva are plotted for all hemocytes (gray bars) and L1+ cells (red bars). Cell counts were calculated as described in Materials and methods.
We next examined ytr mRNA expression in the different ytr alleles. Expression of wild-type size ytr mRNA is severely affected in all ytr alleles. Either there is a lack of detectable transcripts (as in ytr<sup>k13108b</sup> and its imprecise excision lines) or there is a marked reduction in expression coupled with aberrant migration of ytr mRNA (ytr<sup>5E24</sup> and ytr<sup>Ae-3</sup>) (Figs. 3A and B). Sequencing of the genomic region in the ytr<sup>5E24</sup> mutant revealed a single nucleotide change (T to A) in the third intron of ytr compared to Oregon-R DNA. This change is unlikely to be a natural polymorphism, as it was not observed in the EMS mutant pkdt<sup>5K29</sup> that shares the same genetic background as ytr<sup>5E24</sup>. Interestingly, this mutation also causes a reduction in eIF6 transcripts (Fig. 3B), possibly by disrupting a regulatory site shared by the two genes. DNA sequencing of the ytr genomic sequence of ytr<sup>Ae-3</sup> revealed a single nucleotide (G to A) that destroys the splice donor site in the second intron and prevents proper splicing between the second and third exons. Examination of the sequence just downstream of the mutation shows that there is an in-frame termination codon (TGA) which, if used, would lead to the expression of a truncated Ytr protein (Fig. 2C). This mutation had no affect on the level of eIF6 transcripts (data not shown).

**ytr mRNA is dramatically enriched in larval hematopoietic tissues**

ytr mRNA has a dynamic pattern of expression during embryogenesis. It is maternally contributed, and zygotic expression is first detected in invagination furrows and in ventral and head mesoderm. At later stage, it is predominantly detected in the developing neuronal system. At the end of embryogenesis, expression is also found in the area of the presumptive lymph glands and in embryonic hemo-
cytes (Fig. 3C and data not shown). RNA in situ hybridization of larval tissues indicates that ytr is strongly expressed in the lymph glands and a majority of peripheral hemocytes (Fig. 3D). ytr mRNA is also expressed in imaginal discs, albeit at much lower levels (data not shown). We failed to detect ytr expression in other larval tissues. In situ analysis on adult ovaries shows abundant ytr mRNA in the nurse cells until stage 10 after which high levels are found in the oocyte (R. Wynn, unpublished).

ytr Encodes a highly conserved arginine-rich protein that predominantly localizes to the nucleus

Based on sequence homologies performed with the conceptual Ytr protein sequence, we have arbitrarily divided the Drosophila Ytr protein in four separate domains (Fig. 4A). In addition, these searches revealed that Ytr is highly conserved in fission yeast, C. elegans, Arabidopsis, and vertebrates (Fig. 4B). Unfortunately, there is no or little
information to suggest a putative function of Ytr in these various organisms. The N-terminal half of the protein comprises two blocks of charged or serine residues consisting repeated Arg (30% of all residues in the sequence), Ser (8.5%), Asp (5%), and/or Glu (10%) amino acids. This region shows significant homology (40–50%) to domains found in several SR-splicing factors, including U1-70K snRNP protein, SC35, U2AF, as well as RER- and RD-proteins. These repeats have been proposed to mediate direct RNA interaction as well as protein–protein interac-

Fig. 4. Schematic of the Ytr protein and sequence comparison. (A) Several putative domains (based on homology searches) were assigned to the Ytr protein: an N-terminal, R-rich motif (I and II), a short glycine repeat (Gly), an unknown motif with conserved Arg, Ile, and Leu residues (RIL motif) and a unique C-terminal domain (ytr domain). (B) Sequence alignment of the various domains of Drosophila Ytr with orthologs from human, mouse, C. elegans, yeast, and Arabidopsis. Conserved domains are framed and shading highlights conserved amino acid residues. (C) Subcellular localization of HA-mYtr protein in NIH 3T3 cells: (1) diffuse nuclear pattern, (2) punctate nuclear pattern, (3) nuclear and weak cytoplasmic distribution. HA-MITF and HA-Tsp68C: control transfections in NIH 3T3 confirm proper localization of the HA-MITF and HA-Tsp68C proteins to the nucleus and cytoplasm, respectively. In all panels, the nucleus is visualized after DAPI staining, and the protein of interest is detected with anti HA-antibody labeled with fluorescein (see Materials and methods).
tion among different SR-proteins (Patel, 1999; Wu and Maniatis, 1993). The Arg-rich repeats are followed by a polyGly-rich linker which is unique to the fly protein and may provide a flexible hinge that precedes a short stretch of amino acids (referred to as the RIL motif) found in a number of serine/threonine kinases and containing the highly conserved Arg, Ile, and Leu residues. Finally, the C-terminus end of Ytr shows the highest degree of conservation among the various orthologs (Fig. 4B) but shares no significant homology to any other domain in the database. Its function is unknown.

To examine the localization of the Ytr protein, we expressed the mouse Ytr protein tagged with an HA epitope at its N-terminus in NIH 3T3 fibroblasts or COS7 cells. We observed an identical pattern of subcellular localization in both cell types (Fig. 4C and data not shown). Interestingly, we could distinguish three patterns of protein distribution. Consistent with the noted homology to SR-splicing factors, HAMYtr either localized in a diffuse pattern in the nucleus of transfected fibroblasts (Fig. 4C, 1) or was uniformly distributed between the cytoplasm and nucleus (Fig. 4C, 3). Importantly, in about 10% of the transfected cells, we

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Fig. 5. Hemocytes in different ytr alleles. (A) Analysis of lymph gland and circulating hemocytes in larvae-bearing homozygous mutations in ytr (5E24 and ΔP22) was examined by Nomarski microscopy under the indicated magnification, and immunostaining with L1 antibody was performed on circulating hemocytes to reveal lamellocytes. (B) A population of large round hemocytes (black arrows) from ytr mutant larvae often contains two nuclei (DAPI epifluorescence, blue). Binucleated hemocytes from mutant larvae are not actively dividing as evidenced by the lack of antiphosphohistone H3 immunoreactivity (purple, ×120 magnification).
observed a punctate distribution of the protein (spherical doughnuts) in the nucleus (Fig. 4C, 2). These discrete puncta often localized to areas of less condensed chromatin. Control transfections with HA-tag MITF transcription factor (King et al., 1999) and HA-tag tetraspanin protein Tsp68C (S.A. Sinenko and B. Mathey-Prevot, unpublished) expression plasmids confirmed that under our experimental conditions, the two proteins localized to their proper compartment (nuclear and cytoplasmic, respectively).

Loss of Ytr function results in the detection of an altered population of Lz/L1 double-positive hemocytes and binucleated hemocytes

Null and hypomorphic alleles of ytr cause a marked enlargement of the first lobes of the lymph gland (LG), which occasionally contain melanized hemocytes (Fig. 5A). In addition, hemocytes in the mutant LG are larger (ytrAP22) and more heterogeneous in size and shape (ytrSE24) than those found in wild-type LG. Analysis of the circulating hemocytes revealed the presence of numerous big round cells that are normally absent in wild-type and ytr heterozygous larvae (Fig. 5A, data not shown). As their appearance was somewhat reminiscent of lamellocytes, mutant circulating hemocytes were stained for the specific marker L1 expressed by wild-type lamellocytes. As already observed for ytrSE24 (Fig. 1), there was a substantial increase in the percentage of circulating L1+ cells in this and other ytr mutants, ranging from 10% to 35% of total hemocytes (Figs. 5A and 6A). Unlike the restricted expression of the L1 antigen to circulating lamellocytes in wild-type hemolymph, this antigen was detected in ytr mutant hemocytes that showed distinct morphology from that of typical lamellocytes. In particular, a number of small round ytr mutant hemocytes were highly L1 positive (Fig. 5A). In addition, many of the large round mutant hemocytes in lymph glands and in circulation (up to 10% of all hemocytes) contained two nuclei (Fig. 5B). These cells, however, do not express the phosphorylated form of histone H3 (Fig. 5B), indicating that they are not dividing cells but rather exhibit a defect in cytokinesis.

To characterize further the differentiation defect in ytr mutant hemocytes, we monitored the expression of the crystal cell marker Lz in these cells, both by immunocytochemistry (Figs. 6B and C) and using the Lz-Gal4 enhancer trap line (Figs. 6D and E). Normally, Lz is highly expressed in mature crystal cells and to a lesser extent in a rare population of small, circulating hemocytes (presumably crystal cell precursors). Although the production of mature crystal cells was not measurably affected in ytr mutant hemolymph, there was an increase in lymph gland and circulating Lz+ hemocytes. These cells were heterogeneous in size and lacked the characteristic paracrystalline inclusions (Figs. 6D and E). The largest increase was observed in ytrSE24 larval hemolymph. There was a concomitant enrichment of Lz+ cells in the first lobe of the LG of ytrSE24 mutant larvae (as visualized with the Lz enhancer trap Lz-Gal4 and UAS-GFP; Fig. 6B). Interestingly, there was a mild elevation of Lz+ cells in the LG of male ytrSE24 heterozygous larvae, indicative perhaps of a genetic interaction between ytr and the hypomorphic Lz allele of this enhancer trap line (Fig. 6D).

A closer examination of the L1+ and Lz+ hemocytes in ytr mutant revealed that some cells shared a common morphology. To determine whether these cells might coexpress both antigens, the Lz-Gal4 enhancer trap was used to visualize Lz+ hemocytes in wild-type or mutant hemolymph (through expression of GFP), and we counterstained the same cells with the L1 antibody (Fig. 6D). As expected, the strongly GFP-positive (GFP+) cells in wild-type hemolymph are either crystal cells (white arrow in Fig. 6C) or small round cells (presumably crystal cell precursors). Importantly, these cells do not express the L1 antigen. On rare occasion, we noticed dimly GFP+ cells that also expressed L1 (red arrow). These cells differ slightly in morphology from mature lamellocytes that are invariably GFP-negative (black arrow, Fig. 6D). Whether coexpression of the two antigens in these cells defines a distinct or transient population is not known. Regardless, we found up to 20% of L1+ hemocytes that expressed both antigens in ytrSE24 mutant hemolymph (red arrows, Fig. 6C), consistent with the expansion of a rare population of hemocytes or with the overproliferation of bi- or multipotential progenitors in ytrSE24 mutants.

Mouse Ytr corrects ytr mutant hemocytes and leads to full rescue

We next selected a Gal4 enhancer trap P[GawB]5015 that was described in FlyBase as an LG-specific line. Using the GFP reporter gene, we confirmed that this line drove expression in the LG and in about 50% of circulating hemocytes of ytrSE24 mutants (Fig. 7A). Interestingly, and consistent with an elevation of precursor cells in ytr mutants (normally found in the LG), there was no or little GFP expression in ytr heterozygous or in wild-type circulating hemocytes (Fig. 7A and data not shown). In addition, GFP was expressed in the salivary glands and ring gland, two tissues where we failed to detect ytr expression (data not shown). Ectopic expression of Drosophila (d)-Ytr or mouse (m)-Ytr mediated by P[GawB]5015 rescued the hematopoietic phenotype in both ytrSE24/ytrSE24 and ytrSE24/ytrAP22 mutants (Fig. 7A and data not shown). Interestingly, m-Ytr was more efficient at rescuing the phenotype, although this difference may reflect different levels in protein expression. Despite having normal hemocytes and lymph glands, mutant larvae that expressed m-Ytr (or d-Ytr) still died at the pupal stage, suggesting that Ytr function is required in other tissues. To confirm this interpretation, m-Ytr was expressed ubiquitously in mutants through the use of daughterless (da)-Gal4 driver. The hematopoietic defects and lethality were completely rescued in ytr mutants after da-Gal4 mediated expression of m-Ytr (Fig. 7B). The emerging adults showed no obvious phenotype and were fertile (data not shown).
Discussion

Three novel mutations affect normal hemocyte development

The transcription factors, Srp, Lz, Ush, and Gcm, have all been implicated in the specification and function of plasmatocytes and crystal cells (for review, see Evans et al. 2003). However, little is known about the origin and differentiation requirements of the lamellocyte lineage. Mutations in the Jak-kinase Hopscotch (hop<sup>Tum-1</sup> hop<sup>T225</sup> ), in the Toll/cactus pathway (TolI<sup>l(3)mbn-1</sup> and cac<sup>T</sup>D13), as well as in the putative cell adhesion protein I(f3)mbn-1, in ribosomal protein RpS6<sup>amin</sup> or in the nucleosome remodeler factor (NURF301<sup>T</sup>) lead to overproliferation of larval hemocytes accompanied by a large increase in circulating lamellocytes in mutant larvae (Badenhorst et al., 2002; Harrison et al., 1995; Konrad et al., 1994; Qiu et al., 1998; Watson et al., 1992). As these mutations appear to act in a cell autonomous fashion, these genes or pathways have been linked to the regulation of hemocyte development. To gain additional information on genes that affect the lamellocyte lineage, we screened a collection of EMS mutants and identified several complementation groups, which when homozygous, led to the appearance of melanotic masses in larvae. Three of these mutants—yantar, polka dots, and splotchy—were further selected as they exhibited profound anomalies in circulating larval hemocytes. There was a large increase in the relative number of lamellocytes or L<sup>1</sup> cells in each mutant, and the number of circulating hemocytes was significantly increased in ytr<sup>ytr5E24</sup> mutants. Although detection of melanotic masses correlated with altered hemocyte differentiation and overrepresentation of L<sup>1</sup> cells, it did not always result in hemocyte overproliferation as exemplified by the drastic reduction in circulating hemocytes in pkad<sup>SK29</sup> mutants.

Ytr is a conserved arginine-rich protein with nuclear localization

Our genetic mapping and molecular analyses indicate that ytr corresponds to CG18426 located in 60A. Although we have focused on the role of ytr in hematopoiesis, ytr loss of function also results in a marked reduction in imaginal disc size and in a mild loss of actin organization in disc cells (data not shown). The relative enrichment of ytr mRNA in developing tissues (embryonic head and ventral mesoderm, developing gut, embryonic brain, and lymph glands) and in actively proliferating tissues (hemocytes and imaginal discs) coupled with the range of defects observed in ytr mutants suggest that ytr plays a role in regulating growth and differentiation.

Ytr protein shows high evolutionary conservation, and orthologs can be identified in different phylogenic classes of the metazoa. Aside from one report on the ectopic expression of human ytr mRNA in a human lymphocytic cell line as a result of a viral integration (Nakamura et al., 1994), nothing is known about the Ytr proteins in other species. Based on the homology of the R-rich domains in the N-terminal region of Ytr with RS domains found in several SR-splicing factors as well as in RE- and RD-proteins, we speculate that Ytr is involved in nucleic acid binding, either directly or indirectly. Although the two R-rich domains in Ytr lack the canonical RNA-recognition motif (RRM) thought to mediate RNA binding, viral proteins with similar R-rich motifs or RS, RD, and RE dipeptide repeats have been described to be directly involved in RNA recognition and splicing activation (Patel, 1999; Shen et al., 2004). RS domains have also been implicated in protein–protein interaction among different SR-splicing factors, and their phosphorylation have been proposed to promote nuclear import, nuclear retention, as well as activation of these proteins and their complexes (Cazalla et al., 2002; Wu and Maniatis, 1993). Although we have no biochemical proof that Ytr binds RNA (or DNA), its dynamic pattern of subcellular localization and punctate nuclear distribution is reminiscent of that of many SR and arginine-rich proteins (de Graaf et al., 2004; van der Houwen van Oordt et al., 2000). Consistent with this prediction is the fact that several related RNA-binding proteins and splicing factors have been linked to growth and differentiation. For instance, the Drosophila Musashi RNA-binding protein functions in asymmetric cell division downstream of Notch signaling by regulating the translation of the transcription factor TTK69 (Okano et al., 2002) and alternative splicing by SR proteins have been shown to play a role in human T cell development (Wang et al., 2001). Alternatively, the potential homology of the Ytr N-terminal half with the Acinus protein might suggest that Ytr, like Acinus, has chromatin remodeling activity. Interestingly, Acinus is involved in caspase-3-dependent apo-

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**Fig. 6.** Accumulation of Lz<sup>ytr</sup> and L<sup>1</sup> hemocytes in ytr<sup>ytr5E24</sup> mutants. (A) Total peripheral hemocytes were counted as described in Materials and methods. Average total counts (blue bars) are reported for a single larva of the indicated genotype. (B) Hemocytes were immunostained with antibodies directed against the L1 antigen (Mab L1) and Lz protein (Mab α-Lz). Positive cells for each antigen were counted, and counts were plotted as cells/larva (see Materials and methods): L<sup>1</sup> hemocytes (red bars) and Lz<sup>ytr</sup> hemocytes (green bars). (C). Representative panels of wild-type and ytr mutant peripheral hemocytes immunostained with α-Lz Mab. (D) Lz<sup>ytr</sup> cells (green) in anterior lobes of larval lymph glands of the indicated genotypes were visualized with the I(3)Gal4, UAS-GFP reporter line (×20 magnification). (E) Hemocytes were immunostained with Mab L1 and visualized under Nomarski or epifluorescence (GFP) microscopy. In wild-type, third instar larvae, the L1 antigen is restricted to lamellocytes (black arrows) among circulating hemocytes, while Lz (GFP<sup>+</sup>) is strongly expressed in crystal cells (white arrows) and their precursors. Rare Lz<sup>ytr</sup> /L<sup>1</sup> double-positive cells (orange arrow) are present in normal larval hemolymph. High numbers of L<sup>1</sup> (black arrows), Lz<sup>ytr</sup> (white arrows) as well as Lz<sup>ytr</sup>/L<sup>1</sup> double-positive cells (orange arrows) circulate in ytr<sup>ytr5E24</sup> mutant hemocytes (×80 magnification). Third instar larvae from the I(3)Gal4, UAS-GFP and L<sup>1</sup>-Gal4, UAS-GFP, Bc/CyO GFP stocks were used as control for wild-type hemolymph.
ptotic chromosome condensation and is required for erythroblasts maturation and differentiation of monocytes to macrophages in mammals (Sordet et al., 2002). Regardless of the exact mechanism, we propose that the N-terminal region of Ytr is important to promote its nuclear retention where this protein can function in cell differentiation or proliferation by regulating posttranscriptional events through RNA binding or RNA processing, or by participating in DNA remodeling.

The ytr^{SE24} allele is a complex mutation

We do not fully understand how the mutation in ytr^{SE24} results in drastically reduced levels of aberrantly sized ytr mRNA transcripts. One possible explanation is that the mutation creates a (GA)_3 site that could recruit GAFs chromatin remodeling factors known to render DNA sequences more accessible to various nucleases and other factors (Henikoff and Vermaak, 2000; Leibovitch et al., 2002). Recruitment of GAFs to the ytr locus might alter the surrounding chromatin structure, consistent with our observation that eIF6 expression is reduced in ytr^{SE24}, although it is completely unaffected in other ytr alleles. Interestingly, we have found that a short DNA fragment spanning the mutation was difficult to amplify in a PCR reaction. Lastly, in addition to the aberrantly sized transcripts that lacked the third exon, we have recovered hybrid transcripts in ytr^{SE24} mutants that contained ytr 5′ exons fused to a sequence from eIF6 (data not shown). This data confirm that the mutation in ytr^{SE24} is complex and, through its additional effect on eIF6 expression, may explain why the hematopoietic phenotype observed with this allele is quite severe.

Ytr function is required for proper hemocyte differentiation

All ytr mutations result in hematopoietic defects in late third instar larvae characterized by the presence of a large number of L1+ and Lz+ circulating hemocytes. In wild-type hemolymph, the L1 antigen is a specific marker of mature lamellocytes (Asha et al., 2003). Wild-type lamellocytes adopt a variety of cell sizes and shapes in vivo which may reflect transitional stages in cell maturation. The morphology and size of L1+ cells circulating in ytr mutant hemolymph are even more diverse. Aside from typical lamellocytes, there is an abundance of round cells of heterogeneous sizes, including cells that are hard to distinguish from plasmatocytes based on morphology alone. Plasmatocytes, however, do not express the L1 antigen, leaving open the possibility that these L1+ cells may represent lamellocyte precursor cells. Confirmation of this hypothesis will require the use of additional markers of the lamellocyte lineage. Another possibility is that...
the ytr mutation leads to the derepression and ectopic expression of the L1 antigen in some hemocytes. This explanation is made less likely by our observation that many of the L1+ cells in the ytr\textsuperscript{SE24} mutant also express the Lz marker while being morphologically distinct from crystal cells and their precursors. Furthermore, we can detect very rare cells in wild-type hemolymph that similarly coexpress the two markers. We therefore favor the idea that such double-positive cells represent early progenitors of either the lamellocytic lineage or of a normally rare subset of hemocytes distinct from lamellocytes and that these cells are specifically expanded in ytr mutants. Consistent with this notion is our observation that Gal4 expression in the enhancer trap gene P\textsuperscript{[GawB]5015} is normally restricted to rare cells in wild-type hemolymph that similarly coexpress the two markers. We therefore favor the idea that such double-positive cells represent early progenitors of either the lamellocytic lineage or of a normally rare subset of hemocytes distinct from lamellocytes and that these cells are specifically expanded in ytr mutants. Based on these observations, we propose that the loss of Ytr function leads to an expansion and block or delay in maturation of hemocyte precursors. This defect was readily reversed after ectopic expression of wild-type fly and mouse Ytr proteins in mutant hemocytes, a strong indication of the evolutionary conservation and importance of ytr function in Drosophila hematopoiesis.

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