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Identification and Characterization of Genes Involved in Embryonic Crystal Cell Formation During Drosophila Hematopoiesis

Allison B. Milchanowski,*[,1] Amy L. Henkenius,*[,1] Maya Narayanan,* Volker Hartenstein*[,†] and Utpal Banerjee*[,†,‡,2]

**Department of Molecular, Cell and Developmental Biology, ‡Department of Biological Chemistry and †Molecular Biology Institute, University of California, Los Angeles, California 90095*

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ABSTRACT

Parallels between vertebrate and Drosophila hematopoiesis add to the value of flies as a model organism to gain insights into blood development. The Drosophila hematopoietic system is composed of at least three classes of terminally differentiated blood cells: plasmatocytes, crystal cells, and lamellocytes. Recent studies have identified transcriptional and signaling pathways in Drosophila involving proteins similar to those seen in human blood development. To identify additional genes involved in Drosophila hematopoiesis, we have conducted a *P*-element-based genetic screen to isolate mutations that affect embryonic crystal cell development. Using a marker of terminally differentiated crystal cells, we screened 1040 *P*-element-lethal lines located on the second and third chromosomes and identified 44 individual lines that affect crystal cell development. Identifying novel genes and pathways involved in Drosophila hematopoiesis is likely to provide further insights into mammalian hematopoietic development and disorders.

DESPITE the obvious differences in cell type and functionality between vertebrate and Drosophila blood systems, parallels in their developmental mechanisms are remarkable (reviewed by Evans *et al.* 2003). Such shared developmental and functional mechanisms have prompted the use of Drosophila as a model organism to further investigate the genetic control of hematopoietic cell differentiation.

Both vertebrate and Drosophila hematopoiesis involve distinct, terminally differentiated lineages derived from common progenitor cells. Mammalian hematopoietic cells differentiate into two main branches: the lymphoid and myeloid lineages (reviewed by Dzierzak and Medvinsky 1995). Differentiation, function, and lineage hierarchy of Drosophila blood cells, or hemocytes, are most similar to those of the vertebrate myeloid lineage (reviewed by Orkin 2000). The Drosophila hematopoietic system is composed of at least three classes of terminally differentiated hemocytes: plasmatocytes, crystal cells, and lamellocytes, which participate in development and immune response (reviewed by Evans *et al.* 2003; Meister and Lageaux 2003).

Plasmatocytes are the most abundant hemocyte type in Drosophila and are commonly referred to as macrophages. Accordingly, they function to engulf apoptotic cells and debris as well as play a role in immune response by eliminating pathogens (Tepass *et al.* 1994; Lanot *et al.* 2001). Crystal cells, which compose ∼5% of the hemocyte population, participate in immune responses and wound healing through melanization. The paracrystaline inclusions within the cells are thought to contain Pro-Phenoloxidase A1 (ProPO A1; Rizki *et al.* 1980), an enzyme that is similar to tyrosinase and is important in the biosynthesis of melanin (Rizki *et al.* 1985). Unlike plasmatocytes and crystal cells, which are found in all developmental stages, lamellocytes have been observed only in Drosophila larvae and increase in number during immune challenge (Lanot *et al.* 2001; Sorrentino *et al.* 2002).

Drosophila hemocytes have dual sites of origin. Early hemocytes arising from the mesoderm of the embryonic head region are detected throughout development and into adulthood (Holz *et al.* 2003). A split of the hemocyte population into plasmatocytes and crystal cells occurs at an early stage. Crystal cells form a small, cohesive cell group that remains clustered around the embryonic proventriculus (Lebestky *et al.* 2000), whereas plasmatocytes migrate throughout the entire embryo (Tepass *et al.* 1994). A second population of hemocytes that differentiate in the late larva and during metamorphosis to populate the pupa and adult are derived from a second blood-forming tissue, the lymph gland, which is situated next to the dorsal blood vessel (aorta/heart) of the larva.

Over 20 genes have been identified in mammalian blood cell differentiation, including genes that encode transcription factors, recombinases, signaling mole-

[1]These authors contributed equally to this work.

[2]*Corresponding author:* Department of Molecular, Cell and Developmental Biology, 2203 Life Sciences, 621 Charles E. Young Dr. S., University of California, Los Angeles, CA 90095.
E-mail: banerjee@mbi.ucla.edu

cules, transmembrane receptors, and secreted factors (reviewed by Orkin 1996) that can act positively and/or antagonistically in the regulation of hematopoiesis. Many molecules play a role in both vertebrate and Drosophila hematopoiesis, including transcriptional regulators such as GATA, friend of GATA (FOG), and acute myeloid leukemia-1 (AML-1), as well as the signaling transduction molecules Notch, Janus kinase/signal transducer and activator of transcription (JAK/STAT), and NFκB of the Toll/Cactus pathway (reviewed by Evans *et al.* 2003).

Serpent (*srp*) is one of five GATA factors in Drosophila and is required for both embryonic and larval blood development (reviewed by Rehorn *et al.* 1996; Lebestky *et al.* 2000; Fossett and Schulz 2001). A second GATA factor, Pannier (Pnr), is required for development of the heart (Rehorn *et al.* 1996; Gajewski *et al.* 1999) and larval blood (Mandal *et al.* 2004). Srp is the earliest known factor expressed in hemocyte precursors as its expression is first detected in the procephalic mesoderm (Tepass *et al.* 1994; Rehorn *et al.* 1996). Expression of *srp* is necessary for the differentiation of plasmatocytes and crystal cells in the embryo.

FOG is a zinc finger protein that functions as a transcriptional coregulator. Mammalian FOG1 binds directly to GATA-1 and has a similar loss-of-function phenotype as GATA-1 (reviewed by Cantor and Orkin 2001 and Fossett and Schulz 2001; Chang *et al.* 2002). The corepressor C-terminal binding protein (CtBP) and FOG together regulate hematopoietic lineage commitment in mammals. The Drosophila FOG ortholog, U-shaped (Ush), is expressed in all hemocyte precursors throughout embryonic and larval hematopoiesis (Fossett and Schulz 2001). Physical and genetic interaction between Ush and Srp has been demonstrated to repress crystal cell fate in prohemocytes (Fossett *et al.* 2003; Waltzer *et al.* 2003). Accordingly, *ush* is downregulated in crystal cell precursors and *ush* mutants exhibit an increase in crystal cell number.

AML-1, or *Runx1*, was first isolated as a fusion partner in a chromosomal translocation associated with AML (reviewed by Lutterbach and Hiebert 2000; Rabbitts 1994) and is necessary for definitive hematopoiesis in mammals (Okuda *et al.* 1996; Wang *et al.* 1996). Drosophila Lozenge (Lz), a transcription factor that has 71% identity to the Runt domain of the human protein AML-1, is necessary for crystal cell development during embryonic and larval hematopoiesis (Daga *et al.* 1996; Lebestky *et al.* 2000). *lz* has been shown to function downstream of *srp*; however, additional interactions between these transcription factors initiate hemocyte commitment to the crystal cell lineage (Fossett *et al.* 2003; Waltzer *et al.* 2003) by stage 11 of Drosophila embryonic development. An interesting parallel between mammals and Drosophila is the relationship between AML-1 and FOG. In mammals, AML-1 is a positive regulator of myeloid differentiation, while FOG1 is antagonistic. Similarly in Drosophila, Lz is required for proper crystal cell development (Lebestky *et al.* 2000), while Ush acts as a negative regulator of crystal cell differentiation (Fossett *et al.* 2003; Waltzer *et al.* 2003).

srp-expressing cells that differentiate into plasmatocyte precursors also express the transcription factor Glial cells missing (Gcm) (Bernardoni *et al.* 1997), which is the primary regulator of glial cell differentiation in the nervous system (Hosoya *et al.* 1995; Jones *et al.* 1995). There are two *gcm* genes in Drosophila, and Gcm homologs have also been identified in vertebrates (*gcm-1* and *gcm-2*); however, no hematopoietic function has been associated with these (Altshuller *et al.* 1996; Kim *et al.* 1998; Gunther *et al.* 2000; Schreiber *et al.* 2000). Drosophila *gcm-1* and *gcm-2* double mutants show only a 40% reduction in presumptive plasmatocytes, and only a fraction of mature plasmatocyte markers are detected in the remaining cells (Akiyama *et al.* 1996; Alfonso and Jones 2002), suggesting *gcm* is unlikely to be the only determinant of plasmatocyte differentiation.

Signal transduction pathways common to both Drosophila and mammalian hematopoiesis include Notch, JAK/STAT, NFκB, and receptor tyrosine kinases (RTKs). The Notch pathway is important in many Drosophila developmental processes, including cell fate decisions and cell proliferation in the nervous system, mesoderm, and imaginal discs (Artavanis-Tsakonas *et al.* 1999). Notch signaling has also been shown to be required for the development of embryonic and larval crystal cells and in the proliferation of hemocytes (Duvic *et al.* 2002; Lebestky *et al.* 2003). A specific and distinct role for individual Notch receptors has been identified in mammalian hematopoiesis as well (Walker *et al.* 2001; Kumano *et al.* 2003; Saito *et al.* 2003). While it has been well established that both the JAK/STAT and Toll/Cactus pathways are involved in blood cell production during immune response, it also appears that each participates in normal blood development as well (Mathey-Prevot and Perrimon 1998; Qiu *et al.* 1998). JAK/STAT signaling seems to play a specific role in blood cell differentiation and NFκB plays a role in blood cell proliferation (Luo *et al.* 1997). The RTK, vascular and endothelial growth factor receptor (VEGFR), mediates blood cell migration in mammals (reviewed by Traver and Zon 2002), while another RTK, c-Kit, is important for the proliferation and maintenance of myeloid progenitors (Kelly and Gilliland 2002). Similarly, the Drosophila platelet-derived growth factor and VEGF-receptor-related (PVR) protein is expressed in hemocytes and is required for the proper migration (Heino *et al.* 2001; Cho *et al.* 2002) and survival (Brückner *et al.* 2004) of plasmatocytes.

The parallels between vertebrate and Drosophila hematopoiesis have proven flies to be a useful model system in which to dissect the role of the many signaling pathways involved in blood development. Drosophila genetics allows for comprehensive screens for mutations that disrupt a particular biological process. To identify

additional genes involved in Drosophila hematopoiesis, we have conducted a *P*-element screen to isolate mutations that affect crystal cell development in embryos. Crystal cells, composing only 5% of the total hemocyte population (amounting to ~36 cells in a wild-type embryo), are ideal for a screen of this nature because the total number can be accurately determined. In the current screen we utilized the expression pattern of ProPO A1, a highly specific and sensitive marker for crystal cells. Out of 1040 lines screened, we identified 44 with an abnormal number and/or distribution of crystal cells. These lines were subjected to further phenotypic characterization to establish if other developmental defects exist in these mutants. Using antibodies against the Engrailed and Twist proteins we established that none of the 44 mutations had generalized defects in germ-band patterning and gastrulation, respectively. Collagen IV *in situ* hybridization allowed visualization of plasmatocytes and revealed the subset of mutations with defects in both crystal cells and plasmatocytes.

MATERIALS AND METHODS

Fly stocks: All stocks and crosses used were maintained at 25°, unless mentioned otherwise. A collection of lethal lines with *P*-element insertions on the second and third chromosomes from the Bloomington *P*-lethal collection was screened. The lines are referred to by their P-line numbers throughout the study. All available genotypes are listed in Table 1. P lines were balanced over either *CyO* (*Pw*$^{+}$ *Kr-Gal4*, *UAS-GFP*) or *TM3* (*Pw*$^{+}$ *Kr-Gal4*, *UAS-GFP*) balancer chromosomes (Casso *et al.* 1999). The *GFP* balancers allowed the identification of homozygous mutant embryos, which were analyzed in all subsequent experiments except where indicated. The following stocks were obtained from the Bloomington Stock Center: Oregon-R, Canton-S, *ft*^{4}, *stg*^{4}, *lab*^{2}, *twr*^{1}, *put*^{135}, *osp*^{29}, *neur*^{1}, *cbx*^{05704}, *Catsup*^{1}, *syt*^{N6}, *Gug*^{03928}, *dpp*^{H46}, *scw*^{11}, *tkv*^{7}, *sax*^{4}, *wit*^{B11}, *Med*^{5}, *shn*^{1}, *twi-Gal4*, and *UAS-nuclacZ*. Temperature-sensitive alleles for *twr*^{1} and *put*^{135} were collected at the nonpermissive temperature of 30°. *Sec61α [EP(2)2567]* was obtained from the Szeged Stock Center. Mutant alleles obtained from other laboratories include: *srp*^{neo45} and *srp-Gal4* from R. Reuter, *pap*^{53} from J. Botas, *U2af38*$^{\Delta E18}$ from Donald Rio, *corto*^{420} from F. Peronnet, *smt3*$^{l(2)k01211}$ from J. Schnorr, *FRT(3R)CtBP*^{Rev19} from S. Parkhurst, *Sin3A* from G. Rubin, *hrg*^{1} from T. Murata, *Mad*^{10} and *Mad*^{12} from R. Padgett, *brk*^{M68} and *UAS-brk* from C. Rushlow, *ebi*^{E4} from S. L. Zipursky, and *UAS-Rab5*^{S43N} from M. A. Gonzalez-Gaitan.

Phenotypic analysis: Embryo collections and *in situ* hybridizations were performed in 30-well collection boxes as previously described (Hummel *et al.* 1997). Antisense ProPO A1, GFP, and Collagen IV (CIV) digoxygenin-labeled RNA probes were made from 2.3 kb ProPO A1, 0.74 kb GFP, and 1.6 kb CIV cDNAs as previously described (Daga *et al.* 1996). Rabbit antibodies to β-Gal, GFP, Phospho-Histone H3, and Twist were used. Mouse antibody to Engrailed was used. The Engrailed antibody developed by C. Goodman was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development. The GFP and the Phospho-Histone H3 antibody were obtained from Sigma (St. Louis) and Upstate Innovative Signaling Solutions, respectively. *In situ* hybridization and immunohistochemistry protocols were as previously described (Tepass *et al.* 1994).

Counting methods: Crystal cells from 10 embryos for each line were counted at 40× magnification on a Zeiss compound microscope. The average number of cells hybridized to *ProPO A1* per embryo was calculated and graphed using Excel (Microsoft) and Delta Graph (SPSS, Chicago). The number of cells staining for α-Phospho-H3 was determined in the head region of stage 14 embryos ($n = 3$). The head region for this purpose was defined as the tissue demarcated by the anterior edge of the amnioserosa and the anterior end of the embryo.

Molecular analysis: The sequence of genes interrupted by *P*-element inserts was obtained from the Berkeley Drosophila Genome Project (BDGP; http://www.fruitfly.org/p_disrupt/). The locations of the inserts of four P lines not sequenced by BDGP, *P10412*, *P10596*, *P10675*, and *P11066*, were identified using inverse PCR and sequencing. Blast searches were performed using the standard nucleotide-nucleotide BLAST [blastn] program provided by the NCBI database (http://www.ncbi.nlm.nih.gov:80/BLAST/).

RESULTS AND DISCUSSION

Screen for crystal cell mutants: In Drosophila, most components of the hematopoietic developmental hierarchy are still unknown. We have conducted a genetic screen to identify genes involved in this process. This screen utilizes *in situ* hybridization with ProPO A1, a marker specific for terminally differentiated crystal cells. A collection of 1040 *P*-element lethal lines generated and mapped by the Drosophila Genome Project, covering the second and third chromosomes, was screened. To distinguish the homozygous mutant embryos from those of other genotypes, the *P*-element lines were rebalanced with balancer chromosomes containing *Kruppel* (*Kr*)-*Gal4* driving *UAS-GFP* (Casso *et al.* 1999). The screen identified 44 mutants that affect crystal cell development (Figure 1). Forty-two demonstrate a reduction while crystal cell mislocalization is apparent in one line (*P11622*), and there is one line (*P10555*) with both a reduction and mislocalization of crystal cells.

The number of crystal cells present in wild-type flies was determined by counting the number of *ProPO A1*-positive cells in Ore-R, Canton-S, heterozygous nonmutant *10579/CyO*, *Kr-GFP*, and homozygous nonmutant *P10579* embryos. *P10579*, used as a control, contains a *P*-element insertion that does not affect crystal cells. The combined average of the control fly lines established that wild-type flies have a relatively invariant number (36 ± 2.2) of crystal cells located in two bilateral clusters in the head region of stage 14 embryos. The number of crystal cells in the P-line mutants ranges from 3 to 57% of wild type, with the median number of crystal cells in a mutant embryo being 12.5 (Figure 1A). *P11622* was different from the rest in that it showed wild-type numbers of crystal cells that were grossly mislocalized over the entire embryo.

Of the 44 mutants found, 24 of the lines have *P*-element insertions that are within previously characterized genes (Table 1). To verify the location of each *P*-element inser-

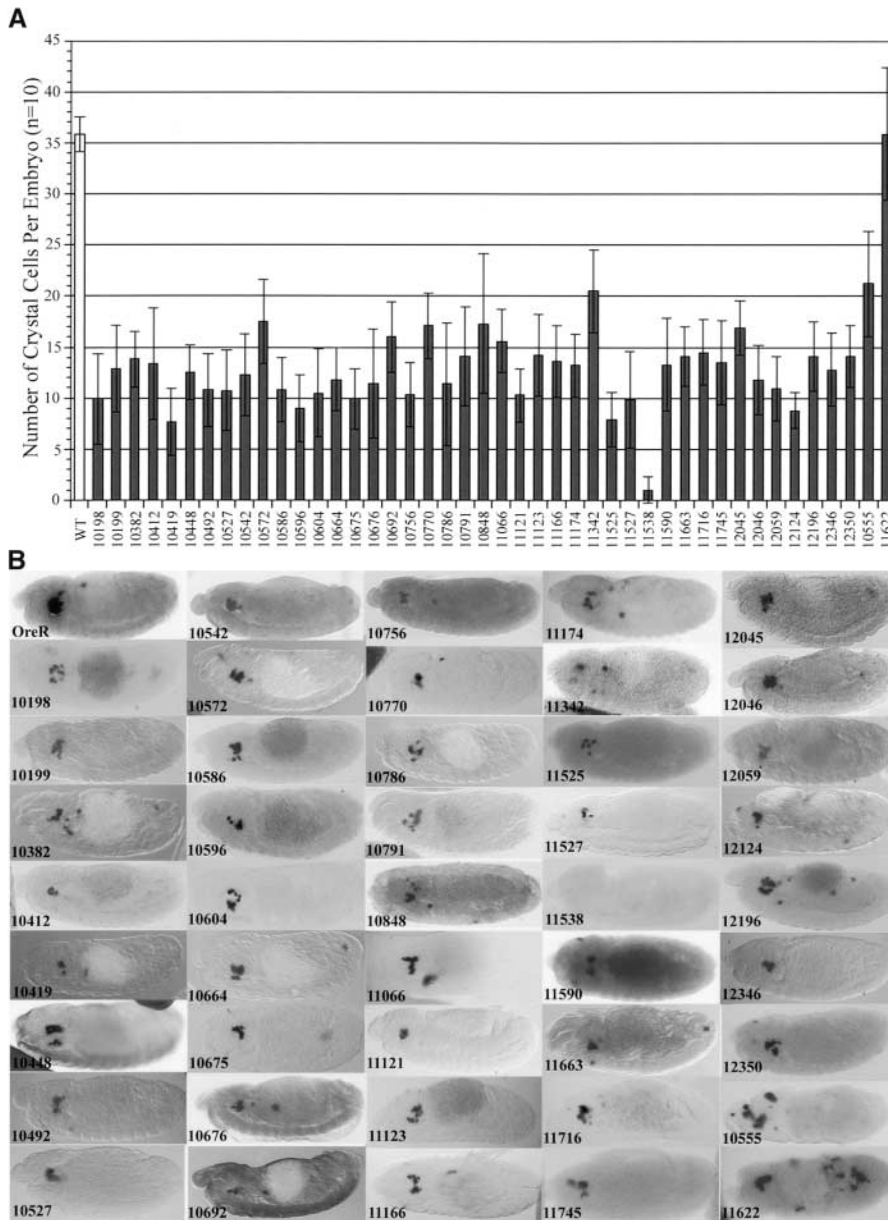

FIGURE 1.—Mutations affecting crystal cell development. (A) Quantitation of the effect of P-line mutants on crystal cell development. The chart shows the average number of crystal cells per embryo ($n = 10$) as marked by *ProPO A1* riboprobe. The wild-type average of 36 is the combined averages of Ore-R, Canton-S, heterozygous nonmutant *P10579/Cyo*, *Kr-GFP*, and homozygous nonmutant *P10579* embryos. (B) Stage 13–14 embryos of wild-type (Ore-R) and mutant P lines hybridized with *ProPO A1* riboprobe marking terminally differentiated crystal cells. Wild-type mature crystal cells can be detected by *ProPO A1* hybridization as early as stage 12 and are grouped in two clusters of 18 each in the head mesoderm. Lines *P10198* to *P12350* show a reduction in the number of crystal cells present. *P10555* and *P11622* show a mislocalization of the mature crystal cells.

tion and its effect on crystal cell development, we tested available loss-of-function mutant alleles of the known genes identified in our screen. Of the 24 known genes, 18 had available loss-of-function alleles, all of which also demonstrate a reduction in crystal cell number, consistent with results from the P lines (Figure 2).

To identify genes disrupted in the remaining 20 P lines, the *P*-element flanking region sequences were obtained from the Drosophila Genome Project when available or were determined in this study. These sequences were used in BLAST searches to identify candidate genes for each of the 20 unknown P lines. One to two candidate genes were identified for 18 of the 20 unknown P lines (Table 1). As with the known genes, the three candidate genes with available loss-of-function alleles were tested for recapitulation of the reduced crystal cell phenotype observed in the P lines. These three are *P10527*, *P10664*, and *P10676*, which mapped to the *Sec61*α, *crossbronx* (*cbx*), and *hiiragi* (*hrg*) loci, respectively. Mutant alleles of these genes all show a reduction in crystal cells consistent with the corresponding P line mutations (Figure 2).

Eleven of the 44 P lines behave as enhancer traps and demonstrate expression in hemocytes and/or crystal cells (Figure 3). Heterozygous and homozygous embryos were examined in this experiment. *P10604*, *P10664*, *P10848*, *P11121*, *P10675*, *P11066*, *P10586*, *P10676*, *P12059*, and *P10756* contain a lacZ within the *P* element that is expressed in embryonic hemocytes in the head region of stage 11 embryos. As examples, *P11066* and *P10586* are shown (Figure 3, A and B). LacZ expression in *P10756* and *P10382* is detected in crystal cells of stage 14 embryos (Figure 3, C and D). These enhancer trap lines nicely illustrate an expression pattern consistent with

TABLE 1

Summary of the mutants identified in the crystal cell screen

P line	Map position	Gene	Candidate gene	Description	Avg. no. of cc
P10198	78 A2–5	*pap*^{L7062}		Component of transcription mediator complex (TRAP240)	9.9
P10199	78 A5–6	*l(3)L5541*^{L5541}	*CG10581*	DUF265, unknown function	12.9
			CG32434	sec7, PH domain	
P10382	24 A1–2	*for*^{k04703}		Protein ser/thr kinase	13.8
P10412	98 C		No CG in area		13.4
P10419	27 C7	*smt3*^{k06307}		Ubiquitin like, nuclear protein tag (*SUMO-1*)	7.6
P10448	21 B7–8	*U2af38*^{k14504}		Component of splicesome	12.5
P10492	29 C1–2	*snRNA:U6atac:29B*^{k01105}		Nuclear mRNA splicing via U12-type spliceosome	10.8
P10527	26 D6–9	*l(2)k03201*^{k03201}	*Sec61α*	Protein transport, cell death	10.7
P10542	46 F1–2	*l(2)k04308*^{k04308}	*CG30011*	pnt/SAM transcription factor	12.3
P10555	55 C9–10	*l(2)08770*^{k04808}	*CG30118*	Mth_Ecto domain	21.2
P10572	37 B8–12	*Catsup*^{k05424}		Regulator of catecholamine metabolism	17.5
P10586	23 B1–2	*syt*^{k05909}		Ca^{2+} phospholipid binding, synaptic vesicle fusion	10.8
P10596	21 B4–6	*l(2)k06019*^{k06019}	*kis*	myb-binding domain, DEAD/DEAH box helicase	9
P10604	47 F1–2	*Fpps*^{k06103}		Sterol biosynthesis, cell division and enlargement	10.5
P10664	46 C1–2	*l(2)k07237*^{k07237}	*cbx*	Ubiquitin-conjugating enzyme	11.8
			CG12744	Zinc finger transcription factor	
P10675	38 B4	*l(2)k07614*^{k07614}	*fok*		9.9
			Klp38B	Chromokinesin, bipoplar spindle assembly	
P10676	43 E15–16	*l(2)07619*^{k07619}	*hrg*	Adenlyltransferase	11.4
P10692	24 D7–E1	*fat*^{k07918}		Contains 34 cadherin repeats	16
P10756	51 D3–5	*l(2)k08015*^{k08015}	*CG10228*	Regulation of nuclear pre-mRNA	10.3
P10770	39 F1–3	*l(2)k08110*^{k08110}	*CG11628*	Guanyl-nt exchange factor, intracellular signaling	17.1
P10786	22 E1–2	*Rab5*^{k08232}		Small monomeric GTPase, Dpp trafficking	11.4
P10791	46 C6–8	*l(2)k08601*^{k08601}	*PKa-R2*	cAMP-dep protein kinase R2	14.1
P10848	30 E1–3	*FKBP59*^{k09010}		Peptidyl isomerase, protein folding	17.3
P11066	53 D11–13	*l(2)k12701*^{k12701}	*CG6301*		15.6
P11121	26 F3–5	*l(2)k14206*^{k14206}	*CG11098*	Putative SMC chromosome segregation ATPase	10.3
			CG13769	Putative leucine-rich ribonuclease inhibitor type domain	
P11123	28 F1–2	*l(2)k14308*^{k14308}	*CG8451*	Sodium-dependent multivitamin transporter	14.2
			CG8419	B-box zinc, C3HC4 ring finger transcription factor	
P11166	21 C2–3	*ebi*^{k16213}		WD40 repeats, F-box, binds E3 ubiquitin ligases	13.6
P11174	36 A4–5	*l(2)k16215*^{k16215}	*CG5953*	MADF domain, transcription factor	13.2
P11342	43 D1–2	*ALDH-III*^{03610}		Oxidizes fatty and aromatic aldehydes	20.5
P11525	99 A5–7	*stg*^{01235}		Tyrosine phosphatase, controls G2/M transition (Cdc 25)	7.9
P11527	84 A1–2	*lab*^{01241}		Homebox transcription factor	9.8
P11538	89 B1–3	*srp*^{01549}		GATA-type zinc finger transcription factor	1
P11590	87 D9–11	*CtBP*^{03463}		Transcription corepressor	13.3
P11622	66 C13/73 D3	*l(3)04069b*^{04069b}	*CG6983*		35.9
			Gug	Transcription corepressor	

(*continued*)

TABLE 1

(Continued)

P line	Map position	Gene	Candidate gene	Description	Avg. no. of cc
P11663	84 A1–2	*twr*^{05614}		Affect eye and macrochaetae	14.1
P11716	82 E5–7	*corto*^{07128b}		RNA transcription factor, component of the centrosome	14.5
P11745	88 C9–10	*put*^{10460}		ser/thr protein kinase, type II TGFβ receptor	13.5
P12045	34 A1–2	*l(2)rK639*^{rK639}	No CG in area		16.9
P12046	35 B1–4	*osp*^{rJ571}		Putative ligand carrier, component of the cytoskeleton	11.8
P12059	57 A3–4	*l(2)s4831*^{s4831}	*CG13434* *CG30153*		10.9
P12124	85 C5–9	*neur*^{j6B12}		E3 type ubiquitin ligase	8.8
P12196	59 E1–2	*l(2)s4830*^{s4830}	*Or59a*	Olfactory receptor	14.1
			CG5357	Putative component of ribosome	
P12346	55 D1–2	*Prp19*^{07838}		Pre-mRNA splicing factor, component of splicesome	12.8
P12350	49 B3–6	*Sin3A*^{08269}		Transcription corepressor	14.1

P lines have been listed in order of their line numbers and are referred to as such throughout this study. Corresponding alleles of each line are listed under Gene. Unknown lines with more than one candidate gene listed have a *P*-element insertion mapped between the loci of two genes. Avg no. of cc, the average number of crystal cells found in homozygous P-line embryos at stage 13–14.

the likely role of these genes in one or more stages of hematopoietic development.

Characterization of isolated mutants: Crystal cells constitute a class of hemocytes derived from the head mesoderm. Genes that, when mutated, cause a change in the number of crystal cells could act directly on the specification of this cell type; alternatively, they could function at an earlier step during the formation of hemocytes or of mesoderm in general. For example, a double mutation of *twist* and *snail* that has no mesoderm also lacks hemocytes, including crystal cells (V. Hartenstein, unpublished data); likewise, in *bicaudal*, where early events in the specification of the anterior body axis fail to take place, head structures, including head mesoderm and all hemocytes, are missing (Tepass *et al.* 1994). Finally, changes in the number of crystal cells could be due to a generalized effect on cell division. To distinguish between these different steps at which the genes uncovered in this screen might act, we used the markers Collagen IV (plasmatocytes; Yasothornsrikul *et al.* 1997), Twist (mesoderm; Furlong *et al.* 2001), Engrailed (metamerically reiterated expression along antero-posterior axis; Bejsovec and Wieschaus 1993), and Phosphohistone-H3 (marker for cells entering mitosis; Hendzel *et al.* 1997).

All mutations showed grossly normal staining with anti-Twist and anti-Engrailed antibodies, indicating that changes in crystal cell number were not caused by a global defect in axis formation, gastrulation, or segmentation (Figures 4 and 5). To assess if the mutations contribute to general cell proliferation defects, we employed the α-Phosphohistone-H3 (Phospho-H3) antibody, which identifies cells in late G2 through anaphase when chromatin is condensed (Hendzel *et al.* 1997). To quantitate the number of cells entering mitosis we chose the head region of stage 14 embryos in which only neuroblasts continue dividing. Choosing this later stage maximizes our chance of detecting general proliferation defects in mutants with significant maternal components, and the relatively small number of cells dividing at this time allows accurate quantitation. Six mutant lines were identified with α-Phospho-H3 as having cell-cycle defects (Figure 6): *P10198*, *P11166*, *P11525*, *P11663*, *P12045*, and *P12196*. These were all shown to have a significant increase in the number of α-Phospho-H3-positive cells, indicating that cells were arrested in G2 or mitosis at a higher than normal rate. Alternatively, the elevated number of α-Phospho-H3-positive cells may elicit an escalated apoptotic response leading to a decrease in the number of differentiated crystal cells. Included in this group are two previously known cell cycle regulators, *ebi* and *stg*. Ebi functions in normal G1 arrest of cells in the peripheral nervous system and central nervous system. Boulton *et al.* (2000) discovered an increase in BrdU incorporation of cells in *ebi* mutants. Failure to arrest in G1 results in an increased number of cells that are Phospho-H3 positive in this mutant. *stg* mutants have been shown to arrest cells in G2 phase, thus accounting for the increase in α-Phospho-H3 staining (Neufeld *et al.* 1998).

We found that six crystal cell-reduction mutant lines also demonstrate a significant reduction in the number

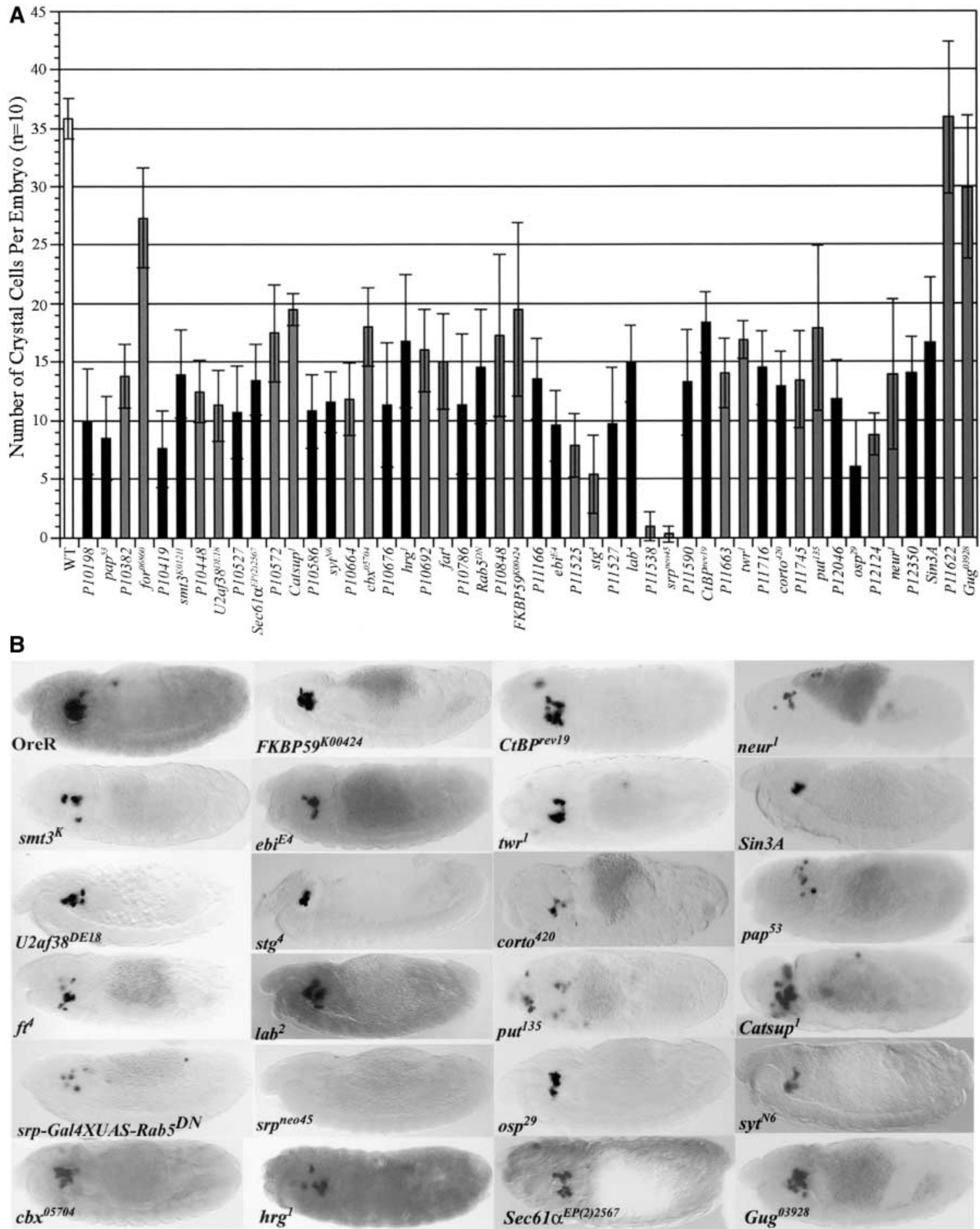

FIGURE 2.—Loss-of-function alleles of known and candidate genes of P-line mutants. (A) Quantitation of the effect of loss-of-function alleles of known and candidate genes on crystal cell development. The chart shows the average number of crystal cells per embryo as marked by *ProPO A1* riboprobe ($n = 10$). The wild-type average of 36 is the combined averages of Ore-R, Canton-S, heterozygous nonmutant *P10579/Cyo*, *Kr-GFP*, and homozygous nonmutant *P10579* embryos. The complete $Rab5^{DN}$ genotype is $srp\text{-}Gal4/UAS\text{-}Rab5^{DN}$. for^{06860} is the strongest allele available; however, is not a null allele, thus accounting for the reduced crystal cell effect as compared to line 10382. (B) Stage 13–14 mutant embryos were hybridized with *ProPO A1* riboprobe. All recapitulate the crystal cell reduction of their corresponding P line except for *Gug*, which is unlikely to be the insertion site of *P11622*, as it does not recapitulate the mislocalized phenotype.

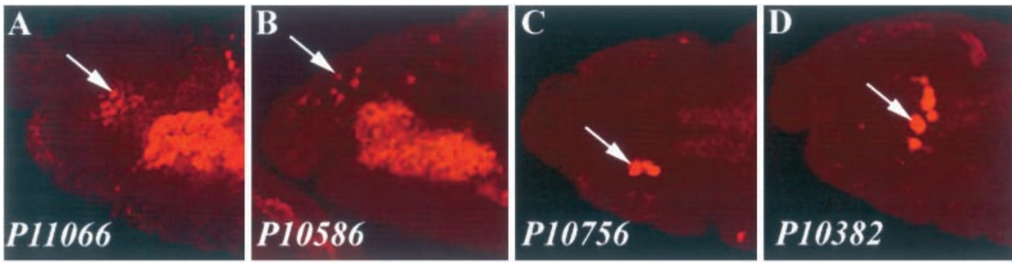

Figure 3.—Enhancer trap expression patterns of P lines. Four examples of P lines contain P-*lacZ* inserts expressing *lacZ* in hemocytes. Embryos of all stages were stained with α-β-gal antibody. Arrows indicate a representative hemocyte in each embryo. (A and B) Stage 11 embryos in which the enhancer trap lines show expression in hemocytes. (C and D) Stage 14 embryos in which enhancer trap expression is evident in mature crystal cells.

of plasmatocytes (Figure 7). These are *P10848*, *P11525*, *P11538*, *P11716*, *P11745*, and *P10676*. Plasmatocytes were found to be mislocalized in *P10555*, where instead of an even distribution throughout the entire embryo, plasmatocytes were clustered in the anterior one-third of this mutant. Similarly, *P11622* also exhibits a mislocalization of plasmatocytes into large clumps throughout the embryo (Figure 7).

Figure 4.—Characterization of the effect of crystal cell mutants on mesoderm development. Stage 8 embryos of wild-type (Ore-R) and mutant P lines stained with α-Twi antibody are shown. All 44 P-line mutants demonstrate proper Twist expression and mesoderm patterning.

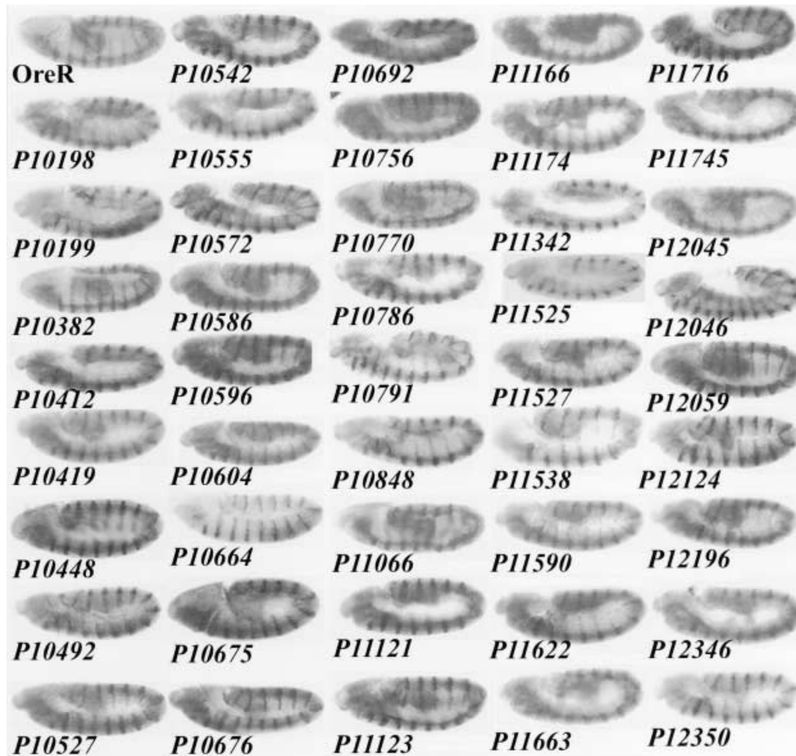

Figure 5.—Characterization of the effect of crystal cell mutants on anterior/posterior segmentation. Stage 10 embryos of wild-type (Ore-R) and mutant P lines stained with α-En antibody are shown. All 44 P-line mutants demonstrate wild-type expression of Engrailed.

Classes of genes that reduce crystal cell development: With the exception of the P line interrupting *srp*, we did not observe a complete elimination of crystal cells in any of our mutants although they show significant reductions. There can be a number of reasons for this partial loss. First, approximately half of the P lines that interrupt previously characterized genes have a maternal complement of RNA. Future analysis of germline clones for each of the P-line mutants will determine if maternal contribution is responsible for the observed partial development of crystal cells. As a test case, we found that *ebi* germline clones have a significantly more severe defect in crystal cells than that seen with the zygotic loss-of-function P line (data not shown). Second, P-line insertions tend to cause hypomorphic rather than complete null mutations. However, we tested all available null alleles corresponding to our mutants and these still eliminate only a fraction of the crystal cell population. Finally, the incompleteness in the number of crystals cells lost could reflect the flexible nature of the blood development system, allowing multiple developmental signal response pathways that lead to cell differentiation.

By excluding general defects in the patterning of the embryo, we were able to show that the P-line mutants likely have a direct role in crystal cell development. Of course, this does not preclude the function of these genes in other tissues. In fact, many genes identified in the screen are pleiotropic. Our results do suggest that the observed defects in hematopoiesis are not secondary consequences of gross patterning defects. The expression pattern of several of the enhancer trap lines in embryonic hemocytes and the fact that eight of the mutants identified affect both crystal cells and plasmatocytes also indicate that these *P*-element insertions interrupt genes involved in blood development. Known and candidate genes isolated in this screen can be placed into four groups: transcriptional regulators, signaling molecules, cell proliferation regulators, and other miscellaneous factors.

Transcriptional regulators: *srp*, a GATA factor homolog required for all hematopoiesis in Drosophila, was found in the screen to ablate crystal cells. This is reasonable as Srp is necessary for the expression of Gcm and Lz, the transcription factors that define the two main branches of Drosophila hematopoiesis (Lebestky *et al.* 2000; Fossett *et al.* 2003; Waltzer *et al.* 2003). Mutations in genes encoding transcriptional corepressors, CtBP and Sin3A, cause a significant reduction in the number of crystal cells. CtBP plays several roles as a corepressor that binds short-range repressors such as Knirps, Snail, Hairless, and Kruppel (Nibu *et al.* 1998). In Xenopus, CtBP interacts with FOG to suppress GATA factors and thereby block erythroid development. U-shaped has also been shown to repress crystal cell development; however, loss of CtBP does not cause an increase in crystal cell numbers as with Ush. CtBP also interferes with both Dpp and Notch signaling pathways (reviewed by Morel *et al.* 2001; Turner and Crossley 2001). Sin3A forms a complex with histone deacytelases, RpAp46/48, histone-binding proteins, and with many other proteins to act as a transcriptional repressor (reviewed by Ahringer

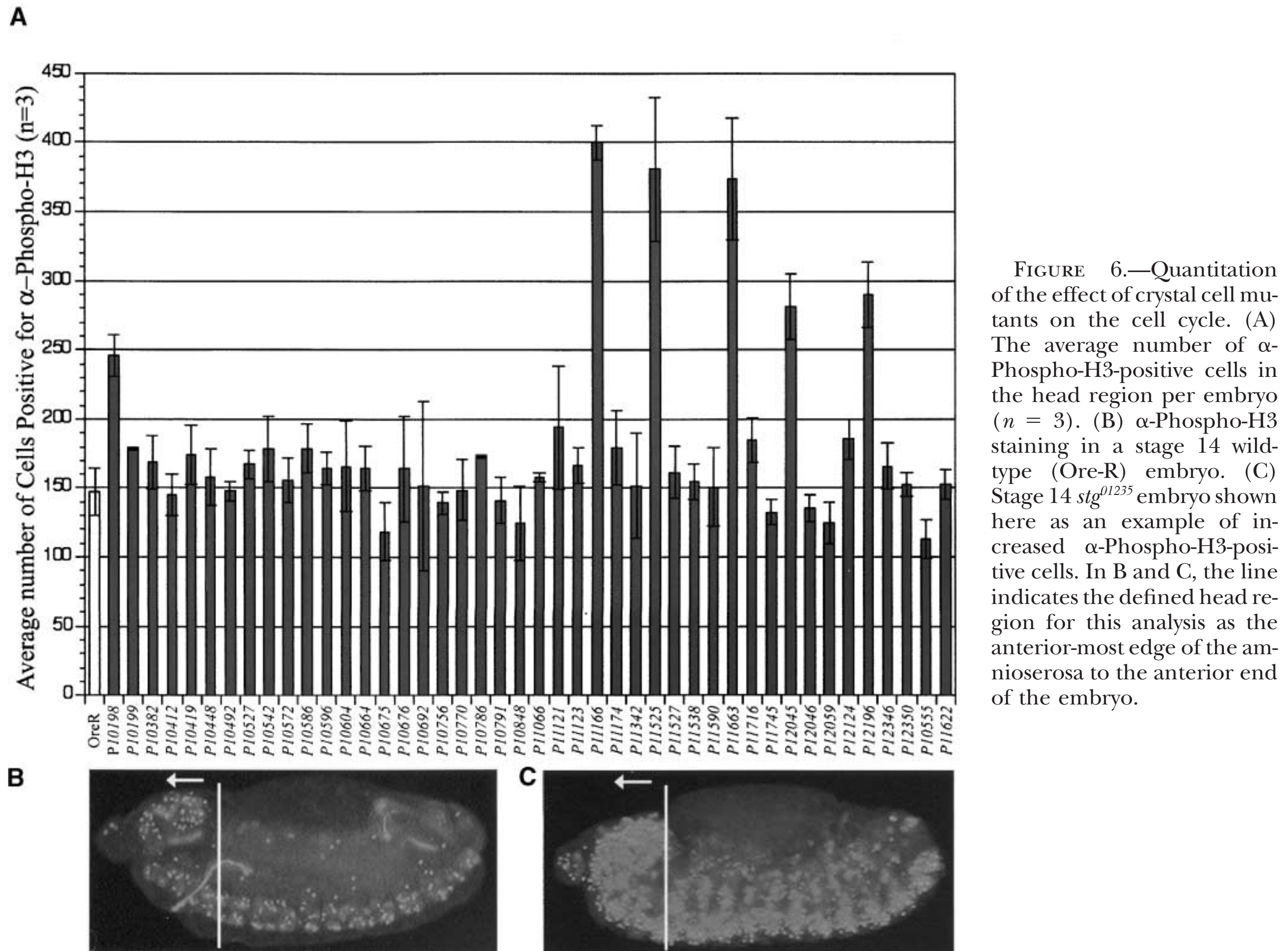

FIGURE 6.—Quantitation of the effect of crystal cell mutants on the cell cycle. (A) The average number of α-Phospho-H3-positive cells in the head region per embryo (n = 3). (B) α-Phospho-H3 staining in a stage 14 wild-type (Ore-R) embryo. (C) Stage 14 *stg*^{01235} embryo shown here as an example of increased α-Phospho-H3-positive cells. In B and C, the line indicates the defined head region for this analysis as the anterior-most edge of the amnioserosa to the anterior end of the embryo.

2000). Thus, Sin3A could play a role in repressing a signaling factor, allowing for the preferential development of the crystal cell lineage over plasmatocytes.

Signaling pathways: One factor with a relationship to the Toll pathway (Smt3) and two with roles in Notch signaling (Neur and Hrg) were detected in the screen. *smt3*^{k06307} and *neuralized* (*neur*^{j6B12}) were found to cause a particularly strong reduction of crystal cells. Smt3 is the Drosophila homolog of SUMO-1, an ubiquitin-like protein, which may conjugate with other proteins and alter their function. One such role of Smt3 is to regulate the nuclear localization and transcriptional activation of Dorsal, a well-characterized component of the Toll signaling pathway, which is involved in both hematopoiesis and immunity (Qiu *et al.* 1998; Wu and Anderson 1998; Bhaskar *et al.* 2000, 2002). The loss of *smt3* may lead to the loss of Dorsal import and interfere with normal NFκB-like signaling, which, one may speculate, is needed for proper crystal cell development. The E3 type ubiquitin ligase, Neur, was discovered as a neurogenic gene required to direct neuroectodermal cells from a neural to an epidermal fate (Boulianne *et al.* 1991; Lai and Rubin 2001). Neur positively regulates Notch signaling in a non-cell-autonomous manner by targeting Delta for internalization and subsequent degradation (Lai *et al.* 2001). Loss of Notch signaling, which regulates Lz expression in crystal cell precursors, is one possible explanation for the decrease in crystal cells caused by *neur*^{j6B12}. *hiiragi* (*hrg*), a candidate gene for 10676, encodes an adenyltransferase that is important in the regulation of the Notch pathway (Bachmann and Knust 1998). Hrg downregulates the transcription of *Serrate* during larval wing development (Murata *et al.* 1996). Therefore, Hrg may be important in the temporal regulation of Notch signaling during crystal cell development. Note that previous work has established that the Notch pathway is essential for crystal cell development (Lebestky *et al.* 2003).

Four mutants could be linked to the Decapentaplegic (Dpp) pathway: *punt* (*put*), *Rab-protein 5* (*Rab5*), *labial* (*lab*), and *poils aux pattes* (*pap*). Drosophila Dpp is a TGF-β family member, and the TGF-β pathway has been known to play a pleiotropic role in all stages of mammalian hematopoiesis (reviewed by Ruscetti and Bartelmez 2001); however, its role in Drosophila hematopoiesis has not been previously explored. Loss-of-function mu-

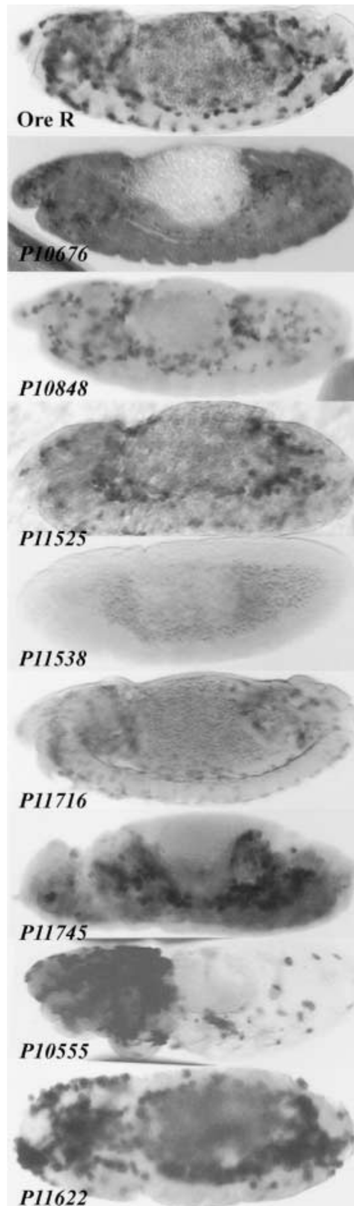

FIGURE 7.—Characterization of the effect of crystal cell mutants on plasmatocytes. Stage 13–14 embryos of wild-type and mutant P lines hybridized with *CIV* riboprobe are shown. *P10676*, *P10848*, *P11525*, *P11538*, *P11716*, and *P11745* show a reduction in the number of plasmatocytes. Crystal cell mislocalization mutants *P10555* and *P11622* also show a mislocalization of the plasmatocytes.

tants in the Dpp pathway, dpp^{H46}, scw^{11}, tkv^{7}, put^{135}, Mad^{12}, Med^{5}, and shn^{1}, all cause significant reductions in the number of crystal cells while mutants in the second type I Dpp receptor, *sax*, and the second type II Dpp receptor, *wit*, do not (Figure 8, A–K).

Dpp acts in an indirect and non-cell-autonomous manner during Drosophila blood development. Active Dpp signaling, marked by pMad staining, is detected in cells adjacent to, but not colocalizing with, Srp-positive cells in the head mesoderm (Figure 8, L–N). *brk*, a negative regulator of Dpp target genes, is able to block crystal cell development when ectopically expressed throughout the mesoderm using the *twi-Gal4* driver, but not when expressed in prohemocytes using a *srp-Gal4* driver (Figure 8, O and P). These studies support an indirect role of Dpp in crystal cell development and a requirement of Dpp signaling in the head mesoderm adjacent to Srp-positive prohemocytes.

To investigate the effect of ectopic *dpp* throughout the mesoderm, we expressed *UAS-dpp* with a *twi-Gal4* driver. Ectopic Dpp signaling in the mesoderm causes mislocalization of crystal cells to further posterior within the embryo (Figure 8Q). A null allele of *brk*, which has been shown to cause an upregulation of Dpp target genes (JAZWINSKA *et al.* 1999a,b; reviewed by NAKAYAMA *et al.* 2000), phenocopied this phenotype (Figure 8R), thereby indicating a role for Dpp target genes in the patterning of the mesoderm to establish a background in which normal crystal cell development may occur. Additionally, put^{10460} also causes a reduction in the number of plasmatocytes in the embryo.

Cell proliferation regulators: Two crystal cell mutants with known roles in cell proliferation are *fat* and *ebi*. A member of the Cadherin superfamily, Fat is important in cell-cell adhesion and plays an autonomous role in the regulation of cell proliferation (MAHONEY *et al.* 1991). Loss of *fat* function may cause a defect in the proliferation of crystal cells due to a misregulation of proliferation signals. *ebi*, which encodes a GTP-binding molecule containing WD40 repeats and an F-box domain, causes a significant reduction in crystal cells. WD40 repeats can bind E3 type ubiquitin ligases and bring them in contact with F-box-bound proteins that are to be degraded. Ebi regulates cell proliferation by limiting the entry of cells into the S-phase of the cell cycle during neuronal development (BOULTON *et al.* 2000) and we show, with α-Phospho-H3 staining, that *ebi* also affects cell cycle regulation during embryogenesis. However, in the regulation of Suppressor of Hairless during photoreceptor development, *ebi* functions downstream of EGF signaling in cell fate specification (TSUDA *et al.* 2002). Given that ebi^{k16213} does not affect plasmatocytes, it is possible that the role of *ebi* in crystal cell development is in patterning the precursor and not in the regulation of cell proliferation.

Other *bona fide* cell cycle factors identified by the screen include String (Stg), a Cdc25 homolog, Centrosomal and chromosomal factor (Ccf or Corto), and Twisted bristle roughened eye (Twr). Stg is a nuclear tyrosine phosphatase that controls the G2/M transition of the cell cycle by dephosphorylating Cdc2, a mitotic kinase (reviewed by EDGAR 1994; EDGAR *et al.* 1994a,b). stg^{01235} causes a strong reduction in the number of crystal cells and also reduces the number of plasmatocytes. Little is known about *twr* except that hypomorphic alleles affect the eye and macrochaetae (LEWIS *et al.* 1980). Corto is necessary for proper condensation of mitotic chromosomes and the maintenance of chromosome structure during mito-

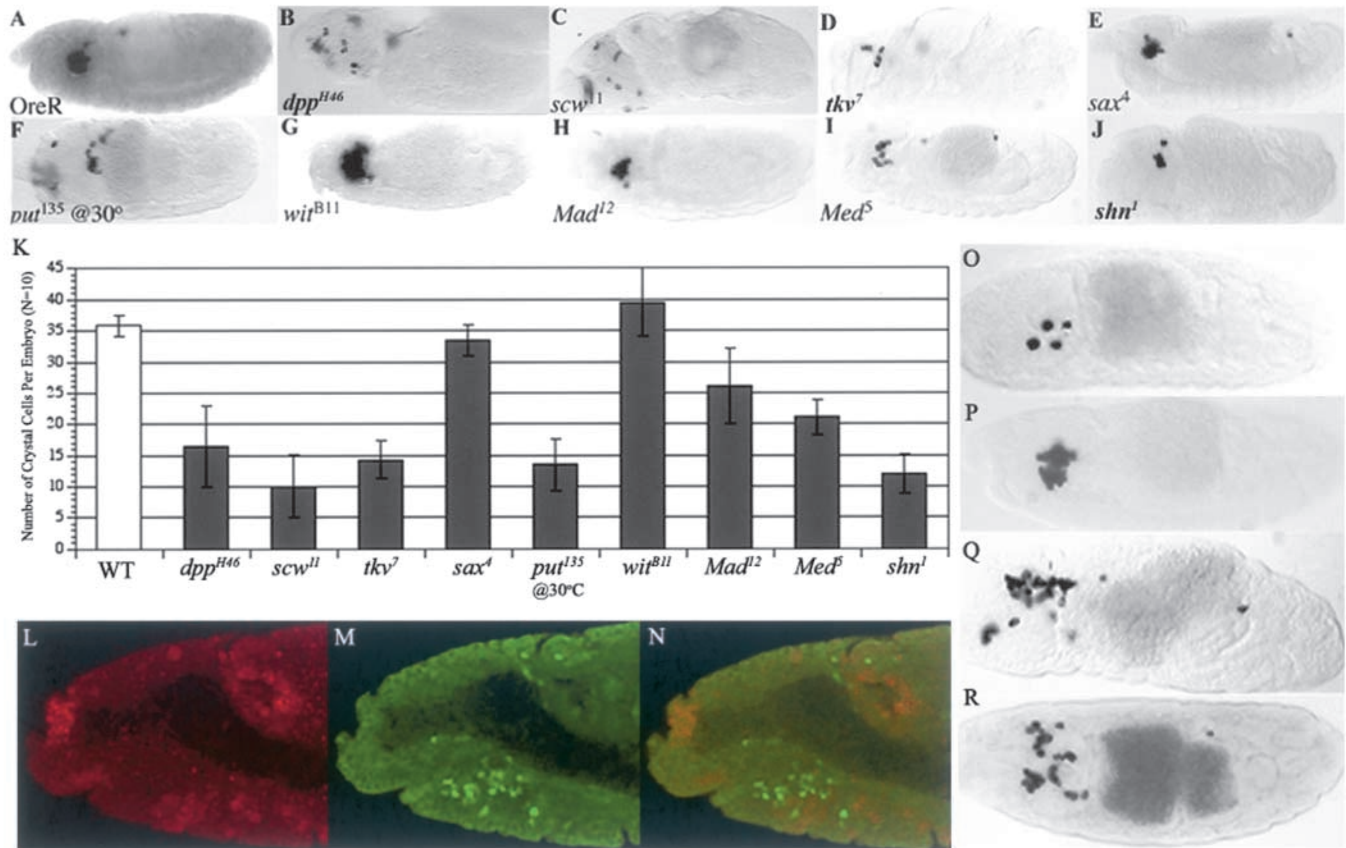

FIGURE 8.—Characterization of the effect of Dpp pathway mutants on crystal cells. (A–J) Stage 13–14 mutant embryos were hybridized with *ProPO A1* riboprobe. The graph in K demonstrates the number of crystal cells found in each mutant ($n = 10$). (L–N) Double staining of stage 11 *srp-Gal4* × *UAS-βGal* embryos with (L) α-pMAD (red) and (M) α-βGal (green) antibodies. (N) The merged image of α-pMAD and α-βGal staining shows α-pMAD-positive cells are adjacent to the α-βGal-positive cells. (O–R) Stage 14 embryos hybridized with *ProPO A1* riboprobe. (O) Ectopic expression of *brinker* driven by *twi-Gal4*. (P) Ectopic expression of *brinker* driven by *srp-Gal4*. (Q) Ectopic expression of *dpp* driven by *twi-Gal4*. (R) *brk*^{M68}.

sis and interphase (Kodjabachian *et al.* 1998). Corto is also a putative regulator of Hox genes (Lopez *et al.* 2001). *pap*^{L7062} and two unknown mutant lines, *P12045* and *P12196*, were also identified by this study to affect the cell cycle; however, their role in this process remains to be investigated.

Other factors: Eleven mutants were identified in the screen that correspond to genes that have interesting functions, but do not fit into any one category: *U2 small nuclear riboprotein auxiliary factor 38* (*U2af38*), *Prp19, foraging* (*for*), *Farnesyl pyrophosphate synthase* (*Fpps*), *FK506 binding protein 59* (*FKBP59*), *Aldehyde dehydrogenase type III* (*Aldh-III*), *outspread* (*osp*), *synaptotagmin* (*syt*), *Sec61*α, *Catecholamines up* (*Catsup*), and *crossbronx* (*cbx*). RNA splicesome components *U2af38* and *Prp19*, both necessary for RNA splicing (Tarn *et al.* 1993; Rudner *et al.* 1996), were found to affect crystal cell development. Loss of RNA splicing can lead to the improper processing of many important developmental proteins. However, it is unclear why the development of hematopoietic cells should be particularly sensitive to the function of these proteins. *for* encodes a serine/threonine kinase involved in larval feeding behavior (Renger *et al.* 1999) and is expressed in both the head mesoderm and mature crystal cells, consistent with a potential role in crystal cell development. *Fpps* encodes a protein required for sterol biosynthesis, which in plants is important in membrane stability, cell growth, proliferation, and respiration (Gaffe *et al.* 2000). *FKBP59* encodes a peptidylprolyl isomerase, which has been indicated to function as a molecular chaperone during protein folding. Consistent with a role in crystal cell development, FKBP59 has been shown to have a unique expression pattern in embryonic lymph glands (Zaffran 2000). Interestingly, FKBP59 is also expressed in a cell type- and developmental stage-specific pattern during mouse male germ cell differentiation (Sananes *et al.* 1998). *Aldh-III*, which causes a weak reduction of crystal cells, is known as the tumor-associated Aldh due to its upregulation in several human tumor types (Park *et al.* 2002). Aldh's are considered to be general detoxifying enzymes that oxidize toxic biogenic and xenobiotic aldehydes (reviewed by Yoshida *et al.* 1998). *osp* encodes a putative transmembrane receptor involved in developmental processes (Ashburner *et al.* 1999). Syt is a calcium phospholipid-binding protein that aids in synaptic vesicle

fusion by facilitating the formation of the SNARE complex (reviewed by Tucker and Chapman 2002). *Sec61α* encodes a protein thought to be involved in protein transport and signal recognition particle-dependent membrane targeting and translocation. Catsup is a negative regulator of tyrosine hydroxylase, the limiting factor in catecholamine metabolism (Stathakis *et al.* 1999). *Cbx* encodes an ubiquitin-conjugating enzyme that is involved in spermatogenesis (Castrillon *et al.* 1993).

These studies have documented several signaling pathways, transcriptional regulators, and other factors that were not previously known to be involved in Drosophila hematopoiesis. We have used the terminal marker (ProPO A1) for crystal cells in our screen, which in principle allows us to identify mutations encompassing the entire span of crystal cell differentiation from the earliest precursor specification to late expression of *ProPO A1*. This larger collection of mutants can now be further classified according to their developmental defects using earlier markers such as Lozenge. In future work we hope to determine the individual role of each of these genes in this important developmental process. Given the conservation of genes known to be involved in mammalian and Drosophila hematopoiesis, it is not unreasonable to expect that a subset of the genes identified in this study will be determined to have functions in hematopoiesis across various species.

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