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A New Hepatocytic Isoform of PLZF Lacking the BTB Domain Interacts With ATP7B, the Wilson Disease Protein, and Positively Regulates ERK Signal Transduction

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Abstract The promyelocytic leukemia zinc finger (PLZF) protein has been described as a transcriptional repressor of the BTB-domain/zinc-finger family, and shown to regulate the expression of Hox genes during embryogenesis and the expression of cyclin A in the cell cycle progression. Here, a 45-kDa isoform of PLZF without a BTB domain was identified via yeast two-hybrid screening using the C-terminal region of ATP7B as bait in our determination of the biological roles of the Wilson disease protein outside of its copper-binding domain. Our immunoprecipitation experiments showed that the hepatocytic isoform of PLZF could specifically interact with the C-terminal region of ATP7B. The immunostaining of HepG2 cells revealed that the ATP7B and PLZF proteins were apparently colocalized into the trans-Golgi complexes. It was also determined that disruption of PLZF expression in the HepG2 cells affected an attenuation of ERK activity in a dose-dependent manner. The hepatocytic activities of ERK kinase were found to be enhanced as the result of PLZF or ATP7B expression, but this enhancement was abrogated by the deletion of the C-terminal region of ATP7B. Furthermore, a transgenic Drosophila strain that ectopically expressed the hepatocytic ΔBTB-PLZF exhibited phenotypic changes in eye and wing development, and these alterations were fully recovered as the result of ATP7B expression, indicating the obvious in vivo interaction between the two proteins. Those PLZF-induced abnormalities were attributed to the enhancement of ERK signaling, as was shown by phenotypic reversions with loss-of-function mutations in ERK signal transduction in Drosophila. These data suggest the existence of a mechanism that regulates ERK signaling via the C-terminus of ATP7B and the ATP7B-interacting hepatocytic PLZF. J. Cell. Biochem. 99: 719–734, 2006.

Key words: PLZF; ATP7B; ERK signal transduction

ATP7B (WND, Wilson disease protein) is a copper-transporting P-type ATPase, which is implicated in Wilson's disease, an autosomal recessive disorder characterized by dysfunctional copper homeostasis [Bull et al., 1993; Tanzi et al., 1993; Petrukhin et al., 1994]. ATP7B is principally expressed in the liver, and several immunocytochemical analyses of ATP7B-expressing cells have revealed that it tends to be localized in the trans-Golgi complexes, in which ATP7B transports copper for subsequent incorporation into apo-ceruloplasmin. However, increases in copper concentration have been shown to elicit the trafficking of ATP7B to cytoplasmic vesicular compartments for the biliary copper excretion and copper homeostasis in the hepatocytes [Lockhart and Mercer, 2001; Huster et al., 2003; Cater et al.,

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2004]. In addition to the hepatic function, recent studies of tumor resistance to platinum-based anticancer drugs including cisplatin have shown that ATP7B is frequently overexpressed in human solid carcinomas, such as ovarian, breast, gastric, and esophageal carcinomas, and that this overexpression can confer resistance to cisplatin [Komatsu et al., 2000; Nakayama et al., 2002; Ohbu et al., 2003].

ATP7B consists of a N-terminal copper-binding domain, a phosphatase domain with a flexible loop, an ATP-binding domain, and a C-terminal portion [Lutsenko and Kaplan, 1995]. Studies regarding the molecular mechanisms of copper transport have revealed an interaction between the N-terminal copper-binding domain of ATP7B and the copper chaperone molecule, HAH1 (human ATX1 homolog) [Klomp et al., 1997; Lin et al., 1997; Hamza et al., 1999]. Little data are currently available, however, regarding the biological functions performed by the other domains, although the ATP7B mutations that have been associated with Wilson's disease are not confined to the N-terminal domain, such as the 7-bp deletion reported by Bull et al. [1993], which induced a frameshift that cause the deletion of the entire C-terminal half of the protein. We conducted the yeast two-hybrid screening protocols in the present study using ATP7B lacking the N-terminal copper-binding domain as bait, in an attempt to determine the biological functions performed by the C-terminal region of ATP7B, and identified that a PLZF (promyelocytic leukemia zinc-finger protein) isoform was interacting with the truncated configuration of ATP7B.

PLZF was identified as a DNA-binding transcriptional repressor which was disrupted in patients of a type of acute promyeolocytic leukemia, in which the protein was found to be deformed into a chimeric fusion protein containing the retinoic acid receptor α [Chen et al., 1993; Li et al., 1997a]. Normal full-length PLZF has been shown to contain an N-terminal conserved protein–protein interaction motif, commonly referred to as the BTB (bric-a-brac, tramtrack, brad complex) domain, as well as a C-terminal DNA-binding motif constructed from nine zinc fingers of the Kruppel type [Godt et al., 1993; Bardwell and Treisman, 1994; Li et al., 1997b]. The expression of PLZF has been reported in a vast array of tissues, including the kidney, liver, and heart, as well as in the developing central nervous system and limb buds [Cook et al., 1995; Reid et al., 1995].

The murine PLZF-targeting disruption and cell culture studies have revealed that the protein acts as a transcriptional repressor of homeobox-harboring Hox genes, via a process of chromatin remodeling, in the modulation of both embryonic limb patterning and apoptotic processes [Barna et al., 2000, 2002; Ivins et al., 2003]. PLZF has also been implicated in the repression of cyclin A to regulate the cell cycle progression. The ectopic expression of PLZF was determined to inhibit entry or progression into S-phase, which ultimately resulted in a marked suppression of growth and increased rates of cell death [Shaknovich et al., 1998; Yeyati et al., 1999]. The nuclear localization of PLZF was affected by the processed HB-EGF (heparin-binding EGF-like growth factor), and the nucleus-localized C-terminal portion of HB-EGF was shown to bind directly to PLZF, and to trigger the nuclear export of the transcriptional repressor [Nanba et al., 2003; Toki et al., 2005]. Moreover, a recent study conducted by Felicetti et al. [2004] suggested that PLZF may also perform the functions of a suppressor of melanoma, independently on the expression of Hox genes or Cyclin A.

Epistatic studies which involved another in vivo PLZF model allowed us to speculate that the protein might perform more varied developmental roles, in addition to the regulation of the Hox genes; the *C. elegans* homolog of PLZF, EOR-1, was shown to be a positively acting component of both the Ras and Wnt signaling pathways with regard to the specification of the fate of P12, as well as the development of the excretory system [Zhang et al., 1999; Howard and Sundaram, 2002; Rocheleau et al., 2002]. EOR-1 was also shown to be involved in the regulation of cell type-specific apoptotic processes, similar to the function of PLZF as a proapoptotic factor in the limb buds of mouse embryos [Hoeppner et al., 2004]. In addition to EOR-1/PLZF, several members of the BTB-domain/zinc-finger family have been known to be implicated in the regulation of Ras/ERK signaling, such as *Drosophila* Tramtrack, Trithorax-like, and mammalian BCL6 [Li et al., 1997b; Tang et al., 1997; Maixner et al., 1998; Shvarts et al., 2002]. The activation of ERK has also been reported in cisplatin-resistant carcinomas which overexpress ATP7B [Persons et al., 2000], and was also known to

result from metallic exposure, including copper treatment, in metal-induced inflammatory response and cell-death events [Wu et al., 1999; Chung et al., 2000].

On the basis of the newly-defined interaction between the copper-binding protein and the possible ERK signaling activator PLZF, we attempted to characterize the heretofore-unknown relationship between PLZF/ATP7B and the ERK signal transduction pathway. Here, possible links between PLZF/ATP7B and ERK signaling were evaluated via in vitro assays in hepatocytic HepG2 cells, which express PLZF or ATP7B, and also by means of epistatic studies regarding ERK signal transduction after the ectopic expression of PLZF in *Drosophila*.

MATERIALS AND METHODS

Yeast Two-Hybrid Screening and PLZF Cloning

A cDNA fragment encoding the ATP loop domain and the C-terminal of human ATP7B was inserted into the pLexA vector and designated pLexA-WDC. EGY48 (p8op-lacZ; pLexA-WDC) cells were transformed with library plasmids (Human Liver MATCHMAKER LexA cDNA Library, Clontech) and screened according to manufacturer's recommendation. After PLZF was identified from the screening, the full-length cDNA of the gene was amplified from first strand cDNA by PCR amplification using the primers, 5′-CCAGATCTATGGATCTGACAAAAATGGG-3′ and 5′-CCCTCGAGTCACACATAGCACAGGTAG-3′. The truncated PLZF cDNA was amplified by PCR using the primers, 5′-CCAGATCTATGGGGGACAAGGTTGAGG -3′ and 5′-CCCTCGAGTCACACATAGCACAGG TAG -3′.

In Vitro Binding Assay, Coimmunoprecipitation, and Western Blot

The ATP loop of ATP7B or the ATP loop and C-terminal region of ATP7A or ATP7B was translated with ^{35}S-methionine and rabbit reticulocyte extract (Promega). The labeled proteins were mixed with maltose-binding protein (MBP)-fused PLZF or MBP, and the mixtures then passed through amylose resin (NEB). The eluted was then separated by 10% SDS–PAGE and exposed to a Fuji BAS1500 phosphorimager.

For immunoprecipitation of endogenous ATP7B or PLZF, HepG2 cells were lysed with RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin) and precipitated with anti-ATP7B or anti-PLZF antibody (2A9, Calbiochem), and Protein A sepharose. The precipitates were separated by 10% SDS–PAGE, transferred onto nitrocellulose membrane, and probed with anti-ATP7B or anti-PLZF antibody. Western blotting was performed using ECL kit (Amersham Pharmacia).

To detect endogenous ΔBTB-PLZF proteins, HepG2 and HL-60 cells were lysed with RIPA buffer. Cell lysates (50 μg) were separated by 10% SDS–PAGE, transferred onto PVDF membrane, and probed with anti-PLZF antibody. The presence of PLZF was detected by ECL plus Western Blotting Detection System (Amersham Pharmacia) according to manufacturer's recommendation.

Immunoflorescent Staining

pEGFP-N1, pEGFP-PLZF, and pEGFP-ΔBTB-PLZF plasmids were transiently transfected into HepG2 cells by lipofectamine plus reagent (GIBCO BRL). The location of γ-adaptin was detected with anti-γ-adaptin monoclonal antibody (A4200) and PE (phycoerythrin)-conjugated anti-mouse IgG (sc-3738, Santa Cruz). The locations of endogenous PLZF and ATP7B proteins in HepG2 were determined using anti-PLZF monoclonal antibody (2A9, Calbiochem) and anti-ATP7B rabbit polyclonal antibody, respectively, as primary antibodies, and anti-mouse IgG-PE and anti-rabbit IgG-FITC (florescein isothiocyanate, sc-2012, Santa Cruz) as secondary antibodies, respectively.

RNA Interference and Immunoprecipitation-ERK Kinase Assay

For double-stranded RNA interference assay, the RNAi vector of ΔBTB-PLZF was constructed by the method of Tavernarakis et al. [2000] with slight modification. The amplified ΔBTB-PLZF cDNA via PCR was ligated to generate the inverted-repeat, inserted into the expression vector pcDNA 3.1(+), and then transfected. The interferenced level of PLZF expression was determined by RT-PCR analysis using three different sets of priming within the coding sequence of ΔBTB-PLZF, and using a set of priming for β-actin mRNA as the internal control.

The HepG2 cells 24 h after cotransfections with pCEP-hERK1 and selected vectors were harvested, and changes in the activity of ERK were monitored. Before harvesting, cells were treated with epidermal growth factor (50 ng/ml) and PD98059 (50 μM) for 1 h. Hemagglutinin (HA)-tagged human ERK1 was immunoprecipitated using anti-HA monoclonal antibody and protein G sepharose beads, and used for the assays. ERK phosphotransferase assays using γ-^{32}P ATP (Amersham Pharmacia) and a peptide substrate (MAP kinase substrate, sc-3011, Santa Cruz) or myelin basic protein (MBP) were performed as previously described [Muda et al., 1996; Sette et al., 1999].

Values obtained from ERK assay using the peptide substrate were normalized to protein content. Transfection efficiency and sample-loading consistency for the assay using MBP were confirmed by western analysis of lysed samples (20 μl) with anti-HA monoclonal antibody (Sigma) and anti-β-actin monoclonal antibody (sc-1615; Santa Cruz). The level of ERK phosphorylation was determined by Western blotting with anti-MAPK monoclonal antibody (Sigma).

Drosophila Genetics

Flies were reared on standard cornmeal medium at 25°C under a photoperiodic regime (12L:12D). Transgenic flies carrying several forms of *dMkp3* were gifts from Chung JK (KAIST, Korea) and other flies *(GMR-GAL4*, *MS1096-GAL4*, *UAS-ras85DN*^{17}, *phl*^{12}, *Dsor1*^{LH}*1*^{10}, *rl*^{1}*)* were obtained from the Bloomington Stock Center. Transformation in *Drosophila* was performed by the injection of pUAST-PLZF, pUAST-ΔBTB-PLZF, or pUAST-ATP7B, and the helper plasmid, pπ25.1, into embryos before the time of pole-cell formation, using an IM-300 microinjector (Narishige) and an Axiovert 25 micromanipulator (Carl Zeiss). Phenotypes from the overexpression of ATP7B, PLZF, or ΔBTB-PLZF were analyzed using *GMR-GAL4* to induce targeted expressions in the developing eye, and *MS1096-GAL4* in the wing.

RESULTS

Interaction Between ATP7B and a Novel Isoform of PLZF

In order to identify any proteins able to interact with the Wilson disease protein, ATP7B, outside of its copper-binding domain, we employed a fragment spanning amino acid residues 995–1465, which contained the ATP binding motif and the C-terminal region, as bait in a yeast two-hybrid screening (Fig. 1A). As is summarized in Table I, 3 known and 1 unknown candidates were detected after the sequence analysis of 28 positive colonies. Among these four, PLZF colonies exhibited the strongest positive signals on the X-gal plates, and the subsequent in vitro pull-down binding assays indicated that the PLZF protein also exhibited specific binding affinity for the ATP7B bait (Fig. 1B, lane 3). The specific interaction between the two proteins was determined to depend on the presence of the C-terminal region of ATP7B, as was shown by the disappearance of the interaction in cases in which the ATP-binding motif alone was applied (lane 6). ATP7A (MNK, Menkes disease protein), a protein most similar to ATP7B, proved unable to interact with PLZF (lane 9).

This interaction was then further confirmed via coimmunoprecipitation (Fig. 1C). The presence of the ATP7B protein was detected via Western blotting using anti-ATP7B antibodies, in to which HepG2 lysates precipitated with anti-PLZF antibody were loaded (lane 2), as is indicated by the arrow. A band appeared on lane 1, at the same molecular weight (165 kDa), where the proteins which were precipitated using anti-ATP7B antibody had been loaded. A PLZF protein (of approximately 42–45 kDa) was detected on lane 6, which contained HepG2

Fig. 1. Interaction between ATP7B and PLZF. **A**: Schematic presentation of the ATP7B. The N-terminal copper-binding domains are depicted by numbered empty boxes and transmembrane domain is represented as the box with vertical stripes. The position of the phosphorylated Asp (D) is indicated by the letter, P in the ATP-binding domain depicted as a gray box. **B**: ATP loop and C-terminal region, ATP loop of ATP7B, or ATP loop and C-terminal region of ATP7A were translated with ^{35}S-methionine and rabbit reticulocyte extract, and then mixed with maltose-binding protein (MBP)-fused PLZF or MBP (indicated as mock) as indicated at the bottom of the figure, and the mixtures then passed through amylose resin. The asterisks represent the labeled proteins of the expected size. **C**: ATP7B and PLZF were precipitated with the antibodies and probed with anti-ATP7B or anti-PLZF antibody as indicated at the top of figure. Arrows indicate the specific signals in each panel. **D**: Cell extracts from HL-60 (lane 1), HepG2 (lane 2), and HepG2 cells transfected with pEGFP-PLZF (lane 3) were probed with anti-PLZF antibody. The asterisk indicates the full-length PLZF in HL-60 of the expected size. Specific signals of ΔBTB-PLZF and GFP-tagged PLZF from HepG2 lysates are indicated by arrowheads.

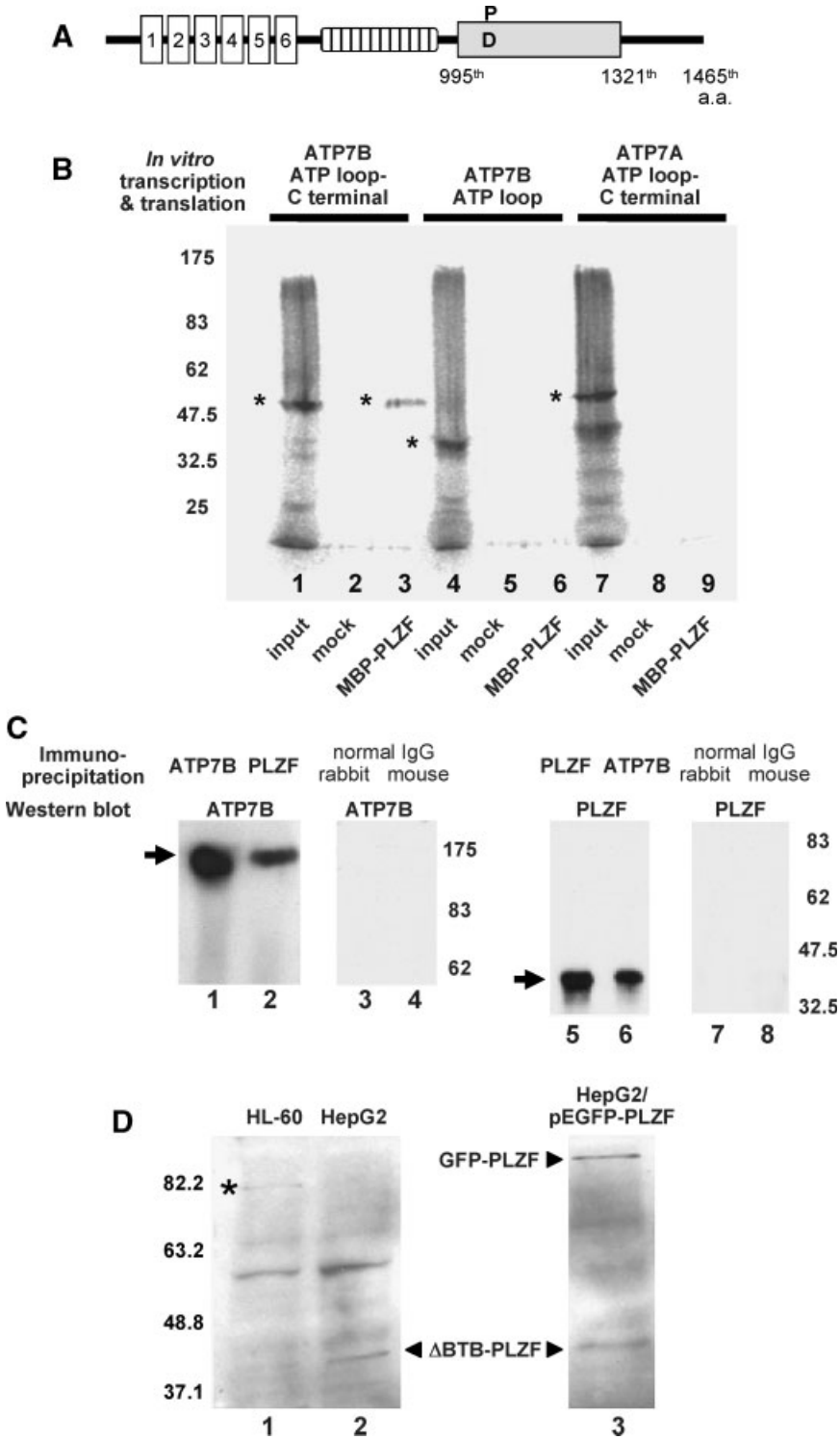

Fig. 1.

lysates that had been precipitated using anti-ATP7B polyclonal antibody. The molecular weight of PLZF was identical to that of the band in lane 5, which was noted to contain HepG2 lysates precipitated using anti-PLZF antibody, thereby indicating a specific interaction between ATP7B and PLZF.

The N-terminal amino acid sequence of both of the bands (Fig. 1C, lane 5, 6) was found to be methionine, and the amino acid was followed by

TABLE I. Summary of yeast two-hybrid screening results

Identified gene[a]	Number of positive clones	Intensity of signals on X-gal plates[b]
PLZF	15	+++
Haptoglobin	7	++
Defensin	4	+
Unknown	2	+

[a]These four candidates were classified according to the patterns after restriction enzyme digestion by AluI and then identified by DNA sequencing.
[b]Intensity shown here was the average of signal intensity from all positive clones in three independent experiments. +++, very strong; ++, strong; +, positive.

six perfectly matching sequences to the known PLZF protein (from the 268th to 274th amino acids of PLZF), thus indicating that a BTB domain-deleted variant of PLZF had been isolated from the HepG2 lysates. The predicted molecular weight of this protein was 45.5 kDa (Fig. 2). Although the presence of the novel PLZF variant had not previously been reported, a PLZF variant which lacked BTB domain had been expected after the previous RT-PCR analysis of alternative splicings of the PLZF gene [Zhang et al., 1999]. The presence of the PLZF isoform was confirmed further in the HepG2 cells via Western blotting, as is shown in Figure 1D. The signals from the HepG2 lysates were observed only at approximately 42 kDa, whereas no signals were detected at the position corresponding to 76–82 kDa (lane 2), in which the full-length PLZF was detected from the HL-60 cell extract (lane 1). Although some non-specific signals were observed, the specificity of the PLZF antibody was verified via Western blotting, using the cell extract from HepG2 transfected with pEGFP-PLZF (lane 3).

Colocalization of PLZF and ATP7B in the Trans-Golgi Complex of HepG2 Cell

Whereas PLZF has been fairly well characterized as a nucleus-localized transcriptional repressor for the regulation of the Hox genes or the progression of the cell cycle, its hepatocytic localization and functions remain unknown. In an unexpected result, the GFP-fused PLZF protein was detected in the trans-Golgi complexes of the HepG2 cells, in which ATP7B is normally localized [Huster et al., 2003], as confirmed by its colocalization with γ-adaptin, a well-known marker for the trans-Golgi network

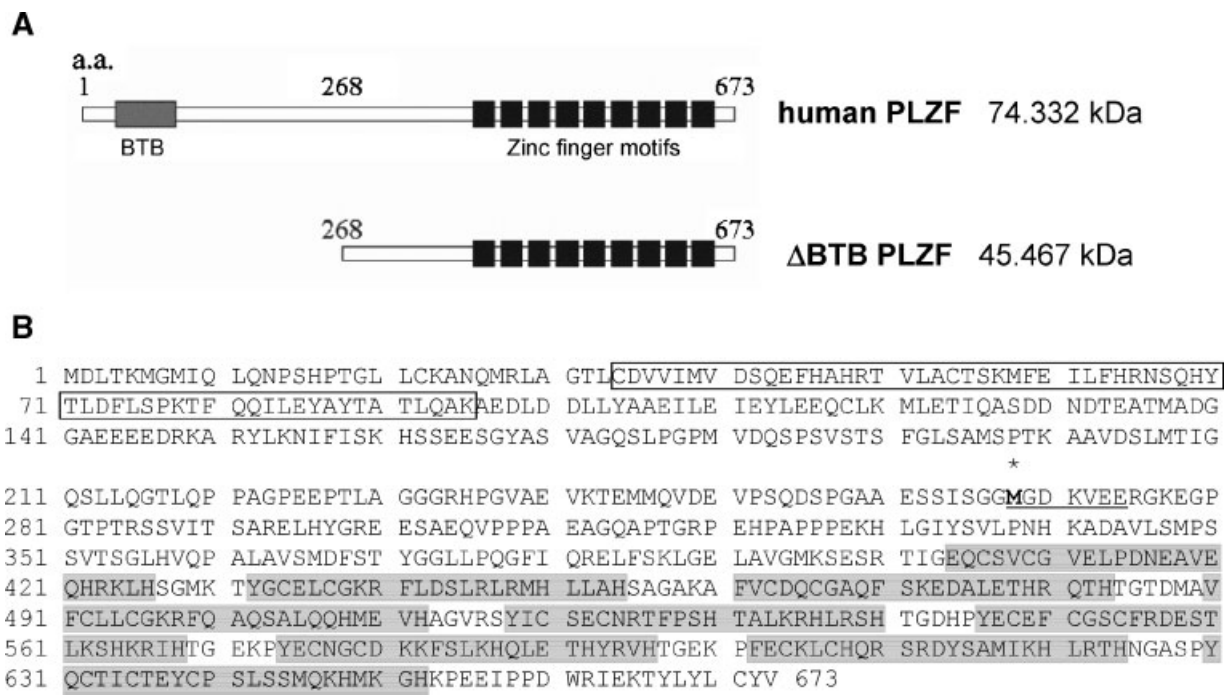

Fig. 2. Comparison of the hepatocytic ΔBTB-PLZF and full-length human PLZF. **A**: The structures of both proteins are represented. The gray box indicates the BTB domain and black boxes represent nine zinc finger motifs. **B**: The start codon of ΔBTB-PLZF is in a bold character with an asterisk and the amino acid sequences obtained from the N-terminal sequencing are underlined. Amino acid sequences of the BTB domain are marked by an empty box and those of zinc finger motifs are shown in the shaded boxes.

[Robinson, 1990] (Fig. 3A). The GFP-fused ΔBTB-PLZF protein, which lacks the BTB domain, was also localized in the trans-Golgi complexes (Fig. 3B).

In order to avoid any possible aberrant localization due to the GFP tag, the locations of the endogenous PLZF and ATP7B proteins were ascertained via the immunostaining of the HepG2 cells with anti-PLZF monoclonal antibody and anti-ATP7B rabbit serum (Fig. 3C). Both of these antibodies were localized in the cytoplasm of the HepG2 cells, and were absent from the nuclei. The superimposition of the confocal images showed the extensive colocalization of both proteins. This suggested that both the ATP7B and PLZF proteins were present in the same subcellular compartment, namely, the trans-Golgi complex of the HepG2 cells.

PLZF and ATP7B Enhanced ERK Kinase Activity in the HepG2 Cells

As PLZF has been determined to possess the molecular properties commensurate with a typical transcriptional repressor, due to its nuclear localization and its BTB domain, which is essential for the recruitment of corepressors [Dhordain et al., 2000; Melnick et al., 2002], we had some difficulty in determining the hepatocytic roles played by the BTB domain-

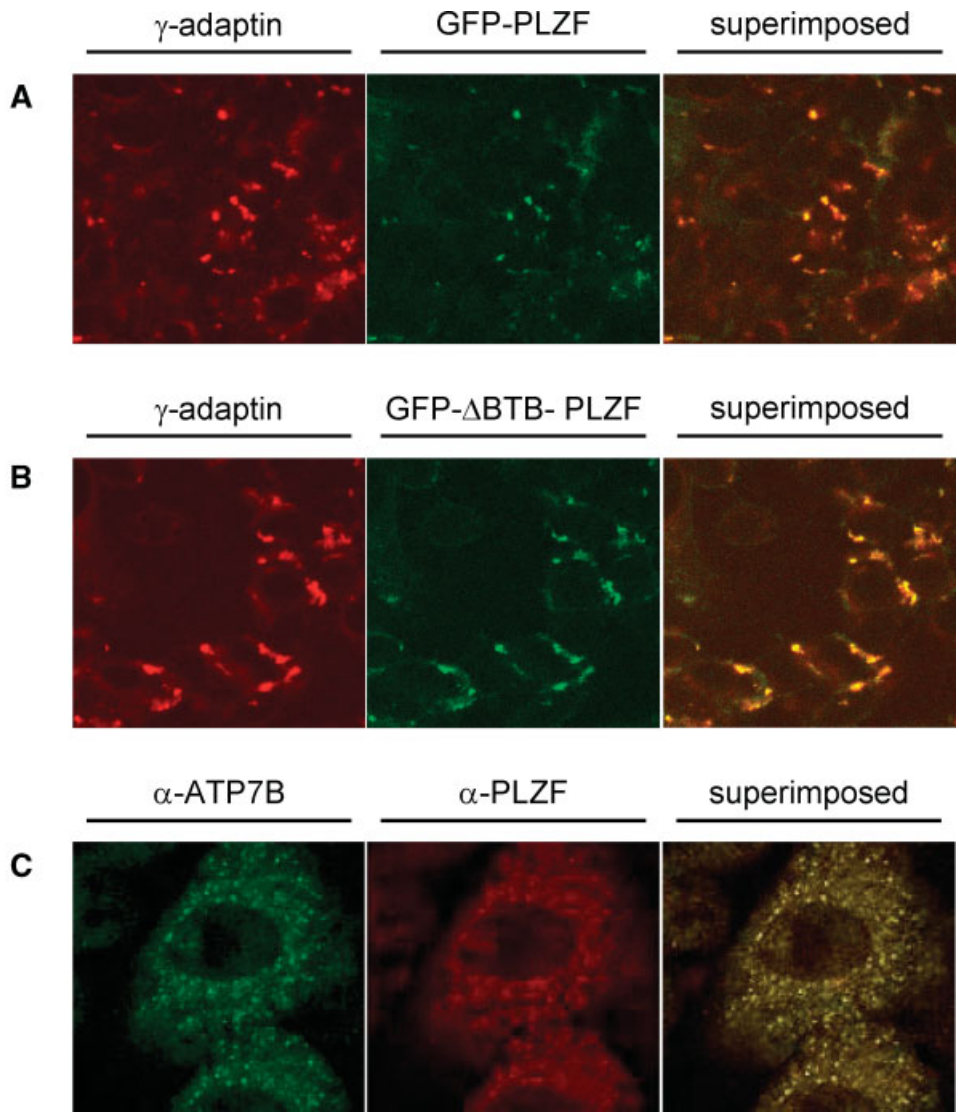

Fig. 3. Colocalization of PLZF and ATP7B proteins in HepG2 cell. **A**: pEGFP-PLZF vector was transfected into HepG2 cells. **B**: pEGFP-ΔBTB-PLZF vector was transfected into HepG2 cells. **C**: The locations of endogenous PLZF and ATP7B proteins in HepG2 cells.

lacking PLZF, which was also localized to the trans-Golgi complexes. As a step toward the functional analysis of the interaction of PLZF with ATP7B, we attempted to assess the possible implications of hepatocytic PLZF in the ERK signal transduction pathway, according to the previous reports regarding the *C. elegans* homolog of PLZF [Howard and Sundaram, 2002].

The expression of the PLZF protein was transiently interfered by the dsRNA-mediated gene silencing, and changes in the activity of ERK were monitored in the HepG2 cells (Fig. 4A). The expression of PLZF mRNA was measured to be diminished by half from the HepG2 cells 8 h after transfection with 200 ng of the RNAi vector of ΔBTB-PLZF, and scarcely detected from the cells 24 h after transfection, whereas the expression of β-actin mRNA was not affected (data not shown, refer to lane 5). The activity of ERK kinase decreased in a dose-dependent manner after the transfection of the pc-ΔBTB-PLZF-IR vector into the HepG2 cells (lane 3–7), but this did not change subsequent to the transfection of the pc-GFP-IR vector, which was used as a negative control (lane 8–12). Therefore, the observed reduction in the activity of ERK was determined to be due to the impaired expression of endogenous hepatocytic PLZF via RNA interference.

We then attempted to determine the effects of exogenous PLZF expressions on ERK activity in the HepG2 cells. The activity of kinase was enhanced slightly via the expression of PLZF or ΔBTB-PLZF (Fig. 4B,C, lanes 3, 5). In addition, ATP7B was also expressed in the hepatocytes, which also resulted in an enhancement of ERK activity. The increase in kinase activity resulting from the expression of ATP7B was found to be more pronounced than the increase in kinase activity which was associated with PLZF expression (lane 7). In a result consistent with the hepatocytic expression of the BTB domain-deleted, the BTB domain of PLZF was not required for the elicitation of ERK activation (lane 5). And the enhancement of ERK activity was also unaffected by the ATP7B expression of the truncated form in which all six copper binding domains had been deleted (lane 9). However, the C-terminal deletion of ATP7B resulted in the definitive abrogation of ERK activity enhancement, showing that the PLZF-interacting C-terminal region of ATP7B is a prerequisite for the activation of hepatocytic ERK (lane 11).

In all of our experiments, the enhancement of ERK activity was inhibited completely as the result of the addition of an MEK inhibitor, PD98059 [Dudley et al., 1995], which indicates that the enhanced ERK activity associated with the expression of PLZF or ATP7B in the hepatocytes depended primarily on the activity of MEK (Fig. 4B,C, lanes 4, 6, 8, and 10). Furthermore, our observation of the phosphorylated-ERK-specific antibodies revealed that the level to which ERK1 was phosphorylated (second row of Fig. 4C) was determined to be intimately related with the enhancement of ERK activity as the result of PLZF or ATP7B expression, whereas the level to which ERK2 was phosphorylated did not undergo any significant changes.

PLZF in *Drosophila* ERK Signal Transduction

In vivo interaction between ATP7B and PLZF was investigated further in *Drosophila* as a model organism, using GAL4-UAS system for the ectopic expression of human ATP7B and the hepatocytic ΔBTB-PLZF [Brand and Perrimon, 1993]. The well-developed *Drosophila* genetics controlling targeted expressions allowed us to monitor the possible interactions according to the phenotypic changes induced

Fig. 4. ERK1 kinase activity in HepG2 cells is regulated by PLZF and ATP7B. **A**: ERK kinase activity of HA-tagged ERK1 was measured using the peptide substrate as described in materials and methods after transfection of RNAi constructs. Activity was normalized to protein content and given in arbitrary units with standard deviations of three independent experiments. HepG2 cells were not transfected (lane 1), transfected with pCEP-hERK1 alone (lane 2), cotransfected with pCEP-hERK1 and 10 ng (lane 3), 100 ng (lane 4), 200 ng (lane 5), 500 ng (lane 6), or 1000 ng (lane 7) of pc-ΔBTB-PLZF-IR, or transfected with pCEP-hERK1 and 10 ng (lane 8), 100 ng (lane 9), 200 ng (lane 10), 500 ng (lane 11), or 1000 ng (lane 12) of pc-GFP-IR. **B**: ERK kinase activity of HA-tagged ERK1 was measured using the peptide substrate as described in materials and methods. Activity was normalized to protein content and given in arbitrary units with standard deviations of three independent experiments. **C**: ERK1 kinase activity assay performed as indicated with ^{32}P-MBP using MBP as substrate. HepG2 cells were transfected and treated as follows: no transfection (lane 1); pCEP-hERK1 (lane 2); pCEP-hERK1 and pcDNA3.1hisA-PLZF (lane 3 and 4); pCEP-hERK1 and pcDNA3.1hisA-ΔBTB-PLZF (lane 5 and 6); pCEP-hERK1 and pcDNA3-ATP7B (lane 7 and 8); pCEP-hERK1 and pcDNA3.1-hisA-mutNATP7B (lane 9 and 10); pCEP-hERK1 and pcDNA3.1-

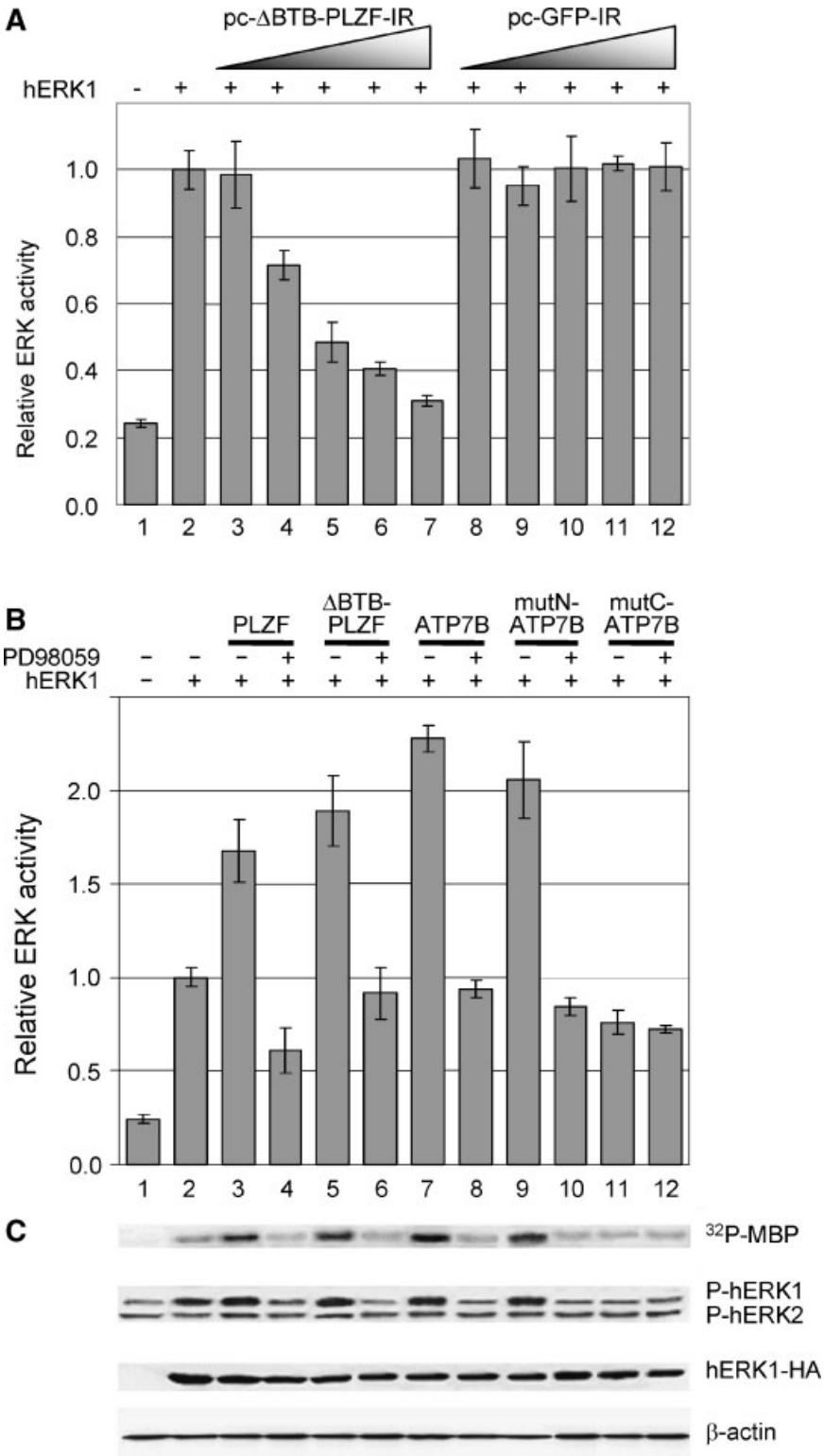
A
pc-ΔBTB-PLZF-IR
pc-GFP-IR
hERK1
Relative ERK activity
B
PLZF
ΔBTB-PLZF
ATP7B
mutN-ATP7B
mutC-ATP7B
PD98059
hERK1
Relative ERK activity
C
32P-MBP
P-hERK1
P-hERK2
hERK1-HA
β-actin

Fig. 4.

as the result of disruptions in eye or wing development.

No apparent phenotypes were noted to result from the ectopic expression of ATP7B (Fig. 5C,G). By way of contrast, we did note major deformities in the eyes and wings of flies that ectopically expressed ΔBTB-PLZF (Fig. 5B,F compared with Fig. 5A,E). The arrays of ommatidia in the compound eyes of these flies were markedly disordered, and the majority of the photoreceptor cells were scattered about, as is shown clearly in the inset. Furthermore, our examination of tangential sections showed that one additional photoreceptor could be openly observed in some of the ommatidia, indicating that PLZF had resulted in the misrecruitment of some photoreceptors (Fig. 6A, arrowheads). Another conspicuous abnormality was observed in the ΔBTB-PLZF-expressing wings under the control of *MS1096-GAL4;* namely, additional ectopic cross-veins noted in the submarginal cell between wing veins L2 and L3, the second and third longitudinal veins (Figs. 5F and 6J, arrows).

These PLZF phenotypes were clearly alleviated by additional ATP7B expression (Fig. 5D,H), evidencing the definitive interaction between PLZF and ATP7B in the in vivo development of organs in *Drosophila*, and also indicating the suppressor role of ATP7B in the PLZF-induced disruption of photoreceptor differentiation and vein formation.

Interestingly, those abnormalities, which were attributed to ectopic PLZF expression, were phenotypically similar to those observed in flies in which the ERK pathways had been activated [Freeman, 1997; Lesokhin et al., 1999; Martin-Blanco et al., 1999]. The activation of Ras/ERK signaling has been shown to induce rough eye phenotypes, as the result of increased photoreceptor misrecruitment in the developing eye imaginal discs, and has also been determined to induce extra-vein phenotypes during the formation of the wing veins. In order

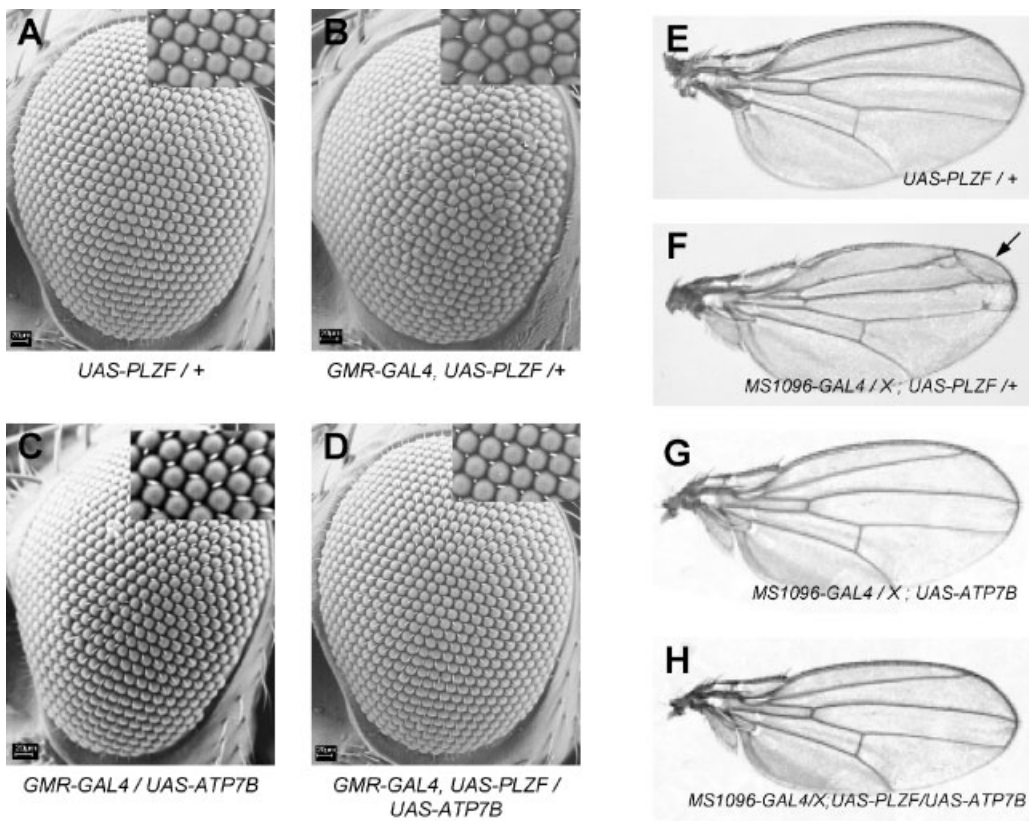

Fig. 5. Genetic interaction between PLZF and ATP7B. **A**–**D**: Eye surfaces of the indicated genotypes were examined by the scanning electron microscopy (SEM; Leo 1455VP; 400 ×). Eye surfaces are shown at higher magnification in insets (1,000 ×). **E**–**H**: Wings of the indicated genotypes were photographed under the optical microscope (20 ×). The ΔBTB-PLZF-induced deformities (**B**, **F**) were fully rescued by coexpression of ATP7B (**D**, **H**). The additional cross-vein is marked with an arrow.

to verify this regulatory role of PLZF in *Drosophila* ERK signaling, which was thought to be most likely to be observed in the hepatocytes, we modified PLZF-induced phenotypes by crossing them with loss-of-function or gain-of-function mutants of ERK signal transduction. The neuronal differentiation of *Drosophila* photoreceptors is initiated with the EGFR activation (encoded by *Egfr)* by binding with Spitz and/or Argos EGFs, and also by induction of the activity of Sevenless RTK (encoded by *sev)*. The activated EGFR signal is transduced along a typical Ras/ERK signaling cascade, which includes *Drosophila* Ras (encoded by *Ras85D)*, Raf (encoded by *phl*, i.e., *pole hole)*, MEK (encoded by *Dsor1*, i.e., *Downstream of raf1)*, and ERK (encoded by *rl*, i.e., *rolled)* [Freeman, 1997].

A dominant negative form of *Drosophila* Ras was coexpressed with ΔBTB-PLZF *(UAS-Ras85D^{N17}/X; GMR-GAL4, UAS-PLZF/+)*, or a loss-of-function *phl* allele was crossed with the PLZF-expressing flies *(phl^{12}/X; GMR-GAL4, UAS-PLZF/+)*, but we did not note any apparent differences in the phenotypes of eyes or wings between these hybrids and those expressing ΔBTB-PLZF only (data not shown) [O'Neill et al., 1994; Lee et al., 1996]. By way of contrast, the heterozygous *rl* or *Dsor1* loss-of-function mutations were determined to result in a reduction in phenotypic alteration from ectopic PLZF expression [Biggs et al., 1994; Hsu and Perrimon, 1994]. Flies harboring these mutations exhibited a lower degree of eye surface scattering, and also exhibited a minimum of additional cross-veins (Fig. 6B,K and C,L, as

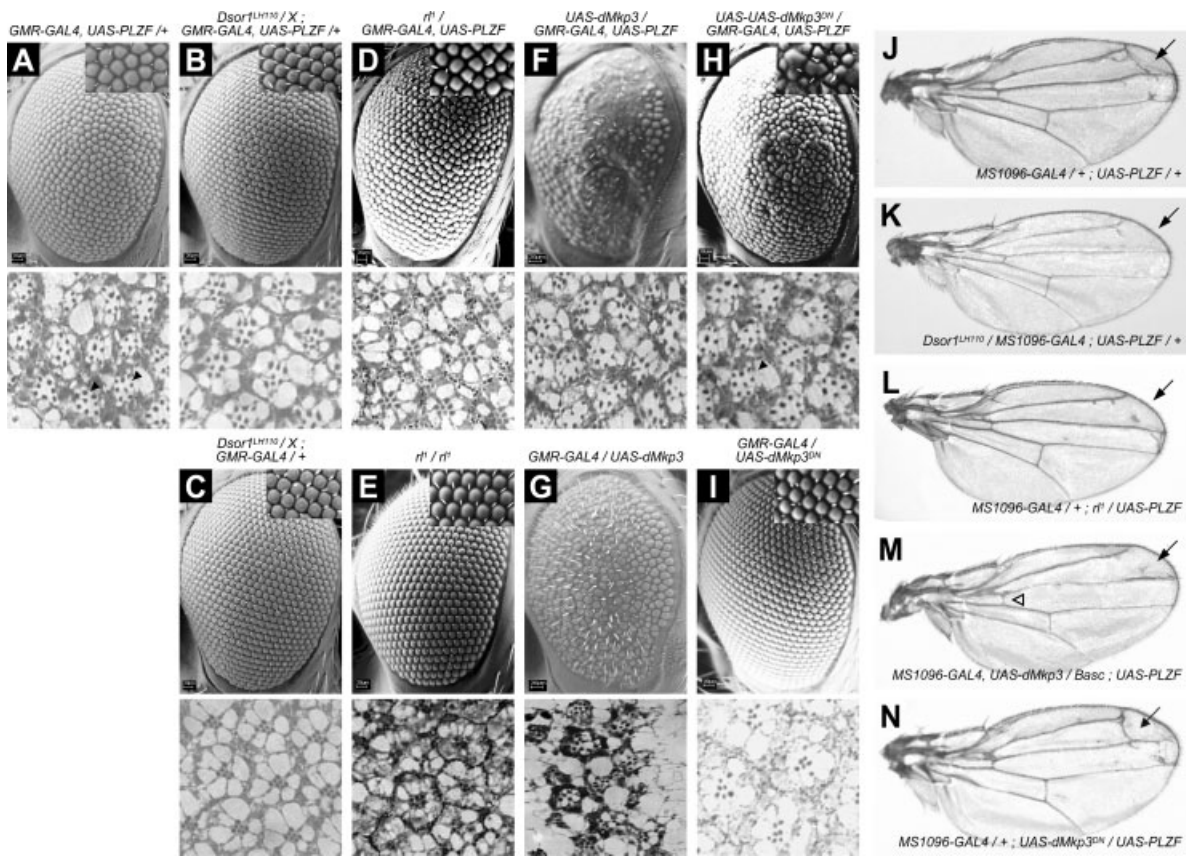

Fig. 6. Genetic interaction between PLZF and *Drosophila* ERK signal components. **A**–**I**: Eye surfaces of the indicated genotypes were examined by SEM (400 ×), and those tangential sections of ommatidia were prepared (1,000 ×). Eye surfaces are shown at higher magnification in insets (1,000 ×). **J**–**N**: Wings of the indicated genotypes were photographed under the optical microscope (20 ×). The activated ERK phenotypes from the ΔBTB-PLZF expression (**A**, **J**) were reduced by one-copy-loss of *Dsor1* (**B**, **K**) or *rl* (**D**, **L**), or by coexpression of *dMkp3* (**F**, **M**). Coexpression of the dominant negative form of DMKP3 strengthened the PLZF-induced phenotypes (**H**, **N**). Ommatidia with an additional photoreceptor are marked with arrowheads, and arrows indicate additional cross-veins or the absence of those. The white arrowhead shows the recovered cross-vein that is frequently missed with the *dMkp3* expression. Note also that

compared with A,J). Tangential sections of Figure 6B (for ΔBTB-PLZF expression in *Dsor1*^{LH110}heterozygotes) and Figure 6C (for ΔBTB-PLZF expression in *rl*^{1} heterozygotes) showed better-organized photoreceptor arrays, with no additional misdifferentiated cells. These modified phenotypes indicated the apparent genetic interactions occurring between exogenous PLZF and *Drosophila* MEK and ERK.

Furthermore, the coexpression of DMKP3 (*Drosophila* ortholog of MKP3) with ΔBTB-PLZF resulted in the complete removal of ectopic cross-veins within the wing (Fig. 6M), and the coexpression of its dominant negative form resulted in a synergistic strengthening of PLZF-induced phenotypes (Fig. 6H,N) [Kim et al., 2002]. Additionally, the photoreceptor-lacking phenotypes associated with wild DMKP3 expression were fully rescued as the result of ΔBTB-PLZF coexpression, as can be observed when comparing the tangential sections of Figure 6F with 6G. The frequently undeveloped cross-veins in the wings of flies expressing *dMkp3* were also recovered when expressing ΔBTB-PLZF (Fig. 6M, white arrowhead), suggesting that exogenous PLZF expression positively modulated ERK signaling during eye and wing development in *Drosophila*, probably in an MEK-dependent but Ras/Raf-independent manner.

DISCUSSION

Using yeast two-hybrid screening, in vitro binding assays, and coimmunoprecipitation, we have observed and characterized the interaction occurring between the C-terminal region of ATP7B and a novel PLZF isoform, in which the BTB domain has been removed. Hepatocytic PLZF and ATP7B were colocalized into the trans-Golgi complexes, and the application of PLZF was observed to enhance ERK activity, as well as the PLZF-interacting C-terminal region of ATP7B, in the HepG2 cells. Finally, ATP7B expression was determined to result in the phenotypic modulation of the ectopic PLZF-induced alterations in the eyes and wings of the model *Drosophila*. These deformities were partially ameliorated as the result of attenuated ERK signaling. These biochemical and genetic results support the notion of an evident interaction occurring between two proteins, probably in the manner in which they regulate ERK signal transduction.

As a result of our yeast two-hybrid screening, which utilized the C-terminal region of ATP7B, the PLZF in which the BTB domain had been deleted was determined to be an interacting protein in the HepG2 cells. A PLZF variant which lacked BTB domain had also been expected after the previous report regarding the genomic organization of the *PLZF* gene [Zhang et al., 1999]. At least four alternative splicings were identified within exon 1 in different tissue distributions, and the BTB domain was also absent in the predicted translation from an isoform of these. However, none of these predicted variants might not correspond to our novel 45-kDa PLZF protein, which is the smallest isoform thus far identified. Whereas it remains unclear as to whether this 45-kDa PLZF protein can be translated from another variant of the alternatively spliced PLZF, or if other modifying mechanisms are employed, the presence of this unexpected 45-kDa PLZF protein was clearly revealed as a result of our coimmunoprecipitation and N-terminal amino acid sequencing (Fig. 1C). Moreover, these endogenous 45-kDa PLZF proteins were identified in the extracts of the HepG2 cells via Western blot analysis (Fig. 1D).

Here, we have also described the enhancement of ERK activity from the hepatocytic expression of PLZF or ATP7B, and the activated ERK phenotypes of flies which express PLZF. Despite these apparent correlations between PLZF/ATP7B expression and enhanced ERK signaling, as evidenced by biochemical and genetic results, the functional consequence of this interaction between PLZF and ATP7B remains to be clearly elucidated. The deletion of the BTB domain and the trans-Golgi-localization of the newly-identified PLZF makes the deduction of the molecular functions of hepatocytic PLZF a much more difficult issue. It remains uncertain as to whether hepatocytic PLZF continues to function as a transcriptional repressor when the BTB domain, which has been reported to be essential to its transcriptional repression, is deleted [Melnick et al., 2002].

Moreover, we registered a discrepancy even in the ERK activity changes caused by the ATP7B expression, between the observed hepatocytic effects and the targeted expression phenotypes in the *Drosophila* organs. Whereas the activity of hepatocytic ERK was clearly positively regulated by ATP7B (Fig. 4B), the

ectopic expression of ATP7B in fruitflies was shown to attenuate the degree to which ERK phenotypes were activated by PLZF (Fig. 5D), referring the suppressor function of ATP7B to the PLZF-induced phenotypes. The latter property of ATP7B can be more simply interpreted, by supposing that its direct binding to PLZF can inhibit the activity of the protein. However, it remains unclear as to whether exogenous human proteins inserted into flies behave in the same manner as those expressed in HepG2 cells. Therefore, flies coexpressing ATP7B and PLZF may not exhibit the same physiological properties as would similarly treated HepG2 cells, and our observed phenotypic modifications should be interpreted as a subsidiary result with regard to the physical interaction between the proteins. This discrepancy would well be traced in a comparative analysis with the fly ortholog of ATP7B. *Drosophila ATP7* encodes for an ortholog of human ATP7B, and acts via copper-regulated trafficking to eliminate copper from the cells [Southon et al., 2004]. On the basis of ATP7B's observed effects with regard to the enhancement of ERK activity in the HepG2 cells, we are unable to dismiss the possibility that *Drosophila* ATP7 may be a positive regulator of ERK signaling, and this will require further study to confirm or disconfirm.

The observed activation of ERK by ATP7B in the present study is significant when considering reports that cisplatin-resistant human solid carcinomas frequently overexpressed ATP7B [Komatsu et al., 2000; Ohbu et al., 2003]. The ERK pathway has also been correlated with resistance to cisplatin, and a specific inhibition of MEK was determined to enhance tumor sensitivity to cisplatin [Persons et al., 2000]. This enhanced ERK signaling appears to be involved in the phosphorylation and stabilization of p53, a well-known regulator of both the G1/S transition in the cell cycle and DNA damage-induced apoptosis. However, the exact relationship existing between ATP7B and the ERK pathway has yet to be determined.

As for the *Drosophila* PLZF ortholog, or the possible gene that corresponds to ectopic PLZF-induced ERK activation, at least 14 members of the BTB-domain/zinc-finger family have been identified in the *Drosophila* genome. Among those characterized or putative proteins, Tramtrack and Trithorax-like have been confidently correlated with Ras/ERK signaling. Tramtrack protein appears to be involved in Ras/ERK signaling during eye development, but this protein suppresses the differentiation of photoreceptors, in marked contrast to the functions of PLZF [Lai et al., 1997]. However, the abrogation of the expression of *Trithorax-like*, a member of the Trithorax group genes which regulate homeotic gene transcriptions in *Drosophila*, were determined to suppress the embryonic lethality induced as the result of inappropriate Ras activation, implying that it may play the role of a transcriptional activator, facilitating Ras signaling [Maixner et al., 1998]. The Broad protein is another BTB-domain/zinc-finger protein, which performs a suppressor function similar to that of the Tramtrack protein in the differentiation of photoreceptors, although its correlation with Ras/ERK signaling has not yet been characterized precisely [Brennan et al., 2001].

However, this protein was determined to be involved in the leg development and the steroid-triggered autophagic cell death, similar to mouse PLZF [Bayer et al., 1997; Lee and Baehrecke, 2001]. Loss-of-function mutations of *abrupt*, the encoding product of which is a protein that is extremely homologous with the Broad protein, caused the development of the same gnarled legs as were seen with the *broad* mutations [Hu et al., 1995]. Of all the *Drosophila* BTB-domain/Zn-finger proteins, the predicted AAF53152 (NCBI) exhibits protein structures most similar to those of PLZF and EOR-1. However, the putative gene products have, unfortunately, not yet been characterized.

The function of PLZF within the ERK pathway was determined in the present study, as a follow-up to the previous studies of the *C. elegans* EOR-1, a positively-acting nuclear component of the Ras and Wnt pathways [Howard and Sundaram, 2002]. However, it should be noted that the hepatocytic PLZF in which the BTB domain had been deleted was localized into the trans-Golgi complexes in a manner different from that of the worm PLZF, which acted as a transcriptional repressor similarly to other BTB-domain/zinc-finger proteins. As was described previously, it remains uncertain as to whether the trans-Golgi-localized proteins might behave as a transcriptional regulator. However, degree to which ERK signaling depends on the expression of PLZF may be commonly obvious between two

proteins: in our studies, PLZF should be essential for ERK activity in the HepG2 cells, as was shown by RNAi assays to downregulate endogenous PLZF (Fig. 4A).

The MEK-dependent and Ras/Raf [Bull et al., 1993]-independent manner of the activated ERK phenotypes of the PLZF flies resembles that of the pulmonary cell's response to metallic exposure of copper [Samet et al., 1998; Wu et al., 1999], in which copper treatment induced ERK activation in a MEK activity-dependent manner, but was scarcely affected by Raf, and only weakly affected by EGFR. Samet and his colleagues discussed the possible involvement of PTPases (protein tyrosine phosphatases, including *Drosophila* Corkscrew, a positive regulator of Ras85D, Phl, and Ksr, cf. Johnson Hamlet and Perkins [2001]) in the metallic activation of MEK [Samet et al., 1999; Wu et al., 1999], and the hypotheses raised in that discussion might be testable in future studies, using regulated expressions of human or fly ATP7B.

To our knowledge, this is the first report for molecular properties of BTB domain-lacking PLZF proteins. Although the functional consequences of the interactions occurring between hepatocytic PLZF and ATP7B have yet to be elucidated, ERK signaling was clearly affected by PLZF or ATP7B expression, probably via the interaction between the two proteins, as is shown by the fact that no ERK activity enhancement was observed in HepG2 cells which expressed an ATP7B variant in which the C-terminus had been deleted (Fig. 4B). Furthermore, transformed fruitflies which expressed hepatocytic PLZF were shown to constitute a good model for the study of the genetic interactions and molecular properties of PLZF, revealing that hepatocytic PLZF played a role as an activating component in ERK signal transduction in the present study.

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