Pendulin, a *Drosophila* Protein with Cell Cycle-dependent Nuclear Localization, Is Required for Normal Cell Proliferation

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Abstract. We describe the dynamic intracellular localization of Drosophila Pendulin and its role in the control of cell proliferation. Pendulin is a new member of a superfamily of proteins which contains Armadillo (Arm) repeats and displays extensive sequence similarities with the Srp1 protein from yeast, with RAG-1 interacting proteins from humans, and with the importin protein from Xenopus. Almost the entire polypeptide chain of Pendulin is composed of degenerate tandem repeats of ~42 amino acids each. A short NH_2-terminal domain contains adjacent consensus sequences for nuclear localization and cdc2 kinase phosphorylation. The subcellular distribution of Pendulin is dependent on the phase of cell cycle. During interphase, Pendulin protein is exclusively found in the cytoplasm of embryonic cells. At the transition between G2 and M-phase, Pendulin rapidly translocates into the nuclei where it is distributed throughout the nucleoplasm and the areas around the chromosomes. In the larval CNS, Pendulin is predominantly expressed in the dividing neuroblasts, where it undergoes the same cell cycle-dependent redistribution as in embryos. Pendulin is encoded by the oho31 locus and is expressed both maternally and zygotically. We describe the phenotypes of recessive lethal mutations in the oho31 gene that result in a massive decrease or loss of zygotic Pendulin expression. Hematopoietic cells of mutant larvae overproliferate and form melanotic tumors, suggesting that Pendulin normally acts as a blood cell tumor suppressor. In contrast, growth and proliferation in imaginal tissues are reduced and irregular, resulting in abnormal development of imaginal discs and the CNS of the larvae. This phenotype shows that Pendulin is required for normal growth regulation. Based on the structure of the protein, we propose that Pendulin may serve as an adaptor molecule to form complexes with other proteins. The sequence similarity with importin indicates that Pendulin may play a role in the nuclear import of karyophilic proteins and some of these may be required for the normal transmission and function of proliferative signals in the cells.

In higher eukaryotes, the regulation of cell proliferation rates is tightly integrated into the developmental program of an organism. In the past few years, it has become clear that many of the central components driving the progression of the cell cycle have been highly conserved during evolution. It appears that perhaps in all eukaryotes, protein kinases that are related to the $p34^{cdc2}$ kinase from the fission yeast *Schizosaccharomyces pombe* are required to trigger the transition from G1 to S and from G2 to M-phase (Nurse, 1990). The activities of $p34^{cdc2}$ and related kinases can be regulated during the cell cycle by changes in their phosphorylation status, by their association with cyclin proteins that serve as activating subunits, or by the binding of inhibitory subunits (for reviews see Solomon, 1993; Dunphy, 1994; Hunter and Pines, 1994; Morgan, 1995). It is thought that developmental and other external inputs can act through these mechanisms to control p34 kinase activites and thereby regulate entry into or exit from the cell cycle. At present, only little is known about the molecular mechanisms that connect developmental events with the regulation of p34 kinase activities and the control of cell proliferation. However, it is obvious that a disruption of these control mechanisms can either lead to a failure of cell proliferation or, in the case of disrupted inhibitory inputs or constitutive activation, to oncogenesis.

The rate-limiting components of mitotic regulation are the most likely targets for developmental inputs and feedback control. Recent studies in *Drosophila* have shown that, at successive stages of embryogenesis, different cell cycle components become rate-limiting, resulting in distinct modes of cell cycle regulation. The first 13 cycles occur as rapid, synchronous divisions in an embryonic syncytium and rely solely on maternal components (Foe and Alberts, 1983). Between nuclear divisions 2 and 7, the overall activity and abundance of cdc2 kinase/cyclin com-

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plexes remain constant during the cycle. During this period, the mitotic cycle may be regulated by the local degradation of cyclin molecules that are associated with components of the mitotic apparatus, i.e., with substrates of cdc2 kinase, and by rate-limiting steps in the re-organization of the mitotic apparatus (Edgar et al., 1994). The exponential increase of these substrates appears to result in a depletion of cyclin levels. This makes cdc2 kinase activity dependent on cyclin re-synthesis during cycles 7 to 13, which could explain the progressive slowing of the mitotic cycle and its dependence on the nucleo:cytoplasmic ratio during this period (Edgar et al., 1986). During interphase of cycle 14, the programmed degradation of the maternal String/cdc25 phosphatase results in phosphorylation and inactivation of cdc2 kinase and, therefore, arrest in G2 (Edgar and O'Farrell, 1990). The subsequent cell cycles 14 to 16 are triggered by zygotic transcription of *string*, which occurs in temporally and spatially defined pulses that appear to be determined by developmental control genes. At the end of cycle 16, most cells of the embryo exit from the cell cycle and arrest in G1. This arrest is, at least in part, due to the transcriptional downregulation of the G1 cyclin E, which is also thought to depend on developmental control genes (Knoblich et al., 1994).

Mitotic divisions resume in a subset of larval cells which proliferate to generate structures of the adult fly. These cells are located in the imaginal discs which give rise to the appendages and other parts of the adult body surface, in groups of histoblasts which will form the abdomen, in imaginal cells of internal organs, in the central nervous system, in the gonads, and in the blood cell producing organs. The mechanisms that determine the spatial and temporal patterns of these divisions have not yet been characterized. Growth factor-like molecules such as Dpp and Wingless, through unknown downstream events, play a role in determining proliferation patterns in imaginal discs, since ectopic expression of these molecules promotes outgrowth of supernumerary structures (Struhl and Basler, 1993; Campbell et al., 1993; Basler and Struhl, 1994; Diaz-Benjumea et al., 1994). An important aspect of mitotic regulation is the termination of proliferation, which occurs in imaginal discs during the first day of puparium formation (Fain and Stevens, 1982; Graves and Schubiger, 1982). This arrest occurs in a disc-autonomous fashion and requires cell–cell interactions (for a review see Bryant and Schmidt, 1990).

The analysis of mutations that affect cell proliferation and the characterization of the corresponding genes can yield new insights into developmental and biochemical mechanisms of growth control. Because gene products with such functions are often abundantly deposited into the egg by the mother, it is quite common that embryos mutant for these genes divide normally, and proliferation is only disrupted during larval stages when the maternal products become limiting. One group of mutations that affect cell proliferation causes a failure of growth, while a second group has the opposite phenotype and results in hyperplastic or neoplastic overgrowth of larval tissues (Gatti and Baker, 1989; for reviews see Glover, 1989; Gateff, 1978; Mechler et al., 1991). Genes that are affected by this second group of mutations are called tumor suppressor genes. In most cases, the overproliferation caused by any of these mutations is limited to a specific larval tissue, such as the imaginal discs, the central nervous system, the ovaries, or the blood cells. However, the molecular characterization of several of these tumor suppressor genes showed that their expression is not restricted to the tissues that display overgrowth in the mutants (Jacob et al., 1987; Lützelschwab et al., 1987; Woods and Bryant, 1991; Mahoney et al., 1991; Boedigheimer and Laughon, 1993; Watson et al., 1992; Konrad et al., 1994). This indicates that the gene products of many tumor suppressor genes are components of more general cellular mechanisms such as cell–cell communication, intracellular signal transduction, or cell cycle control, that are essential to allow growth regulation by tissue-specific signals.

Here we describe a new gene and its product, called Pendulin, that is required for normal cell proliferation. We describe the intracellular distribution of the Pendulin protein, which shuttles between the cytoplasm and the nucleus in a cell cycle–dependent manner. Mutations in the gene that encodes Pendulin, *oho31*, cause abnormal growth in larval tissues and the formation of melanotic tumors, suggesting that Pendulin acts as a tumor suppressor in hematopoietic cells. Like many regulatory components of the cell cycle, Pendulin has been conserved in evolution, and genes with extensive sequence similarities to *Drosophila* Pendulin have been identified from yeast to humans.

Materials and Methods

Isolation and Sequencing of Genomic and cDNA Clones

A 325-bp PstI/RsaI fragment from the cDNA of the pair-rule gene *even-skipped* (*eve*), which encodes the COOH-terminal portion of the *eve* protein (Frasch et al., 1987), was used as a probe to isolate related genomic sequences under low stringency hybridization conditions. Hybridization of an EMBL4 genomic library (obtained from M. Goldberg, Cornell University, Ithaca, NY) was performed as described in McGinnis et al. (1984). Washes were done twice in 4 × SSC for 15 min each at room temperature and twice at 45°C. The 3.7-kb EcoRI fragment of one of the positive phage clones, e2.18, was subcloned into pGEM1 plasmids. This insert was used to isolate cDNAs, including cDNA #7, from an embryonic cDNA library (obtained from L. Kauvar, UCSF, San Francisco, CA). The EcoRI fragment from cDNA #7 was cloned into pGEM1 and a 5′fragment from this cDNA was used to isolate cDNAs with longer 5′ ends from a 0–4 h embryonic cDNA library (Brown and Kafatos, 1988). cDNA #23 was isolated from this second screen. cDNAs and genomic DNAs were sequenced with the Sequenase kit (US Biochemicals, Cleveland, OH) using single-stranded templates. It is unclear whether the low similarities with *eve* are significant. Searches of the SwissProt and GenBank databases for sequence motifs related to Pendulin repeat sequences were performed using the FASTA and FINDPATTERN programs (Devereux et al., 1984).

Northern Analysis

Northern analysis was performed as described in Dohrmann et al. (1990). Total RNA from mutant larvae was isolated with GlassMax RNA Spin Cartridges (GIBCO BRL, Gaithersburg, MD). Extracts from 20 larvae yielded ~50 mg RNA/cartridge. For size determination, an RNA ladder (GIBCO BRL) was hybridized with λ phage DNA.

Antibody Production and Western Analysis

Three different constructs were made to produce bacterially expressed Pendulin proteins. First, a partially digested SalI fragment from cDNA #7 which encodes Pendulin residues 13-522 was cloned into the expression vector pUR290 (Rüther and Müller-Hill, 1983) to express a Pendulin/lacZ fusion protein. Second, a fragment comprising the same portion of the

ORF was cloned as a SmaI (in pGEM1 polylinker)/ClaI fragment into the expression vector pT7-7 (Tabor and Richardson, 1985). For expression of a full-length protein, an NdeI site was introduced at the first ATG with a mutagenic PCR-primer using cDNA #23 as a template. A 380-bp NdeI/NcoI PCR-derived 5′ fragment was ligated together with a ~1.8-kb NcoI/EcoRI 3′ fragment from cDNA #23 into pT7-7 to generate the full-length construct. Proteins were expressed from these constructs and purified as described in Dohrmann et al. (1990). The first immunization of a rabbit was with the Pendulin/lacZ fusion protein, and subsequent boosts were with Pendulin$^{13\text{-}522}$. This fusion protein, or the full length protein, were used for conjugation to CNBr-Sepharose and affinity purification of Pendulin antibodies (Dohrmann et al., 1990).

For Western analysis, embryos and animals of different developmental stages were homogenized in PBS, mixed immediately with SDS/PAGE loading buffer, and boiled. Equal amounts of proteins were loaded on a 10% polyacrylamide gel and after separation the proteins were transferred electrophoretically onto nitrocellulose. Incubation with affinity-purified Pendulin antibodies, alkaline Phosphatase-conjugated secondary antibodies, and visualization with NBT/BCIP were performed according to standard procedures.

Immunofluorescent Antibody Stainings

Fixations and antibody stainings of embryos were performed as described in Frasch (1991). For double stainings, embryos were first incubated with mixed primary antibodies and subsequently with mixed secondary antibodies which were conjugated with FITC or Texas red (Jackson ImmunoResearch, West Grove, PA). For conventional immunofluorescence, DNA was stained with Hoechst dye (0.5 μg/ml). For confocal laser microscopy, embryos were treated with 2 mg/ml RNaseA during the first antibody incubation to allow specific staining for DNA with propidium iodide (20 μg/ml, incubated for 10 min prior to mounting). Embryos were mounted in 90% glycerol, 25 mM Tris/HCl, pH 8, 1% propyl gallate, and 0.05% phenylene diamine. Ganglia were fixed for 30 min in the same buffer as the embryos, rinsed twice with PBS, transferred in series into 100% ethanol, and rehydrated into PBS. The staining procedure was essentially as for the embryos, using affinity-purified Pendulin antibodies that were preabsorbed with fixed tissues from oho31 mutant larvae. A Zeiss Axiophot was used for conventional immunofluorescence, the confocal scanning laser microscope MRC-600 (Bio-Rad) for confocal imaging of embryos, and a Leica confocal microscope for CNS stainings. Figures were processed and assembled using Adobe Photoshop 2.5.1 and printed on a Kodak XL7700 printer. Kodak technical pan was used for all b/w photomicrographs.

Bromodeoxyuridine Stainings of Larval Tissues

Larvae were incubated with bromo-deoxyuridine (BrdU)[1] and processed as described in Truman and Bate (1988), except that tissues were fixed with 4% formaldehyde in buffer B (Frasch, 1991). Incubation with a monoclonal antibody against BrdU (Becton Dickinson, Mountain View, CA) was followed by incubation with a biotinylated anti-mouse antibody and a horseradish peroxidase/avidin complex (VECTASTAIN ABC Elite kit; Vector Labs, Burlingame, CA). Detection was with DAB/H_2O_2 using standard conditions.

Determination of Circulating Hematocyte Concentrations

Hematocyte counts were performed essentially as described by Zinyk et al. (1993). Larvae were anesthetized with ether, placed into 100 ml halocarbon oil on a teflon-well slide (Polysciences, Niles, IL), pierced with a hypodermic needle, and quickly removed. To estimate the volume of the recovered hemolymph, the diameter of the drop was determined with an ocular micrometer and compared with the diameters of drops the volume of which had been determined with glass capillaries. Subsequently, a coverslip was mounted and the cells were counted under phase contrast.

P-element Lines and P-excisions

The P-insertion 155-22 was obtained in the genetic screen described in Azpiazu and Frasch (1993). Insertions near the Pendulin gene were identified by hybridizations of Pendulin sequences to plasmid-rescued DNAs from pools of flies which carried random insertions of P-lacW elements in their genomes. To determine the insertion site, flanking sequences on either side were determined using a primer complementary to inverted repeat sequences and the rescued plasmids as templates. The P-stock *l(2)144/1* was kindly provided by T. Török (Szeged, Hungary). To determine its insertion site, fragments of genomic DNA were amplified by PCR, using a primer complementary to inverted repeat sequences of the P-element that was paired with primers binding to genomic regions of the Pendulin locus, and sequenced.

For excisions of the P-insertion 144/1, jump starter males with the genotype w/Y; P/CyO; Δ2-3, Sb/+ were produced and crossed with w; Sco/CyO females. White-eyed offspring (P-rev/CyO females or P-rev/Sco males) were individually mated with w; Sco/CyO flies to establish 50 lines of P-revertants. Flies from these lines with the excised chromosome in trans to CyO were crossed among each other to test for homozygous lethality. Genomic DNAs from 20 lethal lines were tested for deletions and the absence of P-sequences by genomic Southern analysis and by PCR analysis using primers PK7 and MF35 (Fig. 7 *B*). More precise determination of deletion endpoints was done by restriction mapping of truncated PCR products and by PCR reactions with primers MF35 and DPNde (Fig. 7 *B*).

Results

Molecular Structure and Evolutionary Conservation of Pendulin

The Pendulin gene was discovered in an attempt to isolate novel genes with regulatory roles during embryonic development. One of the genomic clones obtained from this molecular screen (see Materials and Methods), e2.18, mapped to the locus 31A on the second chromosome. In situ hybridizations with whole embryos and oocytes, using a 3.8-kb EcoRI fragment from e2.18 as a probe, suggested initially that this fragment encodes a gene that is involved in the spatial subdivision and segmentation of the early embryo. High levels of Pendulin mRNA were detected in nurse cells, the oocyte, and in cleavage stage embryos (data not shown). During blastoderm, the maternal transcripts disappeared from the terminal regions, and a segmental pattern of Pendulin mRNA was observed between ~20 and 80% egg length. Subsequent studies revealed that, unlike the mRNA, the Pendulin protein maintains its ubiquitous distribution during blastoderm, and immunocytochemical and genetic analyses indicated that Pendulin plays a role in cell proliferation (see below). After gastrulation, high levels of Pendulin mRNA were only observed in neuroblasts and pole cells.

Pendulin cDNAs were isolated and detected transcript patterns in embryos that were identical to those observed with the genomic probe. The size of the longest cDNA, #23 (2,522 bp), is in close agreement with the ~2.6-kb Pendulin mRNA transcripts detected by Northern analysis. The longest conceptual open reading frame initiates with an ATG preceded by a perfect match to consensus sequences for translational start sites (Cavener, 1987), and encodes a polypeptide of 522 amino acids with a calculated molecular mass of 57,802 D and a pI of 5.0 (Fig. 1 *A*, *DPend*). A prominent feature of the polypeptide sequence is a series of degenerate tandem repeats of typically 42 amino acids each, which span almost the entire protein molecule. A total of ten and one half contiguous repeat sequences can be discerned, and the repeats 2 to 9 (amino acids 64 to 474) share the highest sequence similarities

1. *Abbreviations used in this paper*: BrdU, bromo-deoxyuridine; APC, Adenomatous Polyposis Coli; NLS, nuclear localization sequences.

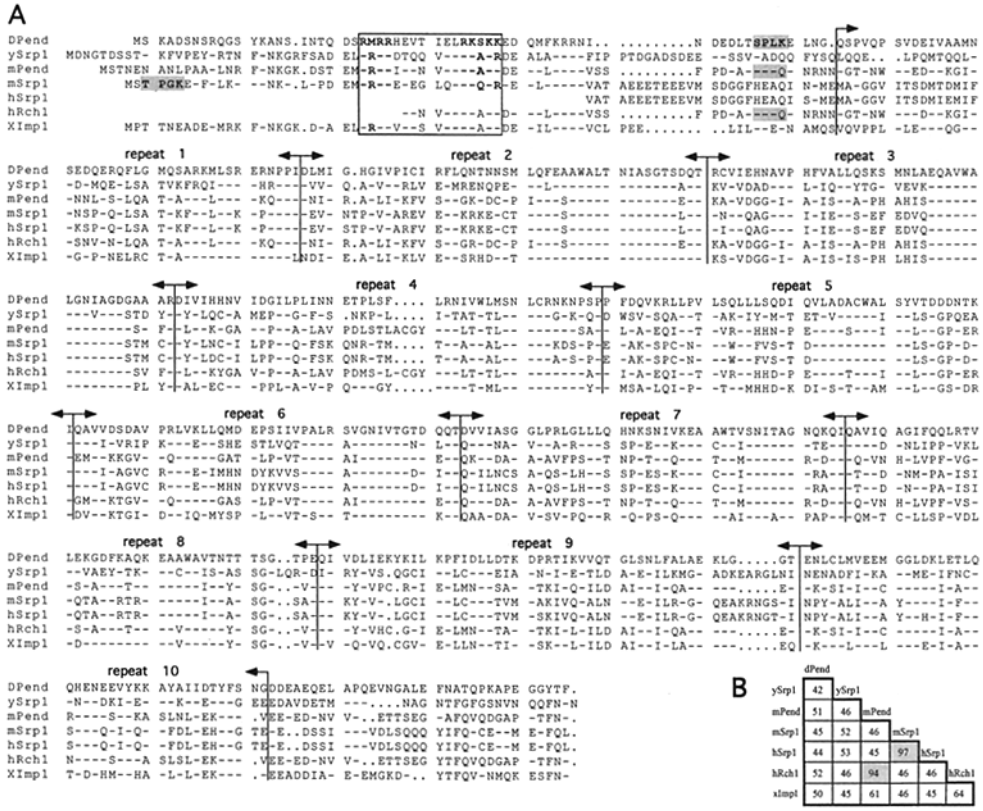

Figure 1. (*A*) Amino acid sequence of the Pendulin gene product (*DPend*) and sequence alignments to related proteins from other species. The positions of internal repeats 1–10 (see Fig. 2) are indicated by vertical lines. Dashes indicate amino acid identities and periods represent gaps introduced to improve the alignments. The putative bipartite nuclear localization sequences are boxed and in bold face. The consensus cdc2 phosphorylation sequences are shaded. (*B*) Percentage of amino acid identities between different Pendulin-related proteins. The numbers for proteins that are likely to represent homologs in mouse and humans are shaded. *DPend*, *Drosophila* Pendulin; *ySrp1*, yeast Srp1; *mPend*, mouse Pendulin; *mSrp1* and *hSrp1*, mouse and human SRP1; *hRch1*, human Rch1; *XImp1*, Xenopus importin1. The sequence data for the cDNA and amino acid sequences of *Drosophila* Pendulin are available from GenBank/EMBL/DDBJ under accession number U12269.

among each other (Figs. 1 *A* and 2). The region covered by these tandem repeats contains predominantly hydrophobic amino acid residues, whereas the NH_2-terminal portion (amino acids 1 to 63) displays a hydrophilic amino acid composition with many basic residues. The NH_2-terminal domain contains a motif resembling the bipartite nuclear localization sequences (NLS) that have been identified in nucleoplasmin, Swi5p, p53, and various other nuclear proteins (Fig. 1 *A*; Robbins et al., 1991). Similar to nucleoplasmin, Swi5p, p53, and other proteins, the putative nuclear targeting sequence of Pendulin is adjoined by a potential phosphorylation site for cdc2 kinase (Fig. 1 *A*; Moll et al., 1991; Addison et al., 1990).

Polypeptides that share between 42 and 52% amino acid sequence identities with *Drosophila* Pendulin have been identified in *Saccharomyces cerevisiae* (Srp1: Yano et al., 1992; Küssel and Frasch, 1995), in the mouse (Pendulin: Küssel and Frasch, 1995; and mSRP1: Cortes et al., 1994), in humans (Rch1: Cuomo et al., 1994; hSRP1: Cortes, et al., 1994; and NPI-1: O'Neill and Palese, 1995) and in *Xenopus* (importin: Görlich et al., 1994) (Fig. 1 *A* and *B*). Yeast *SRP1* was initially isolated as a suppressor of RNA polymerase I mutations (Yano, et al., 1992), and the human homologs were isolated through their interactions with the V(D)J recombination inducing protein RAG-1 (Cuomo, et al., 1994; Cortes, et al., 1994) or with influenza virus nucleoprotein (O'Neill and Palese, 1995). *Xenopus* importin was isolated based on its activity to promote nuclear import of karyophilic proteins (Görlich et al., 1994). All of these proteins share a structure of ten and one half repeats with *Drosophila* Pendulin and contain short, less well conserved, polypeptide sequences at their NH_2 and COOH termini that are not part of the repeat structures (Fig. 1 *A*). However, in the NH_2-terminal portions of all members of this protein family from which complete sequences are available, we identified consensus sequences for bipartite nuclear localization signals (NLS's; Fig. 1 *A*, *boxed*). With the exception of yeast SRP1, the NLS's of all of these proteins are adjoined by a consensus sequence for cdc2/Cdc28 phosphorylation (Fig. 1 *A*, *shaded*). These findings demonstrate that Pendulin-like genes have been conserved during evolution and suggest that the biochemical functions of their gene products are required in processes that are common to all eukaryotes.

Pendulin Is a Member of a Superfamily of Proteins with Armadillo (Arm) Repeats

A search of the SwissProt and GenBank databases detected additional protein sequences that display repeat structures related to those of Pendulin, albeit with considerably lower degrees of similarities than the ones de-

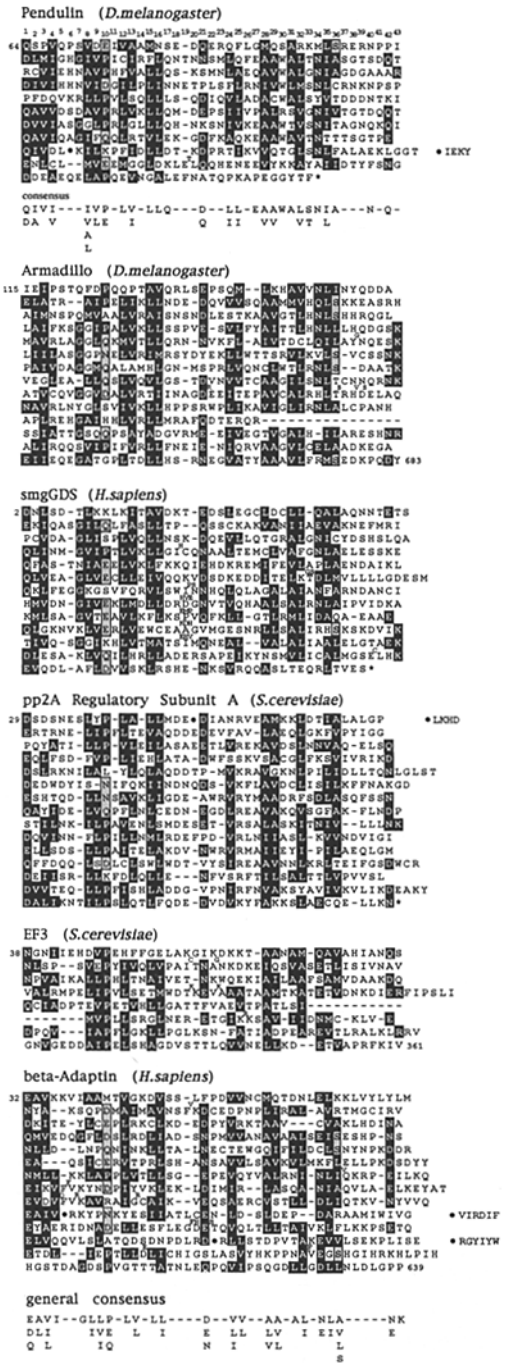

Figure 2. Sequence alignments of Pendulin and other proteins that contain Arm repeats. Identical or related amino acid residues that occur at corresponding positions in repeats of the different proteins are highlighted in black. Related amino acids were grouped as follows: (*V*, *L*, *I*, *A*, and *M*); (*E*, *D*, *Q*, and *N*); (*R* and *K*). At positions where two groups are prevalent, amino acids of the less abundant group are marked with shaded boxes. Amino acids in the consensus sequences for the Pendulin repeats (*top*) and for all proteins shown (*bottom*) are ordered from top to bottom according to their prevalence and residues that were present in no more than 10% of the repeats were omitted. Single residues or pairs of amino acids that were looped out to maximize alignments are shown in small letters above the corresponding lines, and larger groups of such residues are symbolized by a dot and shown to the right of the lines. Asterisks indicate COOH termini of polypeptides.

scribed above. As shown in Fig. 2, these include the *Drosophila* segmentation gene product Armadillo and its vertebrate homologs, β-catenin and plakoglobin (Riggleman et al., 1989; McCrea et al., 1991; Franke et al., 1989); the mammalian guanine nucleotide exchange factor smgGDS (Kikuchi et al., 1992); regulatory subunits of protein phosphatase 2A from yeast and humans (van Zyl et al., 1992; Hemmings et al., 1990); the yeast translation elongation factor EF3 (Qin et al., 1990); and the conserved adaptor molecules from coated pits, α- and β-adaptin (Kirchhausen et al., 1989; Robinson, 1989; Ponnambalan et al., 1990). All of these proteins contain 8–15 tandem repeats, consisting of ~42 amino acids each with amino acid residues at particular positions being conserved between the repeats and among the different proteins (Fig. 2, *black boxes*). Thus, *Drosophila* Pendulin and its counterparts from mouse, humans and yeast are members of a larger superfamily of proteins with Armadillo-like repeats (Arm repeats). The similarities among several members of this superfamily, including two additional proteins which are the protein kinase substrate p120 (Reynolds et al., 1992) and the tumor suppressor Adenomatous Polyposis Coli (APC) (Groden et al., 1991; Kinzler et al., 1991), have also been noted by Peifer et al. (1994).

Pendulin Is Expressed in Two Phases of Development

In Northern analysis of poly(A)$^+$ RNA from different developmental stages, we detected a single band corresponding to a 2.6-kb mRNA. High levels of Pendulin mRNA transcripts are present in 0–2 h embryos (Fig. 3 *A*). Because there is only a small amount of transcription during these stages and in situ hybridization detected strong staining for Pendulin mRNA in oocytes (data not shown), it is likely that these mRNAs are maternally derived. In agreement with the results from in situ hybridization, the mRNA levels decrease significantly after four hours of embryonic development and only faint signals are observed in late stage embryos and young larvae. Increasing Pendulin mRNA levels are evident in second instar larvae and are followed by dramatic increases of expression levels in pupal stages. The highest transcript levels are observed in adults, particularly in females, in which expression occurs presumably in the germ line.

We used affinity purified polyclonal antibodies raised against a bacterially expressed Pendulin polypeptide to analyze the developmental profile of the Pendulin gene product. In Western analysis with whole extracts, these antibodies detected a major band of 54 kD, which can often be resolved into a closely adjacent doublet (Fig. 3 *B*). The bacterially produced, full-length protein migrates with a similar mobility. High levels of Pendulin protein are detected during the first half of embryogenesis, reaching a maximum between 4 and 8 h of development. The protein is already present in 0–2-h old embryos, presumably being supplied maternally or translated from maternal mRNA. At this developmental stage, and more weakly in adult females, an additional band is seen that migrates with a significantly slower mobility (57 kD). We do not know whether this is a modified, maternal form of Pendulin, or a different gene product with common antigenic epitopes. Between 2 and 8 h after fertilization, several faster migrat-

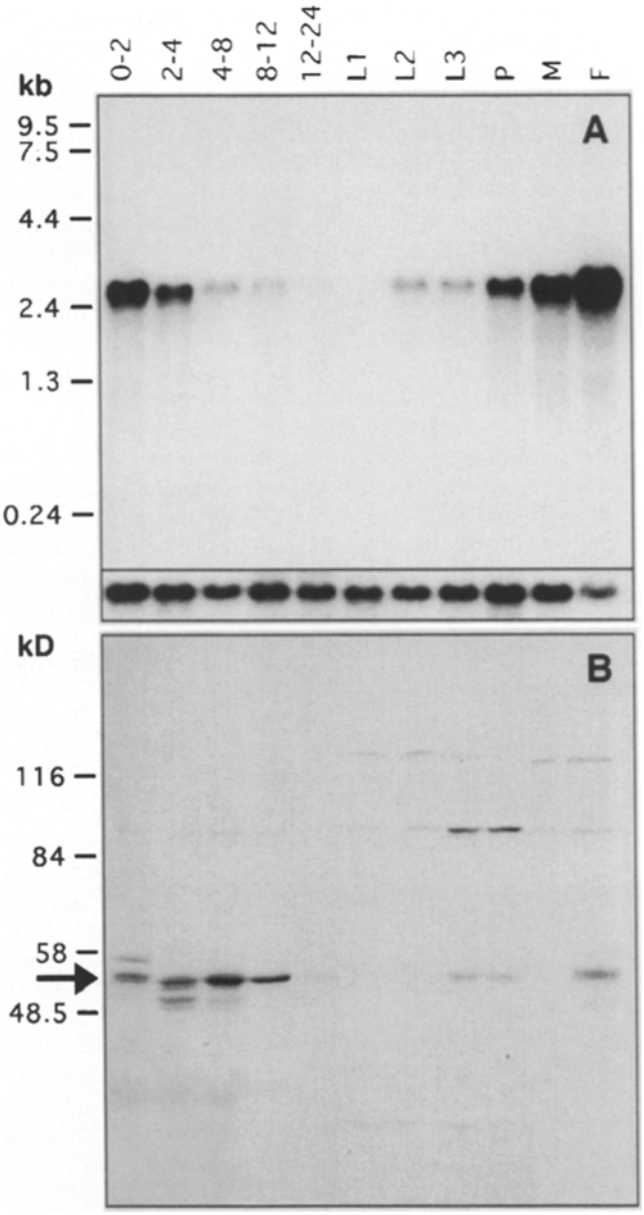

Figure 3. Northern and Western analysis of Pendulin mRNA and protein expression. (*A*) Poly(A)$^+$ RNA from 0- to 2-h, 2- to 4-h, 4- to 8-h, 8- to 12-h, 12- to 24-h embryos, from first (*L1*)-, second (*L2*)-, and third (*L3*)-instar larvae, from pupae (*P*), and from adult males (*M*) and females (*F*) was tested. Pendulin mRNAs migrate at 2.6 kb. The relative amounts of poly(A)$^+$ RNA in each lane were estimated by hybridization with an α-tubulin probe (*bottom*). (*B*) Total protein homogenates (25 μg protein/lane) from the same stages as in *A* were tested on a Western blot with affinity-purified Pendulin antibodies. The antibodies recognize a major band, or doublet, at ~54 kD and several minor species above or below the main band. Two bands above 58 kD that are recognized by the antibodies are of unknown identity.

ing bands appear, which are likely to be in vivo degradation products of Pendulin. Only a faint band of Pendulin is detected between 12 h of embryogenesis and second instar larval stages, but protein expression increases again from the third larval instar onwards. Unlike adult females, very little protein is detectable in adult males, despite the presence of high levels of mRNA. These differences in the profiles of the mRNA and protein levels indicate that Pendulin protein expression is controlled by both transcriptional and posttranscriptional mechanisms. In summary, Pendulin is expressed in a maternal/early embryonic phase, and again during morphogenesis in late larval and pupal stages.

Pendulin Translocates from the Cytoplasm into the Nucleus in a Cell Cycle-dependent Fashion

We used immunofluorescence to analyze the subcellular distribution of Pendulin and observed that the protein changes its localization during the cell cycle. Fig. 4 (top four rows) shows confocal images of nuclei and their surrounding cytoplasm during the 12th nuclear division cycle of blastoderm embryos stained with Pendulin antibodies. During interphase, the Pendulin protein is located in the cytoplasm and excluded from the nuclei. Upon entry into mitosis, Pendulin rapidly migrates into the nuclei. During prophase, when the chromatin condenses, there is increased staining intensity in the nuclei. Because we estimate that this transition occurs in the range of a few seconds (see below), this higher intensity is probably due to an increased access of antibodies to their epitopes rather than to net protein synthesis. The Pendulin protein does not accumulate on the chromatin in the nuclei, but rather it is distributed throughout the nucleoplasm. At metaphase and anaphase, the nuclear staining intensity decreases, and while Pendulin is still located in the areas around the chromosomes, it is excluded from the spindle apparatus. The protein is distributed into the daughter nuclei along with the chromosomes, and telophase nuclei display higher staining intensities, similar to prophase nuclei. During decondensation of the chromatin, when the nuclei enter interphase, Pendulin rapidly redistributes into the cytoplasm. Until the 13th nuclear division cycle, residual levels of Pendulin persist in the cytoplasm during mitosis, but during subsequent cell divisions, the protein shifts quantitatively into the nuclei at mitosis (Fig. 4, *bottom row*). Because of the lower number of nuclei and the high levels of maternal Pendulin in earlier stages, the nuclear compartment might be saturated and unable to take up all of the Pendulin protein before cell cycle 14.

We examined how early during mitosis does the translocation from the cytoplasm into the nuclei occur. Fig. 5 *A* shows a field of nuclei at the transition from interphase to the 13th mitosis from an embryo that was stained for Pendulin (left) and for nuclear lamins and DNA (right). Since mitosis occurs in a wave-like fashion in blastoderm embryos, the nuclei in the bottom section of the picture are still in interphase, those in the middle are in the process of entering mitosis, and the ones in the top section are in early prophase and display condensed chromatin. The Pendulin protein initiates nuclear translocation during a very early stage of mitotic entry, at a time when the nuclear envelope is still intact (Fuchs et al., 1983; Frasch et al., 1988) and chromatin condensation is not yet visible (Fig. 5 *A*). In some of the transitional nuclei, accumulation of Pendulin can be observed at the nuclear envelope. Since a mitotic wave proceeds from the poles to the equator of the embryo in ~30 s and the field in Fig. 5 *A* comprises

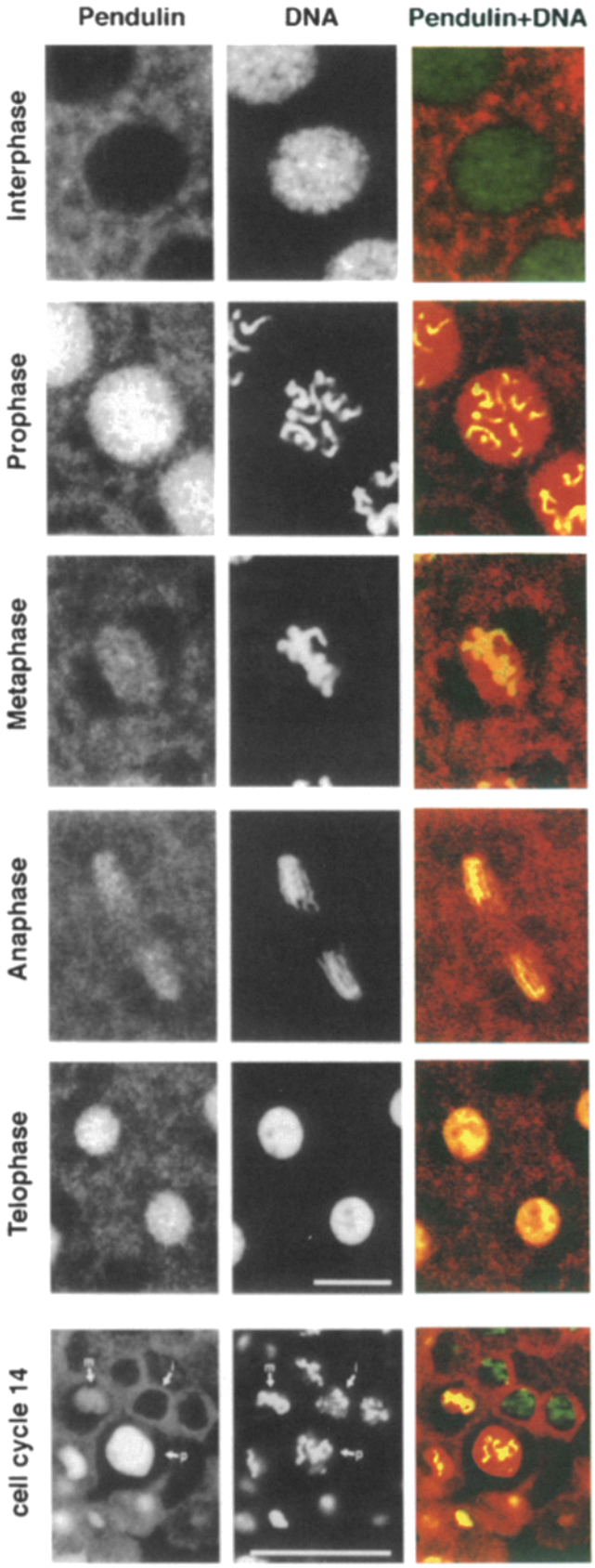

Figure 4. Subcellular redistribution of Pendulin during the cell cycle. Embryos were stained with Pendulin antibodies (*left*) as well as propidium iodide to visualize chromatin (*middle*), and analyzed by confocal microscopy. The overlay of the two stainings (*right*) shows Pendulin distribution in red and chromatin in green. (*Top five rows*) Mitotic stages from embryos undergoing nuclear cycle 12, high magnification views. Pendulin is cytoplasmic during interphase and predominantly nuclear during mitosis. Bottom row: View of a mitotic domain in cell cycle 14. In interphase cells (*i*), Pendulin is localized strictly in the cytoplasm, whereas in prophase (*p*) and metaphase (*m*), the cytoplasm is depleted of Pendulin and the protein is exclusively nuclear. Bars: (top five rows)10 μm; (bottom row) 25 μm.

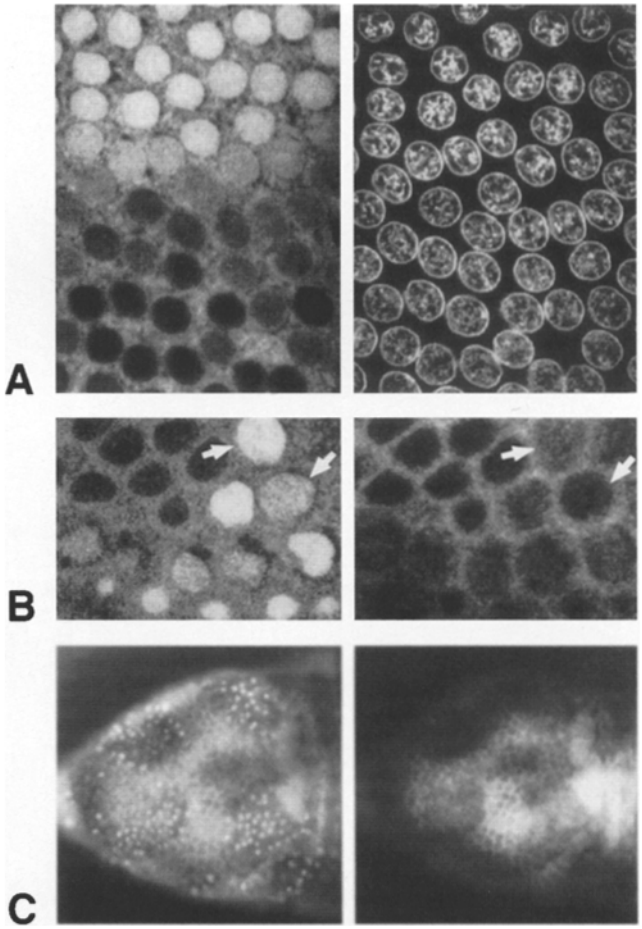

Figure 5. Nuclear entry of Pendulin at the G2/M-phase transition. (*A*) Confocal microscopy of an embryo stained for Pendulin (*left*) and for lamin plus DNA (*right*). Shown is a mitotic wave in nuclear cycle 12, with nuclei on the bottom section being in interphase and those in the top section in prophase. Transitional stages are seen in the middle region, where the nuclei contain increasing levels of Pendulin, and where some staining is evident at the nuclear envelope. (*B*) Confocal microscopy of a mitotic domain (cell cycle 14), stained for Pendulin (*left*) and cyclin B (*right*). A nucleus in the early stage of G2/M-phase transition (arrows to the right) has accumulated low levels of Pendulin and weakly stains for cyclin B. A nucleus that has progressed further (arrows to the left) is stained more strongly for both proteins, indicating a simultaneous redistribution of Pendulin and cyclin B into the nucleoplasm. (*C*) Conventional immunofluorescence microscopy with Pendulin antibodies (*left*) and cyclin B antibodies (*right*). Shown are mitotic domains in the dorsal head region of an embryo undergoing cell cycle 14. In the mitotic domains, Pendulin is nuclear, and cyclin B is being degraded.

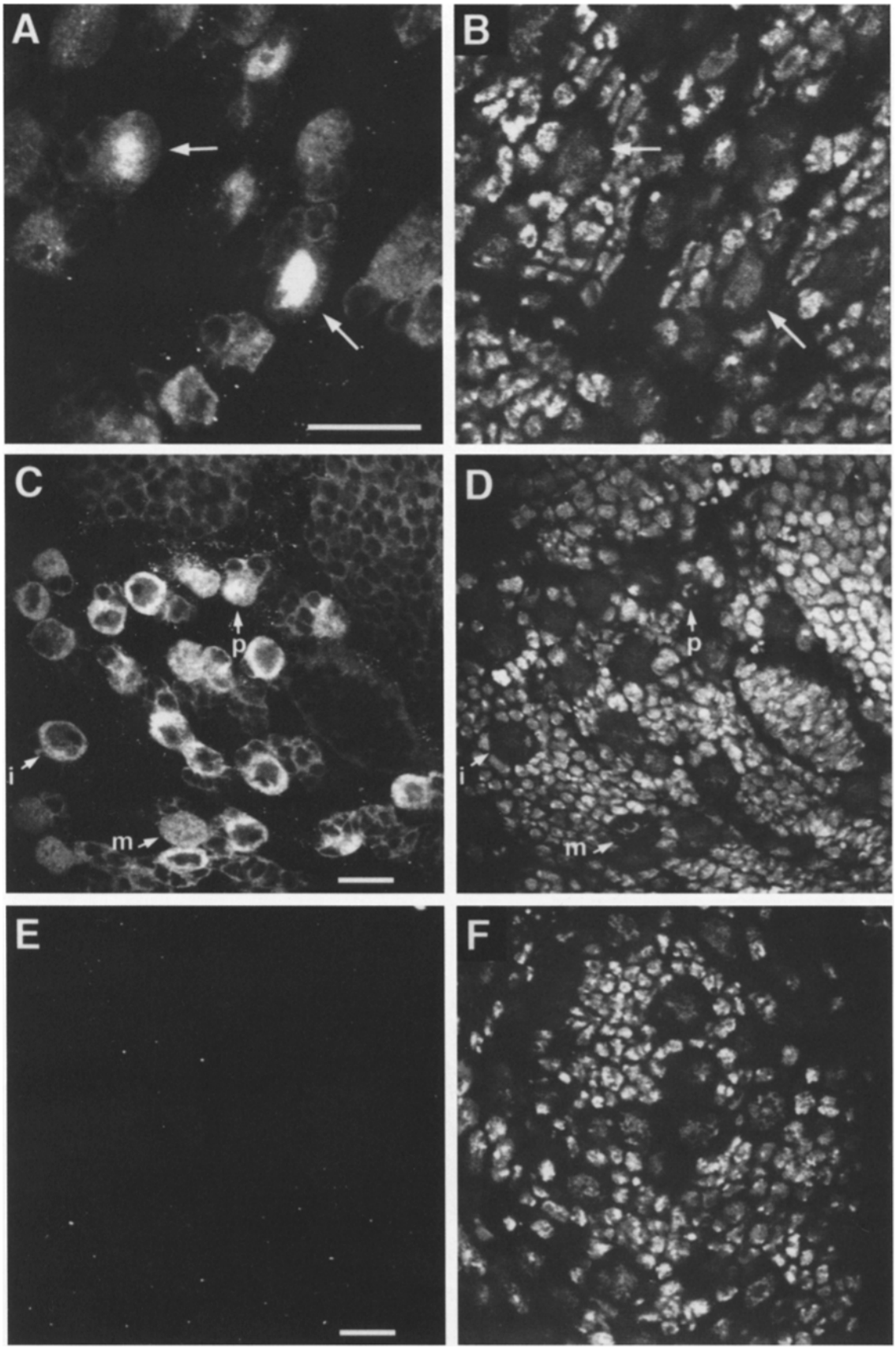

Figure 6. Pendulin expression and localization in larval brains. (*A*) and (*C*) show dorsal views of brain lobes from wild type larvae stained with Pendulin antibodies (confocal images). *B* and *D* show the corresponding fields stained with propidium iodide to visualize all nuclei (note that the nuclei of neuroblast are larger and less condensed during interphase than those of the ganglion cells). Pendulin is detected at high levels in neuroblasts and at much lower levels in their daughter neurons. Intermediate levels of Pendulin are seen in proliferating neurons of the optic lobe region (*C*, *top*). Arrows in *A* identify neuroblasts in early prophase with predominantly nuclear localization of Pendulin. Arrows in *C* show neuroblasts in interphase (*i*), prometaphase (*p*), and metaphase (*m*), which display subcellular distributions of Pendulin signals similar to those shown for embryonic cells in Fig. 4. (*E*) Pendulin antibody staining of a brain from an *oho31*$^{144/1.3}$ mutant larva does not detect any protein. (*F*) propidium iodide staining of the same field as in *E*. Bars, 10 μm.

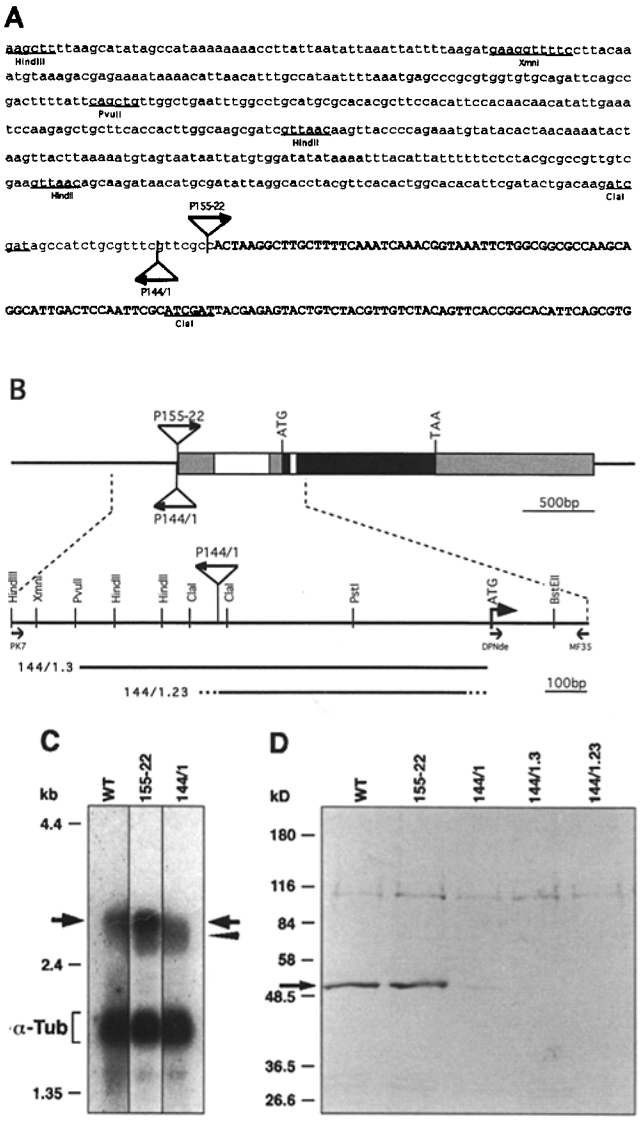

Figure 7. P-insertions in the Pendulin gene and P-excisions. (*A*) Precise insertion sites of the two P-elements, P155-22 and P144/1, near the 5′ end of the Pendulin gene. Sequences that correspond to those at the 5′ end of cDNA #23 are in bold capital letters. Arrows indicate the 5′ to 3′ orientation of the P-lacW elements. (*B*) Schematic diagram of the Pendulin gene. The boxed region indicates transcribed sequences, white boxes represent intron sequences, and black boxes represent translated portions (genomic sequences downstream from the MF35 primer binding site were not determined and may contain additional small introns). The diagram below shows a restriction map of the genomic flanking regions of P144/1 that was used in the excision screen. The location of binding sites for the primers PK7, DPNde, and MF35 that were used for the analysis of P-excisions is indicated. The bars indicate deleted sequences in the excision mutants *oho31*$^{144/1.3}$ and *oho31*$^{144/1.23}$. (*C*) Northern blots with poly(A)$^+$ RNA from 3rd instar wild type larvae (*WT*) and from larvae homozygous for the insertions P155-22 and P144/1. Arrows indicate the position of wild type Pendulin mRNA and the arrow head indicates the median position of Pendulin mRNAs from *oho31*$^{P144/1}$ larvae. Exposure times of the filter were adjusted to obtain equal signals for the α-tubulin probe used as a standard (*WT*: 100 h, 155-22; 16 h, 144/1; 55 h) (*D*) Western blot with homogenates from wild type and *oho31* mutant larvae. Proteins from larval ganglia of third instar wild type and *oho31* mutant larvae were probed with Pendulin antibodies. Only minute amounts of Pendulin protein (54-kD band, arrow) are detected in the *oho31*$^{P144/1}$ mutants, whereas the viable *oho31*$^{P155-22}$ line expresses normal levels. In the deletion mutants *oho31*$^{144/1.3}$ and *oho31*$^{144/1.23}$, no Pendulin protein is detected. Homogenates from ten phenotypic wild type ganglia and twelve homozygous mutant ganglia were loaded per lane to equalize protein amounts.

~1/4 of an embryo, the events from the bottom to the top take place within ~15 s (Foe and Alberts, 1983). To correlate the timing of nuclear entry of Pendulin with other cell cycle events, we performed double-stainings with cyclin B antibodies. At the transition between G2 and M-phase, a fraction of the cyclin B pool enters the nucleoplasm (Lehner and O'Farrell, 1990). Comparison with the Pendulin distribution shows that the two proteins enter the nucleoplasm at the same point in the cell cycle, and there is a good correlation between the levels of nuclear cyclin B and those of nuclear Pendulin (Fig. 5 *B*, *arrows*). These results demonstrate that the nuclear translocation of Pendulin is very rapid and starts at the transition from G2 to M-phase of the cell cycle. After the 13th mitosis, divisions take place in well-defined domains of cells (Foe, 1989). As a consequence, in post-gastrulation embryos, Pendulin is observed in fields of nuclei that correspond to these domains (Fig. 5 *C*, *left*). Since the cyclin proteins are degraded during mitosis, these same domains appear darker after staining for cyclin B (Fig. 5 *C*, *right*).

In late stage embryos, Pendulin protein is not detectable in epidermal and mesodermal, post-mitotic cells, while it persists in the central nervous system, where cells continue to proliferate (data not shown). A striking pattern of Pendulin expression is observed in the CNS of third instar larvae, when a set of larval neuroblasts divide as stem cells to generate the neurons of the adult fly (Truman and Bate, 1988; Ito and Hotta, 1992). These neuroblasts express high levels of Pendulin protein, whereas much lower protein levels are detected in newly generated ganglion cells that are adjacent to the mother cells (Fig. 6, *A–D*). No Pendulin protein is detected in ganglion cells located further away from neuroblasts, which were presumably born during earlier divisions. These data show that Pendulin is expressed predominantly, if not exclusively, in the neuroblast stem stells and gradually disappears from postmitotic neurons. In the dividing neuroblasts, Pendulin shuttles between the cytoplasm and the nucleus in a cell cycle-dependent fashion identical to that described above for blastoderm embryos (Fig. 6, *A–D*, *arrows*). The specificity of these antibody stainings was demonstrated by stainings of brains from a Pendulin protein-null mutant line (see below), which were negative (Fig. 6, *E* and *F*). Taken together, these observations suggest that the function of Pendulin is mainly required in proliferating cells.

Pendulin Is Required to Maintain Normal Rates of Cell Proliferation

To study the function of Pendulin, we attempted to generate mutations in the gene. We performed a random insertional mutagenesis followed by plasmid rescue of the P-elements and their flanking genomic sequences, which were subsequently assayed by hybridization with Pendulin probes

(Walter, N., E. Hafen, and M. Noll, manuscript in preparation). One out of 24,000 independent P-lines tested was found to carry an insertion, P155-22, at position -1 with respect to the nucleotide sequence of cDNA #23 (Fig. 7, *A* and *B*). Surprisingly, animals homozygous for this insertion are viable and express Pendulin protein at normal levels. Imprecise excisions of this P-element to create small deletions in the Pendulin gene were unsuccessful, because a second P-element is located closeby at 30F (near *big brain*) and double excisions gave rise to only large deletions that affect many other genes as well. A single P-insertion at the Pendulin locus at 31A was isolated independently in a screen for lethal insertions on the second chromosome (Török et al., 1993), and was kindly provided to us by T. Török. We found that the P-insertion of this line, *l(2)144/1*, had occurred six nucleotides upstream and in the opposite orientation from P155-22 (Fig. 7, *A* and *B*). P144/1 results in larval lethality, and precise excisions cause reversion to the wild type (Török et al., 1993). Homozygous larvae from this line fail to pupariate and develop overgrown lymph glands and melanotic tumors (Török et al., 1993). Based upon this phenotype, the locus that encodes Pendulin is now called *overgrown hematopoietic organs-31* (*oho31*). By Northern and Western analysis, we examined whether this P-insertion affected the expression of Pendulin. In larvae homozygous for the lethal P144/1 insertion, Pendulin mRNAs are expressed at almost the same levels as in wild type larvae. However these mRNAs migrate with a slightly higher mobility and appear more heterogeneous in length as compared to wild type mRNAs, which may indicate transcriptional initiations downstream from the normal start site (Fig. 7 *C*). In larvae homozygous for the viable P155-22 insertion, Pendulin mRNAs were detected at higher levels and with similar lengths as in the wild type (Fig. 7 *C*). Thus it appears that P-lacW insertions can allow transcriptional activation from cryptic start sites, the location of which may depend on the orientation and flanking sequences of a particular insertion (see also Bellen et al., 1992). In homogenates from P144/1 homozygous larvae (data not shown) and in brain extracts from such animals, we observed a massive reduction of Pendulin protein levels when compared to wild type larvae, indicating that the truncation of Pendulin mRNAs caused by P144/1 results in a strong decrease in the translation of this protein (Fig. 7 *D*). In contrast, Pendulin protein was translated at essentially normal levels in P155-22 homozygous larvae, thus explaining the viability of this line (Fig. 7 *D*).

To obtain protein-null mutants for Pendulin, the P144/1 insertion was mobilized via transposase in order to create imprecise excisions that delete parts of the Pendulin gene. The two largest deletions that were identified in this screen, $oho31^{144/1.3}$ and $oho31^{144/1.23}$, extended very close towards the presumed translational start site and thus deleted the 5′ UTR of the Pendulin gene. Since the 3′ end of the closest gene upstream from the Pendulin gene (encoding a cuticle protein, Frasch, M., unpublished results) is located at a distance of ~1 kb from the original site of the P144/1 insertion, at least the $oho31^{144/1.23}$ deletion disrupts the Pendulin transcription unit only. By Western analysis and immunofluorescence, no Pendulin protein was detected in brains from larvae that were homozygous for these two deletions (Figs. 7 *D* and 6 *E*). Together, the results from the precise and imprecise excisions of P144/1 show that the *oho31* mutant phenotypes are due to disruptions of the Pendulin gene.

The phenotypes of the P144/1 insertion line and excision mutants (data not shown) with strongly reduced amounts of Pendulin protein are indistinguishable from those of protein-null mutants that are homozygous for $oho31^{144/1.3}$ and $oho31^{144/1.23}$. The most dramatic consequence of the severe reduction, or loss, of Pendulin function in *oho31* mutants is the formation of multiple melanotic tumors in the larvae (Fig. 8, *A* and *B*). These tumors become obvious in mutants at 2–4 d after their heterozygous siblings have pupariated. It appears that the tumors result from the excessive proliferation of hematopoietic cells in these animals, since the lymph glands, where blood cells are formed, are greatly enlarged (Török et al., 1993; see below). In the wild type, hematocytes differentiate in response to foreign agents, such as parasites and infections, and subsequently encapsulate them and become melanized (Rizki, 1978; Rizki and Rizki, 1980). In mutants with overproliferating hematocytes these cells, called plasmatocytes, appear to differentiate precociously into podocytes and lamellocytes which encapsulate themselves or invade other larval tissues in the absence of external stimuli, thus forming melanotic tumors (Sparrow, 1978; Gateff, 1978). Small regions of the salivary glands (just posterior to the imaginal rings) and the genital discs appear to become invaded and encapsulated preferentially by tumorous blood cells in *oho31* mutants (Fig. 8 *A*, *arrows*; Fig. 8 *C*). The levels of circulating hematocytes in the hemolymph of mutant larvae were greatly elevated (Fig.8, *D* and *E*). $oho31^{P144/1}$ mutant third instar larvae contained a mean of 18,000 hematocytes/ml of hemolymph (SD = 13,000, n = 11) whereas in wild type larvae, we counted a mean of 2,000 cells/μl only (SD = 1,200, n = 5). While the hemolymph of wild type larvae contained mostly plasmatocytes, the majority of hematocytes in mutant larvae consisted of differentiating podocytes (Fig. 8, *D* and *E*). The observed phenotype indicates that, in hematopoietic cells of wild type animals, Pendulin serves a function as a tumor suppressor.

oho31 mutant larvae fail to pupariate and die between 10 and 23 d after hatching (Török et al., 1993). Because of the peculiar intracellular distribution of Pendulin during the cell cycle and its specific expression in developmental stages and cells that are mitotically active, we examined whether Pendulin has a function in cell proliferation of larval tissues other than the blood cells. Western blots and immunofluorescence with tissues from wild type larvae detected high levels of Pendulin in imaginal discs and the CNS (Figs. 6 and 7, and unpublished results). Both tissues have high mitotic activities and in the CNS, Pendulin expression appeared to be largely restricted to proliferating neuroblasts (Fig. 6). To compare the proliferative activities of neuronal and imaginal disc cells in normal and mutant larvae, we analyzed the levels of bromo-deoxyuridine (BrdU) incorporation into nuclei from these tissues. When BrdU was given to heterozygous (i.e., phenotypically wild type) larvae during the whole 3rd instar (72 to 115 h after hatching at 23°C) or as a pulse in late 3rd instar (91 to 115 h), the majority of nuclei in imaginal discs incorporated the nucleotide analog (Fig. 9, *B* and *D*). In contrast, the

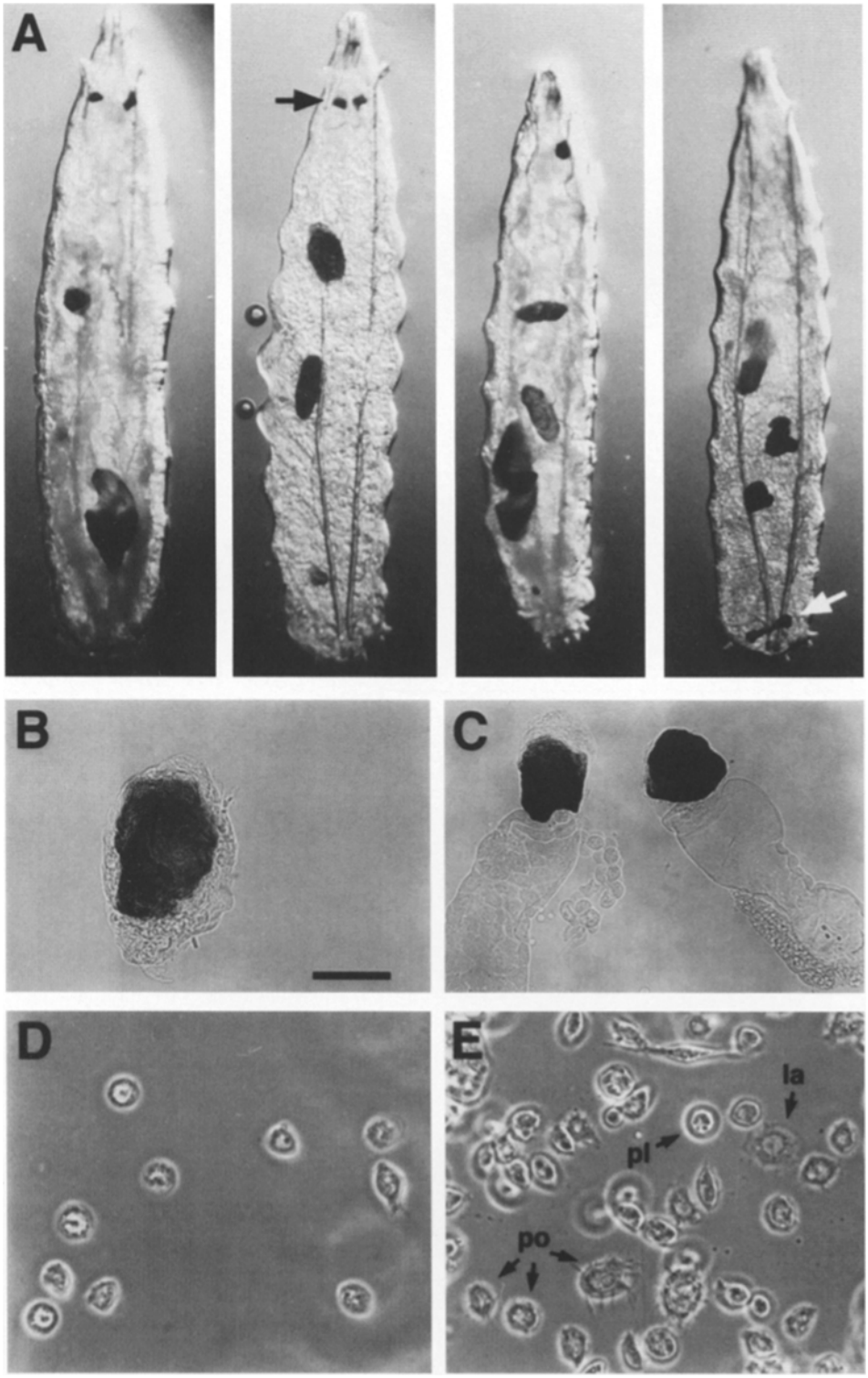

Figure 8. Melanotic tumors in *oho31*$^{P144/1}$ mutant larvae. (*A*) Mutant 3rd instar larvae (live animals), 5 to 10 d after pupariation of heterozygous sibs. Each larva contains several melanotic tumors. Some tumors are attached to a specific region of the salivary glands (*black arrow*) or in the region of the genital discs (*white arrow*). (*B*) High magnification, bright field view of a blood cell tumor, showing melanotic cells in the interior. (*C*) High magnification view of melanotic tumors attached to anterior portions of the salivary glands. This area of the gland contains small polytenized nuclei, while the imaginal cells are located more anteriorly and are not melanized (not shown). (*D* and *E*) Phase contrast views of circulating hematocytes in the hemolymph from wild type (*D*) and *oho31*$^{P144/1}$ mutant 3rd instar larvae. Wild type hemolymph contains predominantly plasmatocytes (*pl*), whereas hemolymph from mutants contains an increased number of blood cells which mainly consist of podocytes (*po*) and some lamellocytes (*la*). Bar, 0.2 mm.

density and number of nuclei in discs from homozygous *oho31* mutant larvae that were given BrdU between 72 h and 115 h, were clearly reduced when compared to larvae with a wild type copy of the Pendulin gene (Fig. 9 *A*). As a result of the reduced growth rate, the mutant imaginal discs are much smaller than their normal counterparts. BrdU incorporation into mutant discs during late 3rd instar is even further reduced, and the imaginal discs fail to grow to normal size during the extended larval period (Fig. 9 *C*). These growth defects are accompanied by abnormal morphogenesis of the imaginal discs (Fig. 9, *A* and *C*). BrdU incorporation in the CNS of wild type larvae reveals precisely defined patterns of neuroblast and ganglion mother cell divisions (Truman and Bate, 1988; Ito and Hotta, 1992). When heterozygous larvae were pulse-labeled with BrdU in late 3rd instar (see above), strong incorporation was found in the outer proliferation centers of the optic lobe anlagen and in symmetrical fields of dividing neuro-

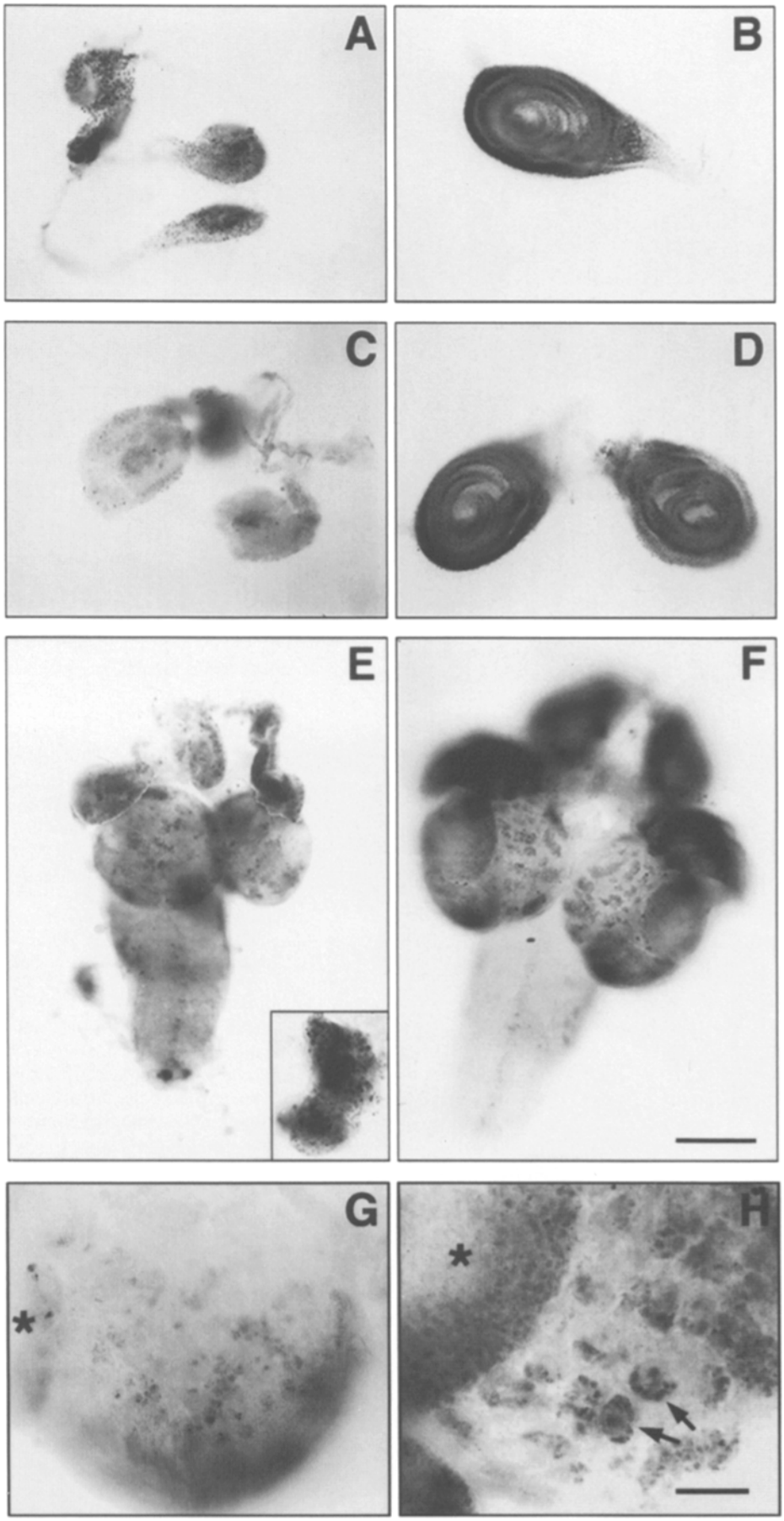

Figure 9. Morphology and BrdU incorporation of imaginal discs and central nervous systems from normal and *oho31*$^{P144/1}$ mutant larvae. Imaginal discs from homozygous mutants (*A* and *C*) and from heterozygous larvae (*B* and *D*) were stained for BrdU incorporation. The larvae from *A* and *B* were fed BrdU between 72 h and 115 h after hatching and then processed. The larvae from *C* and *D* were fed BrdU from 91 h after hatching and heterozygous larvae (*D*) were processed at 115 h (wandering stage), mutant larvae (*C*) after tumor formation (3 d after pupariation of sibs). BrdU incorporation in mutant imaginal discs is reduced in comparison to normal discs, particularly in late 3rd instar. Mutant imaginal discs are smaller than normal and have aberrant shapes. (*E*) shows an *oho31* mutant CNS and eye-antennal discs, stained for BrdU incorporation as in *D*. The imaginal discs were photographed at a different plane of focus than the CNS. BrdU incorporation in the CNS and the eye imaginal discs from mutant larvae is reduced and occurs in abnormal patterns. The tissues are reduced in size. *E* (*inset*) shows a piece of a ruptured lymph gland from an *oho31* larva. The animal was fed BrdU from 91 h of development and stained for BrdU incorporation 5 days later. The lymph gland is hypertrophied and displays high levels of BrdU incorporation (magnification as for the central nervous systems). (*F*) shows the CNS and eye-antennal discs from a heterozygous larva after treatment as in *B*. *G* and *H* show high magnification views of brains with genotypes and stained as in (E) and (F). The arrow in (F) indicates an example of a cluster of labelled neurons that are derived from the same neuroblast. *Asterisk*, optic lobe region. Bars: (*A-F*) 100 μm; (*G* and *H*) 20 μm.

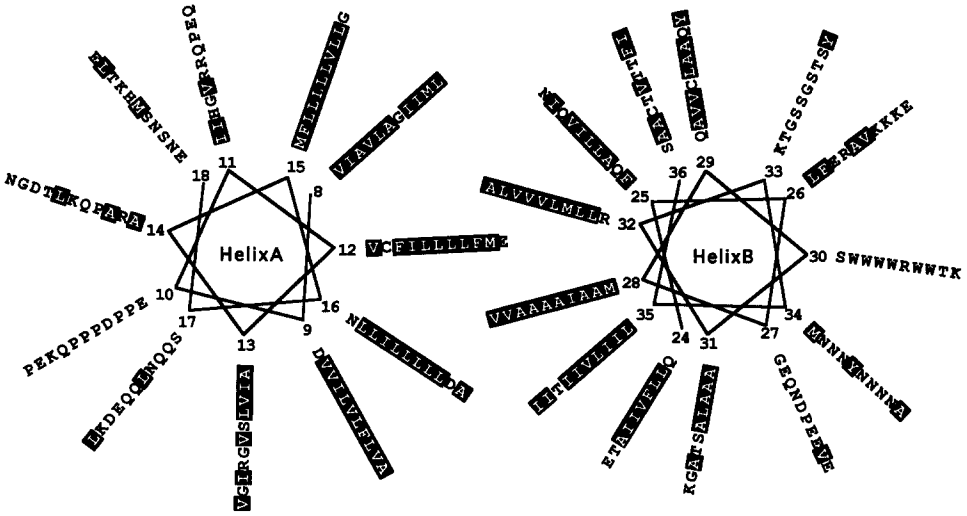

Figure 10. Helical wheel projections of amino acids 8 to 18 (*Helix A*) and 24 to 36 (*Helix B*) from all Pendulin repeats (numbering as in Fig. 2). Hydrophobic amino acids (*black boxes*) are largely positioned on one side of each helix and charged amino acids are prevalent on the opposite sides. This arrangement would allow for interactions between the two helices from each repeat through hydrophobic residues, as well as for the stacking of the helices from consecutive repeats, as proposed by Ruediger et al. (1994) for the A subunit of PP2A. These authors predicted that the prolines (in Pendulin at position *10*) would introduce a bend in helix A.

blasts in the two brain lobes (Fig. 9, *F* and *H*; note the similarities to the Pendulin expression pattern shown in Fig. 6 *C*). In contrast, in brains from homozygous mutant larvae that were BrdU treated for the same period, the analog was incorporated in many fewer nuclei, and these are arranged in irregular patterns (Fig. 9, *E* and *G*). The reduced proliferation rate of neuroblasts and ganglion mother cells is consistent with the smaller size of the mutant CNS when compared with the wild type of the same age. Although we observe continued neuronal cell division in the CNS of mutants during their extended larval period, the division patterns remain irregular, and the brain lobes assume abnormal morphologies. BrdU incorporation in the imaginal rings of the salivary glands is reduced as well (data not shown). Thus, Pendulin is also required for regular cell proliferation in imaginal discs, the CNS, and in other imaginal cells to allow normal morphogenesis of adult structures. In contrast to the reduced BrdU incorporation in the imaginal discs and the CNS, high levels of incorporation were found in the lymph glands of *oho31* mutants, as was expected from the observed hypertrophy of this tissue (Fig. 9 *E*, *inset*).

Discussion

Cell Cycle-dependent Nuclear Translocation of the Pendulin Protein

The translocation of Pendulin from the cytoplasm into the nuclei at the transition from G2 to M-phase was the first indication that this protein may be required to regulate or execute cell cycle progression. The timing of the nuclear translocation and the presence of a consensus sequence for cdc2 phosphorylation, which is near a potential NLS, suggest a mechanism whereby activated cdc2 kinase directly phosphorylates Pendulin and triggers its nuclear import. There are indeed several other examples of proteins whose nuclear translocation depends on phosphorylation sites that are located close to their NLS (for a review see Boulikas, 1993). In some of these proteins, such as Swi5, phosphorylation inhibits nuclear entry, whereas in the case of viral Jun, phosphorylation is thought to induce nuclear translocation during G2 (Moll et al., 1991; Chida and Vogt, 1992). If nuclear import of Pendulin were similarly triggered by phosphorylation of the cdc2 site, it would be one of the early targets of cdc2 kinase, since the activating cyclins A and B are also cytoplasmic in G2 and enter the nucleoplasm along with Pendulin (Fig. 5; Lehner and O'Farrell, 1989, 1990; Whitfield et al., 1990). Nuclear targets of $p34^{cdc2}$, such as lamins and histone H1, become phosphorylated only after these translocation events, which is consistent with the observation that the nuclear envelope appears still intact and the chromatin not yet condensed at the time when Pendulin becomes nuclear. The detection of two closely co-migrating Pendulin bands in embryos could reflect forms that are differentially phosphorylated at this site. Analysis of Pendulin variants that have a mutated cdc2-consensus site or NLS in transgenic animals will allow us to test this proposed mechanism of nuclear translocation and may distinguish between nuclear and cytoplasmic functions of this protein. Unlike *Drosophila* and mouse Pendulin, the yeast homolog Srp1p lacks a cdc2 consensus sequence near the NLS. The NLS of the yeast protein seems to be permanently exposed, or functional, since this protein is located in the nuclei throughout the cell cycle (Küssel and Frasch, 1995). The protein products of two other *Drosophila* genes that are required for normal mitosis also translocate from the cytoplasm into the nuclei at the onset of mitosis. The serine/threonine kinase encoded by the *polo* gene and the Swallow protein appear to migrate into the nuclei at about the same time as Pendulin (Llamazares et al., 1991; Hegdé and Stephenson, 1993). Like Pendulin, both of these proteins have bipartite NLS consensus sequences, but they lack an adjacent cdc2 phosphorylation site (Hegdé and Stephenson, 1993; and own observations). Whereas mutations in *polo* and *swallow*

cause aberrations in chromosome segregation and spindle abnormalities, we have not detected any mitotic defects in *oho31* embryos or larvae that lack Pendulin (see Fig. 6 *E*). It is possible that such defects, if any, are too subtle to be detected by examining the chromatin distribution. At least the hematopoietic cells appear to be able to complete normal mitosis in the absence of Pendulin because they continue to proliferate in mutants. Thus, the *oho31* phenotype suggests that mutation of Pendulin primarily affects the rate of cell division rather than execution of a mitotic process.

Tumorigenesis and Growth Arrest in Pendulin Mutants

Mutation of Pendulin causes obvious phenotypes only in mitotically active tissues of the larvae such as the imaginal discs and imaginal rings, the nervous system, and the hematopoietic organs. The normal appearance of post-mitotic cells, which are mostly polyploid or polytene in larvae, in *oho31* mutants and the viability of mutant larvae during their extended lifetime indicate that Pendulin function is only required in cells that are still capable of dividing. This notion is consistent with the observed disappearance of Pendulin mRNA and the strong reduction of protein levels in post-mitotic embryonic and larval cells. The maternal pools of Pendulin mRNA and protein seem to be sufficient to allow normal cell divisions in embryonic stages of *oho31* mutants, and cell proliferation is only affected in third larval instar when maternal protein levels are presumably reduced below a critical threshold. A similar explanation has been suggested for the larval or pupal lethality of many mitotic mutations in *Drosophila* (Gatti and Baker, 1989; Glover, 1989). Elimination of maternal Pendulin by producing females with germ line mosaics is expected to uncover the functions of this protein in oocyte and embryonic development.

The disruption of Pendulin function has two opposing effects with respect to growth rates of different tissues: Whereas hematopoietic cells over-proliferate, the rate of imaginal and neuronal cell division is reduced as compared to wild type. This differential effect could reflect the utilization of distinct mechanisms for proliferation control in these tissues. Hematopoietic cells divide in a stem cell-like fashion and are released from the lymph gland into the hemolymph. Their proliferation rate is thought to be regulated by hormonal and perhaps also by immunogenic stimuli, although these events have not yet been characterized in detail (Rizki, 1978; Rizki and Rizki, 1980; Zinyk et al., 1993). In contrast, dividing cells in imaginal discs and in the nervous system remain in contact with one another and, at least in discs, cell–cell contacts are crucial to regulate cessation of proliferation (Bryant and Schmidt, 1990). Accordingly, several of the cloned tumor suppressor genes that affect cell proliferation in imaginal discs but not of hematopoietic cells encode proteins located on or near the cell surface (Lützelschwab et al., 1987; Mechler et al., 1991; Mahoney et al., 1991; Woods and Bryant, 1991; Boedigheimer and Laughon, 1993). As observed in *oho31* mutants, the overgrowth phenotypes of mutations in the large majority of the ~22 known loci that act as blood cell tumor suppressor genes are restricted to the hematopoietic cells as well (Gateff, 1978; Watson et al., 1991; Török et al., 1993). It is therefore conceivable that hematopoietic cells utilize signal transduction pathways for growth regulation that are distinct from those in solid tissues. Mutation of Pendulin or of other blood cell tumor suppressors might result in a constitutive positive signal for hematopoietic cell proliferation, whereas in imaginal discs they disrupt transmission of signals that stimulate mitosis. Similar conditions could also explain the tissue-specificity of human tumor suppressor genes. For instance, mutation of NF1, a GTPase-activating protein, appears to create a constitutive proliferative signal in neuronal tissues only, although the protein is expressed in other tissues as well (Xu et al., 1990; Daston and Ratner, 1992).

Pendulin is the third blood cell tumor suppressor from *Drosophila* that has been molecularly characterized. The other two are *air8*, which corresponds to the ribosomal protein S6, and *l(3)mbn*, which is a cytoplasmic protein with repeat sequences related to those in cytokeratins (Watson et al., 1992; Steward and Denell, 1993; Konrad et al., 1994). Although the ribosomal protein S6 has been implicated in the control of cell growth and proliferation, it is still unclear how these proteins function to suppress blood cell tumors. The molecular cloning and characterization of additional genes from this class combined with biochemical experiments will help to determine whether any of their products functionally interact with Pendulin.

Evolutionary Conservation and Cellular Function of Pendulin

The Pendulin-like proteins are members of a larger superfamily of proteins that share Arm repeats with one another (Fig. 2; Peifer et al., 1994). The properties of other members of this superfamily provide some clues to the possible biochemical functions of the Pendulin family. Several of these proteins, including Armadillo, appear to be components of signal transduction pathways and interestingly one of them, human APC, acts as a tumor suppressor as well (Kinzler et al., 1991). The occurrence of protein–protein interactions appears to be a common theme among the proteins of this superfamily. For example, the NH_2-terminal domains of α- and β-adaptin which consist of Arm repeats have been shown to complex with at least two polypeptides, AP17 and AP50 to form the clathrin-associated complex AP-2 (Keen, 1990). The Arm domain of β-catenin has been found to interact with APC, and β-catenin as well as Armadillo and plakoglobin form complexes with cadherins and α-catenin (Rubinfeld et al., 1993; Su et al., 1993; Kemler, 1993; Peifer, 1993). Like Pendulin, two other members of this superfamily, the mammalian guanine nucleotide exchange factor smgGDS and the regulatory A subunit of phosphatase 2A, consist almost entirely of Arm repeats. These two proteins physically interact with their catalytic partners and their Arm domains must be involved in mediating such protein–protein interactions. In the case of PP2A, extensive in vitro studies of these interactions have been performed (Ruediger et al., 1992; Ruediger et al., 1994). Based on the results of these experiments, as well as on structural studies and helical wheel analysis, a model was proposed in which each repeat domain of the A subunit forms two antiparallel amphipatic helices and the repeats are stacked in a linear fash-

ion. This arrangement would lead to a rod-shaped molecule, to which the B subunit of PP2A associates through the NH_2-terminal repeats and the C subunit through the COOH-terminal repeats (Ruediger et al., 1994). The loops connecting the antiparallel helices of each repeat, which are all located on the same face of the rod in this model, appear to play critical roles for these associations. As shown in Fig. 10, helical wheel projections of the Pendulin repeat sequences produce very similar results as for the A subunit of PP2A. The amino acids on the hydrophobic sides of each helix include the most highly conserved residues in each repeat from Pendulin and from other Arm repeat proteins (Fig. 10, see Fig. 2). This indicates that Pendulin and the other members of this superfamily share a common tertiary structure with one another, which may be similar to the one proposed for the A subunit of PP2A. Biochemical analysis with normal and mutant versions of several of these proteins, including PP2A and human SRP1, suggest that the Arm repeats serve as modules for protein–protein interactions (Ruediger et al., 1992; Cortes et al., 1994). Since the residues in the intra-repeat loops, which appear to be involved in these interactions, are not highly conserved, each protein of the Arm repeat superfamily would be expected to interact with different binding partners. Pendulin consists almost entirely of Arm repeats, except for the short NH_2-terminal domain which may be involved in nuclear translocation. Thus, we propose that Pendulin might serve as an adaptor molecule that functions to assemble other proteins into complexes.

What might be the cellular functions of Pendulin? The strong sequence conservation over the whole extent of the polypeptide chain of Pendulin-like proteins from different species suggests that they share basic functions that are required in all eukaryotes. So far the data point clearly to roles in maintaining the structure and function of the cell nucleus. Thus, a mutation in the yeast homolog *SRP1* suppresses a mutation in RNA polymerase I (Yano et al., 1992). Since the Srp1 protein is not predominantly located in the nucleolus, but rather distributed throughout the nucleus (Küssel and Frasch, 1995), it is possible that this genetic interaction is indirect and does not reflect a biochemical interaction between these two molecules. Depletion of the Srp1 protein from yeast cells that carry a disruption in the *SRP1* gene causes a mitotic phenotype and defects in nuclear integrity. These cells arrest during M-Phase and frequently display aberrations in chromosome or nuclear segregation as well as in spindle morphology (Küssel and Frasch, 1995; Yano et al., 1994). Neither *Drosophila* nor mouse Pendulin were able to complement the *SRP1* mutation (Küssel and Frasch, 1995), suggesting that there is some functional divergence among Pendulin-like proteins in the different species. The association of the Pendulin-like proteins Rch1 and SRP1 from humans with the recombination-activating protein RAG-1 is yet another indication for a nuclear function of this protein family (Cuomo et al., 1994; Cortes et al., 1994). Although it has been shown that the RAG-1 protein is located in the nucleus, its exact role in recombination is still obscure (Silver et al., 1993). It has been suggested that Rch1 and hSRP1 might play a structural role in the nucleus to allow recombination (Cuomo et al., 1994; Cortes et al., 1994), but further analysis of Rch1 and RAG-1 is required to better understand the significance of their interactions. The recent isolation and functional analysis of the *Xenopus* protein importin may provide important clues to the cellular function of Pendulin. Importin is required to mediate the first step of nuclear protein import, which is the binding of karyophilic proteins to the nuclear envelope. Because of the sequence similarities between importin and Pendulin (Fig. 1) it is conceivable that Pendulin plays a role in nuclear protein import as well. The observation that Pendulin can be specifically cross-linked to NLS-containing polypeptides provides additional evidence for such a function (Adam, S., personal communication). Pendulin is neither detectable in post-mitotic cells of wild type animals nor in overproliferating hematocytes and other cells of *oho31* mutants during their extended larval period, all of which presumably require protein import into their nuclei. Therefore, Pendulin is unlikely to be responsible for the nuclear import of NLS-containing proteins in general. Rather, Pendulin might mediate nuclear import of a specific subset of proteins that are required in mitotically active cells. Some of these karyophilic proteins may function in the control of cell proliferation, which could explain the observed phenotypes of *oho31* mutants. Perhaps there exist additional proteins of the Pendulin family in *Drosophila* that mediate import of other nuclear proteins in both mitotic and post-mitotic cells. The analysis of potential interactions between Pendulin and two *Drosophila* proteins that are thought to mediate the second step of nuclear protein import, BJ1/RCC1 (Frasch, 1991) and Ran (Frasch, M., unpublished results), as well as the genetic or biochemical identification of novel proteins that interact with Pendulin, will be crucial to clarify the role of this protein in nuclear import or in other events that are required for the maintenance of normal proliferation rates.

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