Central role of Prominin-1 in lipid rafts during liver regeneration

Prominin-1, a lipid raft protein, is required for maintaining cancer stem cell properties in hepatocarcinoma cell lines, but its physiological roles in the liver have not been well studied. Here, we investigate the role of Prominin-1 in lipid rafts during liver regeneration and show that expression of Prominin-1 increases after 2/3 partial hepatectomy or CCl4 injection. Hepatocyte proliferation and liver regeneration are attenuated in liver-specific Prominin-1 knockout mice compared to wild-type mice. Detailed mechanistic studies reveal that Prominin-1 interacts with the interleukin-6 signal transducer glycoprotein 130, confining it to lipid rafts so that STAT3 signaling by IL-6 is effectively activated. The overexpression of the glycosylphosphatidylinositol-anchored first extracellular domain of Prominin-1, which is the domain that binds to GPI130, rescued the proliferation of hepatocytes and liver regeneration in liver-specific Prominin-1 knockout mice. In summary, Prominin-1 is upregulated in hepatocytes during liver regeneration where it recruits GP130 into lipid rafts and activates the IL6-GP130-STAT3 axis, suggesting that Prominin-1 might be a promising target for therapeutic applications in liver transplantation.

The liver is a pivotal organ for maintaining homeostasis by regulating metabolism, drug detoxification and bile transportation. Hepatocytes, the major parenchymal cells in the liver, could be damaged by various factors such as surgical operation, alcohol, virus and chemicals, which leads to a decrease in liver mass. To maintain homeostasis, the liver has a unique capability to recover its original mass. Many studies for cytokines or growth factors have tried to contribute therapeutic approaches to promote liver regeneration. For example, there are antibody, agonist or antagonist therapy targeting for specific signaling pathway in liver regeneration. In addition, some studies have attempted to use a cell population with stemness for a cell therapy. Understanding the molecular mechanisms in liver regeneration is important for application in the field of liver disease therapy.

A 2/3 partial hepatectomy (PHx) is a well-characterized experimental model for liver regeneration in rodents. Mice recover most of their liver mass 7 days after PHx. During liver regeneration, quiescent hepatocytes proliferate by several cytokines and growth factors, such as epidermal growth factor (EGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), tumor necrosis factor-α (TNFα), and interleukin-6 (IL-6). IL-6 is a pleiotropic cytokine in the body. After PHx or other liver injuries, gut-derived factors such as lipopolysaccharides (LPS) activate Kupffer cells and resident liver macrophages to secrete IL-6. Secreted IL-6 binds to the interleukin-6 receptor (IL-6R) and then forms a signaling complex consisting of IL-6R and interleukin-6 signal transducer glycoprotein 130 (GP130) in hepatocytes. The complex initiates
several downstream signaling pathways, including Janus kinases (JAKs), signal transducer and activator of transcription 3 (STAT3), MAP kinases and the PI3 kinase pathway.

IL-6 knockout impairs hepatocyte proliferation and induces liver necrosis after PHx in mice, preventing liver mass recovery. As a result, IL-6 knockout significantly increases mortality after surgery. Thus, a single injection of IL-6 rescues this phenotype in a 6-knockout mice. In addition, liver-specific Stat3 knockout impairs the DNA synthetic response in hepatocytes and decreases the expression of G1 phase cyclins such as cyclin D1 and cyclin E. Consistent with the important role of the IL-6 signaling pathway during liver regeneration, liver-specific knockout of suppressor of cytokine signaling 3 (SOCS3), a negative regulator of the STAT3 pathway, exhibits prolonged activation of STAT3 and enhances hepatocyte proliferation, resulting in accelerated liver mass replenishment after PHx.

Promin-1 (PROM1), also known as CD133, is a penta-span transmembrane glycoprotein. PROM1 is associated with distinct detergent-resistant lipid rafts and is found in membrane protrusions such as filopodia and microvilli. PROM1 has been studied as one of the most widely used cancer stem cell (CSC) markers in various human tumors, including the liver. In addition to cancer stem cells, PROM1 is also expressed in normal stem cells, including hematopoietic stem cells and vascular endothelial cells, in the brain, kidney, digestive track, and liver. Specifically, PROM1 has been known to express in the liver (human and mouse), including canals of Hering, bile ducts and hepatocytes. Indeed, PROM1 regulates the glucagon and TGF-β signaling pathways in the liver by interacting with radixin and SMAD7, respectively.

Because PROM1, a marker for hepatic progenitor cells, is also upregulated in hepatocytes after liver injury, the upregulated PROM1 might regulate various signaling pathways related to hepatocyte proliferation. Here, we observed a significant increase in the expression of PROM1 in hepatocytes during liver regeneration after PHx or CCl4 injection. Liver-specific Prom1 knockout (Prom1/LKO) mice showed impaired liver regeneration because of reduced hepatocyte proliferation. Mechanistically, we found that the increased expression of PROM1 in hepatocytes confined GP30 to lipid rafts and facilitated activation of STAT3. These results demonstrated that PROM1 plays an important role during liver regeneration through the IL6-GP30-STAT3 signaling pathway.

Results

PROM1 is upregulated in hepatocytes during liver regeneration

To investigate the expression of PROM1 during liver regeneration, we performed 2/3 partial hepatectomy (PHx) in wild-type mice. We found that the mRNA level of PROM1 increased after PHx by qRT-PCR (Fig. 1a). The mRNA level of PROM1 peaked 48 hours after PHx and then gradually decreased. Consistently, immunoblotting confirmed that the protein level of PROM1 increased 24, 48, and 120 hours after PHx (Fig. 1b and Supplementary Fig. 1A). Next, we determined which cells expressed PROM1 in the liver by PROM1 double immunofluorescence with hepatocyte nuclear factor 4α (HNF4α or Ki-67 as a specific marker of hepatocytes) or cytokeratin-19 (CK19 as a specific marker of ductal cells). (Fig. 1c, d). PROM1 was mainly expressed in ductal cells of sham liver, whereas it was expressed in hepatocytes of PHx liver. In our previous report, we confirmed that PROM1 was expressed in microvilli of primary hepatocytes using an electron microscopy. Thus, we tried to confirm the localization of PROM1 in the microvilli of hepatocytes in PHx liver by correlative light and electron microscopy (CLEM). As shown in Supplementary Fig. 1B, immunogold-labeled PROM1 was localized in the microvilli of hepatocytes in wild-type (Prom1/Lf) liver, but not in Prom1/LKO liver. These data showed that PROM1 was localized in the microvilli of hepatocytes from PHx liver.

To further clarify the cells types expressing PROM1 during liver regeneration, we generated a lineage-tracing mouse in which tdTomato (tdTom) was expressed by tamoxifen in PROM1-positive cells (Fig. 1e) and observed the expression of tdTom after PHx in the liver. Consistent with the immunofluorescence data, the expression of tdTom significantly increased in HNF4α-expressing hepatocytes but not in CK19-expressing ductal cells after PHx (Fig. 1f). Indeed, -41% of HNF4α-expressing hepatocytes expressed tdTom (Fig. 1g). These data demonstrate that the expression of PROM1 significantly increases in hepatocytes during liver regeneration after PHx.

PROM1 deficiency impairs liver regeneration in mice

To determine the role of PROM1 in the process of liver regeneration, we compared the livers of wild-type (Prom1/L+) and liver-specific Prom1 knockout mice (Prom1/L−/−) after PHx. As a result of measuring the remnant liver-to-body weight ratio following PHx, liver regeneration of Prom1/L−/− mice was impaired compared to that of Prom1/L+ mice (Fig. 2a). Prom1/L−/− mice recovered their original liver mass almost 5 days after PHx, whereas Prom1/L+ mice did not. Compared with Prom1/L+ mice, the liver-to-body weight ratio was significantly lower in Prom1/L−/− mice 48 and 120 hours after surgery.

To investigate hepatocyte proliferation between Prom1/L+ and Prom1/L−/− mice during liver regeneration, we confirmed cell cycle-related genes (Cyclin A, B, D, E, and PCNA) in PHx livers by qRT-PCR and immunoblotting. The levels of cyclin mRNAs were reduced in Prom1/L−/− livers more than in Prom1/L+ livers (Fig. 2b). Consistently, the expression of cell cycle-related proteins in Prom1/L−/− mice decreased compared to that in Prom1/L+ mice after PHx (Fig. 2c, d). We also analyzed hepatocyte proliferation by H&E staining and double immunofluorescence along with Ki-67 (as a cell proliferation marker) and HNF4α-expressing hepatocytes (Fig. 2e-g). As shown in Fig. 2f, Ki-67 expression in Prom1/L+ mice increased more than that in Prom1/L−/− mice after PHx. Indeed, PROM1 deficiency decreased the number of Ki-67-positive cells by ~50% (Fig. 2g). These results suggested that the liver-specific deletion of PROM1 decreased hepatocyte proliferation and impaired liver regeneration after PHx.

Liver-specific PROM1 deficiency reduces liver regeneration in mice injected with CCl4

To further investigate the effects of PROM1 deficiency on the proliferation of hepatocytes in the regenerating liver, we analyzed the liver after injecting CCl4 into mice. As with liver regeneration by PHx, PROM1 expression also increased after CCl4 injection. PROM1 mRNA increased over ~10-fold in the liver by CCl4 injection (Fig. 3a). PROM1 double immunofluorescence with HNF4α or CK19 showed that major cells expressing PROM1 were hepatocytes after CCl4 injection (Fig. 3b, c).

Next, we compared the expression of cell cycle-related proteins in the livers of Prom1+ and Prom1−/− mice after CCl4 injection. PROM1 deficiency significantly decreased the expression of Cyclin A, Cyclin B, and PCNA, as determined by immunoblotting (Fig. 3d, e). Hepatocyte proliferation and apoptosis were confirmed by H&E staining and TUNNEL assay in the livers of Prom1+ and Prom1−/− mice after CCl4 injection (Fig. 3f and Supplementary Fig. 2). PROM1 deficiency decreased the number of Ki-67-expressing cells without changing apoptosis after CCl4 injection by ~80%, as determined by immunofluorescence (Fig. 3g, h, and Supplementary Fig. 2). Taken together, these data suggested that PROM1 deficiency attenuates hepatocyte proliferation during liver regeneration in the CCl4 model.

PROM1 increases IL-6 signaling during liver regeneration

Hepatocyte proliferation in the early stage of liver regeneration requires the JAK-STAT, PI3K, MAPK, and β-catenin signaling pathways initiated by different mitogens, such as IL-6, EGF, HGF and Wnt. To examine the signaling pathways affected by PROM1, we observed the expression and activation of these mitogenic signaling molecules after PHx by immunoblotting. PROM1 deficiency significantly decreased the phosphorylation status of STAT3 and ERK but not the phosphorylation status of AKT or GSK3β (Fig. 4a, b). In the CCl4 model, PROM1
Fig. 1 | The expression of PROMI in hepatocytes increases after partial hepatectomy. A 2/3 partial hepatectomy was performed in 8-week-old male wild-type mice. a The relative mRNA level of PROM1 in sham and PHx livers (n = 3 for sham, n = 5 for 24 h, n = 7 for 48 h, n = 5 for 120 h, p = 0.045). b Immunoblotting for PROM1 in wild-type livers 48 hours after PHx (n = 2 for sham, n = 9 for PHx). c, d Double immuno-fluorescence for PROM1 and HNF4α (c) or CK19 (d) in sham and PHx livers. e Prom1-Cre/ERT2; Rosa26-loxP-STOP-loxP-tdTomato mice were generated for lineage tracing of cells expressing PROM1 in the liver. PHx was performed 1 day after tamoxifen injection. The mice were analyzed 7 days after sham (n = 4) or PHx (n = 4). f Representative images of tdTom double immuno-fluorescence with HNF4α or CK19 in sham and PHx livers. g The percentage of tdTom-expressing cells was statistically determined from total HNF4α- or CK19-expressing cells (p = 6.197×10^-4 for HNF4α). Scale bar = 100 µm. Two-sided student t-test; *p < 0.05, **p < 0.001, n.s, nonsignificant. Data are expressed as the mean ± SEM with individual values. Source data are provided as a Source Data file.
Fig. 2 | Liver-specific deletion of Prom1 in mice impairs liver regeneration after partial hepatectomy. A 2/3 partial hepatectomy was performed in 8-week-old male Prom1f/f and Prom1LKO mice. a Ratio of liver-to-body weight on the indicated days after PHx (n = 4 for sham, 12 h, and 120 h, n = 5 for 24 h, n = 7 for 48 h, p = 0.034 for 48 h, p = 0.005 for 120 h). b The relative mRNA levels of cell cycle genes (Ccnb, Ccne, Ccnb) 48 hours after PHx (n = 8). Each mRNA level was normalized by 18 S rRNA (p = 0.041 for Ccnb, p = 0.029 for Ccnb, p = 0.039 for Ccne). c Immunoblotting for PROM1 and cell cycle proteins (Cyclin A, B, and E, and PCNA) 48 hours after PHx. d Statistical analysis of the band intensity in c (n = 3 independent samples, p = 0.032 for Cyclin A, p = 4.508 × 10^−5 for Cyclin B, p = 0.042 for Cyclin E, p = 0.049 for PCNA). The band intensity of each protein was normalized to that of β-actin. e Representative H&E staining in the liver 48 hours after PHx. Mitotic cells are indicated by arrows. The experiment was repeated independently three times with similar results. f Representative double immunofluorescence for Ki-67 and HNF4α in the liver 48 hours after PHx. g Statistical analysis of the number of Ki-67-expressing cells after PHx (n = 3, p = 1.149 × 10^−6). The number of Ki-67-positive cells was normalized to the number of DAPI-stained dots. Scale bar = 100 µm. Two-sided student t-test; *p < 0.05, **p < 0.01, ***p < 0.001. Data are expressed as the mean ± SEM with individual values. Source data are provided as a Source Data file.
Fig. 3 | Liver-specific deletion of Prom1 in mice impairs liver regeneration after CCl₄ injection. Eight-week-old male Prom1⁺⁺ and Prom1⁻KO mice were intraperitoneally injected with vehicle (n = 3) or CCl₄ (n = 5) for 48 hours. The liver was analyzed by qRT-PCR, immunoblotting and immunofluorescence. a The relative mRNA level for PROM1. The mRNA level of PROM1 was normalized by 18 S rRNA (p = 0.028). b, c Double immunofluorescence for PROM1 and HNF4α (b) or CK19 (c). The experiment was repeated independently three times with similar results. d Immunoblotting for PROM1, Cyclin A and B, and PCNA. e Statistical analysis of the band intensity in d (n = 3, p = 0.033 for Cyclin A, p = 0.029 for Cyclin B, p = 0.005 for PCNA). The band intensity of each protein was normalized to that of β-actin. f Representative H&E staining in the liver. Mitotic cells are indicated by arrows. The experiment was repeated independently three times with similar results. g Double immunofluorescence for Ki-67 and HNF4α. h Statistical analysis of the number of Ki-67-expressing cells (n = 3, p = 4.821 × 10⁻⁸). The number of Ki-67-positive cells was normalized to the number of DAPI-stained dots. Scale bar = 100 µm. Two-sided student t-test; *p < 0.05, **p < 0.01, ***p < 0.001. Data are expressed as the mean ± SEM with individual values. Source data are provided as a Source Data file.
deficiency also decreased the phosphorylation of STAT3 (Fig. 4c, d).
Since IL-6 signals are known to activate both STAT3 and ERK, these results led us to investigate the IL-6 signaling pathway in more detail.
IL-6 ELISA showed that PROM1 deficiency did not change the serum level of IL-6 after PHx or CCl4 injection (Fig. 4e). In addition, PROM1 deficiency did not change the expression level of growth factors such as EGF and HGF as determined by qRT-PCR (Supplementary Fig. 3A, B). Thus, these data allow us to rule out the effect of PROM1 on IL-6, EGF and HGF production and secretion during liver regeneration. Next, we confirmed that PROM1 overexpression statistically increased IL-6-induced phosphorylation of STAT3 by ~2-fold in HEK 293 cells and by ~6-fold in primary hepatocytes obtained from Prom1LKO mice (Fig. 4f, g).
To further confirm the association between PROMI and the IL-6 signaling pathway, we observed whether liver regeneration impaired by PROMI deficiency was rescued through adenosine overexpression of constitutively activated STAT3 (Stat3c) in Prom1\textsuperscript{fl/fl} mice. As determined by qRT-PCR and immunoblotting (Supplementary Fig. 4A–C), cyclins A, B, and E and PCNA were significantly increased by Stat3c overexpression. Consistent with these data, Ki-67 and HNF4 were decreased in PHx livers (Fig. 5a, b). PROM1 was expressed in lipid rafts of sham livers. However, GP130 was not expressed in lipid rafts of both sham livers. In contrast to sham livers, both PROM1 and GP130 were found in lipid rafts of Prom1\textsuperscript{fl/fl} mice. We confirmed that PROMI deficiency reduced the expression of GP130 in lipid rafts of PHx livers. PROMI-positive hepatocytes accounted for ~1% of total hepatocytes in sham liver and ~40% in PHx liver (Fig. 1f, g). Thus, PROMI expression level is too low to recruit GP130 to lipid rafts on this result, the expression of GP130 in liver lipid rafts (Fig. 5c).

Because giant plasma membrane vesicles (GPMVs), which are isolated from mammalian cells without using detergent, are useful for identifying raft proteins, we tested whether PROMI and GP130 are recruited into raft phase of GPMV. HEK 293 cells were co-transfected with Prom1-GFP or glycosylphosphatidylinositol-anchored GFP (GPI-GFP) and stained with Dil (1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) for a non-raft marker. As shown in Supplementary Fig. 5A, GPMVs were successfully isolated and purified from HEK 293 cells co-transfected with Prom1-GFP or glycosylphosphatidylinositol-anchored GFP. Western blot analysis showed that Prom1, GP130, and β-actin were co-localized with the GPMVs (Fig. 5c). We further confirmed that there are two types of rafts, PROM1-enriched and GPI-anchored rafts (Supplementary Fig. 6A, B). As shown in Fig. 6c, EX1 was prominently expressed in lipid rafts in PHx liver. Indeed, PROMI overexpression in Prom1\textsuperscript{fl/fl} sham livers increased the expression of GP130 in lipid rafts (Fig. 5c).

The first extracellular domain of PROMI is required for the interaction with GP130 and the regulation of the STAT3 signaling pathway

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The first extracellular domain of PROMI is required for the interaction with GP130 and the regulation of the STAT3 signaling pathway

To determine the domain required for the interaction between PROMI and GP130, we generated various deletion mutants of PROMI (Fig. 6a).

A co-immunoprecipitation assay using these mutants showed that all deletion mutants of PROMI still interacted with GP130 (Fig. 6b). Based on this result, the first extracellular domain of PROMI (PROMI-EX1) would be an important region for the interaction between the two proteins. To examine the possibility, we generated GPI-anchored PROMI-EX1 (PROMI\textsuperscript{GPI-EX1}) in which the first transmembrane domain was substituted with a GPI anchor and observed the interaction between PROMI\textsuperscript{GPI-EX1} and GP130. We identified the GPI anchor from PROMI\textsuperscript{GPI-EX1} after treatment of phosphatidylinositol-specific phospholipase C (PI-PLC) which releases GPI-anchored proteins from plasma membrane (Supplementary Fig. 6A, B). As shown in Fig. 6c, EX1 itself interacted with GP130.

PROMI is not co-localized with alkaline phosphatase, a GPI-anchored protein, although both proteins are raft proteins, suggesting that there are two types of rafts, PROMI-enriched and GPI-anchored protein-enriched rafts. To further address this question, cell-surface immunostaining for PROMI and PROMI\textsuperscript{GPI-EX1} was performed in HEK 293
cells overexpressing either or both of untagged PROM1 and FLAG-tagged PROM1\textsuperscript{GPI-EX1}. PROM1 was labeled with an anti-PROM1 antibody that binds to EX2 and EX3 whereas PROM1\textsuperscript{GPI-EX1} was labeled with an anti-FLAG antibody. As shown in Supplementary Fig. 6C, PROM1 and PROM1\textsuperscript{GPI-EX1} were not co-localized with each other. In addition, WGA was co-localized with PROM1 but not with PROM1\textsuperscript{GPI-EX1} (Supplementary Fig. 6D). All these data suggested that PROM1 and PROM1\textsuperscript{GPI-EX1} were not co-localized in the same type of lipid rafts.

Since GPI-anchored proteins are expressed in lipid rafts, we examined whether PROM1\textsuperscript{GPI-EX1} enhances the STAT3 signaling pathway. Exogenous PROM1\textsuperscript{GPI-EX1} itself increased the activity of STAT3 in HEK 293 cells (Fig. 6d, e). Taken together, the first extracellular domain...
of PROM1 is required for binding to GPI30 and regulating the GPI30-STAT3 signaling pathway.

The expression of GPI-anchored PROM1-EX1 rescues liver regeneration in PROM1-deficient mice after partial hepatectomy

To evaluate whether PROM1 is required in vivo function in liver regeneration after PHx, we observed recovery of liver mass and hepatocyte proliferation after adenosine overexpression of PROM1-GPI in a proem-GPI mice. PROM1-GPI was overexpressed in the liver, as determined by qRT-PCR and immunoblotting (Fig. 7b, c). The overexpression of PROM1-GPI alone was sufficient to increase the liver-to-body weight ratio at 24 and 48 hours after PHx in proem-GPI mice (Fig. 7a). As determined by qRT-PCR and/or immunoblotting for CYC A and B, PCNA and phospho-STAT3, H&E staining, and immunofluorescence for Ki-67, the overexpression of PROM1-GPI statistically increased hepatocyte proliferation via STAT3 phosphorylation compared to the overexpression of LacZ (Fig. 7b–h and Supplementary Fig. 7). In addition, GPI30 was relocated into lipid rafts after the overexpression of PROM1-GPI (Fig. 7i). These data suggested that PROM1-GPI has a crucial role in refining GPI30 into lipid rafts and mediating an IL-6-GPI30 axis, thereby promoting liver regeneration.

Discussion

PROM1 is well known as a marker for cancer stem cells and normal stem cells. Recent studies have revealed its ability to regulate various cellular signal transduction pathways by interacting with PI3K, HDAC6, radixin, and SMAD723,28,30. Indeed, PROM1-deficiency leads to the prevention of glucagon-induced gluconeogenesis via inactivating the function of radixin as a kinase-anchoring protein (AKAP)22, and aggravation of bile duct ligation (BDL)-induced liver fibrosis via SMAD7 degradation30, indicating that PROM1 has different functions in the liver. Here, we demonstrated that PROM1 is also necessary for regulating IL-6 signaling during liver regeneration. We found that the expression of PROM1 dramatically increased in hepatocytes during liver regeneration after PHx or CCl4 injection. Hepatocellular PROM1 facilitated the IL-6 signaling pathway by interacting with GPI30 in lipid rafts. As a result, PROM1 promoted the proliferation of hepatocytes during liver regeneration (Fig. 9). Thus, this study is the first to elucidate the function of PROM1 in liver regeneration and is expected to provide a deeper understanding of liver regeneration and liver transplantation therapy.

Lipid rafts are defined as a membrane domain resistant to non-ionic detergent because they are tightly packed with glycosphingolipids and cholesterol27–29. The tight packaging of glycosphingolipids and cholesterol also induces phase partitioning in GPI304. However, phase partitioning in GPI is different from DRM for many multi-span transmembrane proteins due to the disruption of the cytoskeleton network, lipid bilayer asymmetry, and protein-protein interaction in GPI30. In previous our report21, PROM1 interacts with cortical actin by radixin. Since cortical actin might be disrupted during GPI30 isolation, it seems that we failed to observe raft recruitment of PROM1 in GPI30.

GPI30 is recruited from nonraft to lipid rafts after ciliary neurotrophic factor (CNTF) treatment in neural cells (IMR-32 cells)43 whereas it is always found in lipid rafts independent on ligand activation in mouse embryonic neural precursor cells21, Madin-Darby canine kidney cells21, and Hep3B cells30. Our data showed that GPI30 was found in lipid rafts in PROM1-overexpressing Prom1KO sham liver, indicating GPI30 localization in lipid rafts is dependent on PROM1 but not IL-6 activation.

During liver regeneration after PHx and CCl4 injection, PROM1 was highly upregulated, as determined by qRT-PCR, immunofluorescence, and immunoblotting. In addition, PROM1 upregulation was dramatically demonstrated in PROM1 lineage tracing mice (Prom1Cre/Rosa26tdTomato, Rosa26Gp130marmouse mice), in which cells express tdTomato under the control of the PROM1 promoter. Because hepatocellular PROM1 upregulation is also observed after bile duct ligation (BDL)29 and a lithocholic diet (data not shown), various liver damages might lead to hepatocellular PROM1 upregulation. Many extracellular and intracellular factors, such as HIF-1α, TGFβ1, p53 and mTOR, regulate the expression of PROM121,45. A previous study reported that STAT3 promotes the transcription of PROM1 in hepatocellular carcinoma46. Therefore, we hypothesized that the IL-6-STAT3 signaling pathway might be necessary for upregulating PROM1 in hepatocytes, and then, the upregulated PROM1 would form a ‘positive loop’ because PROM1 promotes the IL-6-STAT3 signaling pathway.

PROM1 interacts with various signaling molecules through its different domains. The cytoplasmic C-terminal domain of PROM1 binds to PI3K and radixin, maintaining cancer stem cell properties and regulating glucagon-induced PKA activity, respectively28,30. The first intracellular loop of PROM1 binds to HDAC6 and SMAD7, regulating Smad3 signaling and TGFβ signaling, respectively28,30,47. Here, we demonstrated that the first extracellular domain of PROM1 binds to GPI30. Furthermore, lipid raft-targeted GPI30 alone is sufficient to replace the function of full-length PROM1, which recruits GPI30 into lipid rafts and then facilitates IL-6-induced STAT3 phosphorylation, leading to hepatocyte proliferation and liver regeneration.

The PROM1-positive population in various tumors has self-renewal and differentiation potential and chemotherapy or radiotherapy resistance21,24. Although most cancers are removed through cancer therapy, only a small number of surviving PROM1-positive cells can proliferate and cause cancer to recur. Thus, PROM1 has been considered a very important target protein for cancer therapy. Because PROM1 expression was upregulated at the early stage of liver regeneration (within 48 hours after PHx) and then returned to sham liver levels at the termination stage of regeneration (7 days after PHx, data not shown), hyperplasia or tumorgenesis might not occur during liver regeneration.

In addition to IL-6, GPI30 is involved in various signaling pathways of IL-6 family cytokines, such as IL-11, leukemia inhibitory factor (LIF), oncostatin M, and ciliary neurotrophic factor47. Therefore, the PROM1-GPI30 axis could be a potential therapeutic target for human diseases induced by these cytokines. For example, a PROM1-neutralizing antibody targeting PROM1-EX1 is a good candidate for alleviating inflammatory diseases caused by these cytokines.
Methods

Animal studies

Whole-body Prom1 knockout mice (Prom1\textsuperscript{Cre/ERT2-nlacZ}) were purchased from The Jackson Laboratory (Stock No: 017743, Bar Harbor, ME, USA) and backcrossed with C57BL/6 N mice for five generations. Liver-specific Prom1 knockout mice were generated by crossing Prom1\textsuperscript{fl/fl} C57BL/6 mice (ToolGen, Seoul, Korea) with Alb-Cre C57BL/6 mice containing the Cre recombinase sequence driven by the albumin promoter (The Jackson Laboratory, Bar Harbor, ME, USA). Prom1 lineage tracing mice were generated by crossing Prom1\textsuperscript{Cre/ERT2-nlacZ} C57BL/6 mice with Rosa26\textsuperscript{tdTomato} C57BL/6 mice containing the tdTomato sequence prevented by the loxP-flanked STOP cassette (Stock...
Fig. 6 | The first extracellular domain of PROM1 interacts with GP130 and regulates the STAT3 signaling pathway. a Structures of PROM1 deletion mutants. EX extracellular domain, TM transmembrane domain, GPI glycosylphosphatidylinositol, GFP green fluorescence protein. b, c Co-immunoprecipitation between each PROM1 mutant and GP130. HEK 293 cells were transfected with various FLAG-tagged PROM1 mutants (1-133, 1-456, 1-812, and PROM1GPI-EX) or full-length PROM1 (1-856) and His-tagged GP130 for 48 hours. Each experiment was repeated independently three times with similar results. d, e HEK 293 cells were transfected with empty vector (EV) or FLAG-tagged PROM1GPI-EX for 48 hours. After purification was performed by double cesium chloride-gradient ultracentrifugation. Viral particles in cesium chloride (density=1.345) were collected and washed with washing buffer (10 mM Tris pH 8.0, 2 mM MgCl₂, and 5% sucrose). Purified adenoviruses (0.5 × 10⁹ pfu) were intravenously injected into the tails of mice.

RNA isolation and quantitative RT–PCR

Total RNA was extracted from liver tissues using an easy-spin™ total RNA extraction kit (Intron Biotechnology, Korea) according to the manufacturer’s protocol. Total RNA (4 µg) was used for cDNA synthesis using random hexamers, oligo dT primers, and reverse transcription master mix (EBT-1511, EBT-1512; ELPIS Biotech, Korea). Quantitative real-time PCR was performed using the cDNAs and each gene-specific oligonucleotide primer in the presence of TOReal qPCR premix (RT500M; Enzymics, Korea). The following real-time PCR conditions were used: an initial denaturation step at 95 °C for 15 min, followed by 50 cycles of denaturation at 95 °C for 10 sec, annealing at 58 °C for 15 sec, and elongation at 72 °C for 20 sec. Each PCR product was evaluated by melting curve analysis for quality control. The qRT-PCR data were collected using LightCycler 480 software 1.5.0 (Roche). Supplementary Table 1 shows the sequences of the gene-specific primers used for qRT-PCR.

Immunoblotting and immunoprecipitation

To extract whole cell lysates, the livers were homogenized with a tissue homogenizer and harvested. The homogenized tissues were lysed with radioimmunoprecipitation assay buffer (50 mM Tris-CI pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and protease- and phosphatase-inhibitor cocktail (P3100, P3200; Gendepot, USA)) on ice for 30 min. Whole-cell lysates were extracted from supernatant by microcentrifugation at 14,000 rpm for 10 min at 4 °C. The whole cell lysates were quantified by BCA assay. The normalized protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred to a nitrocellulose membrane and incubated with the primary antibodies of interest (Supplementary Table 2) followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Supplementary Table 3). The protein band signals were visualized by chemiluminescence detection using an EZ-Western kit (DG-W500; Dogenbio, Korea).

For immunoprecipitation, homogenized tissues or cells were lysed with buffer containing 25 mM HEPES, 150 mM NaCl, 1% NP-40, 10 mM MgCl₂, 1 mM EDTA, 2% glycerol, and protease inhibitor cocktail (P3100; Gendepot) on ice for 30 min. Whole-cell lysates were extracted from the supernatant by microcentrifugation at 14,000 rpm for 10 min at 4 °C. The whole cell lysates were quantified by BCA assay. One milligram of protein in whole cell lysates was incubated with specific primary antibody overnight at 4 °C, followed by incubation with 60 µg of Protein A- or G-agarose bead slurry (1134515001, 11243233001; Roche, Germany) for 4 hours at 4 °C. The bead precipitates were washed with buffer containing 25 mM HEPES, 150 mM NaCl, 1% NP-40, 10 mM MgCl₂, 1 mM EDTA, 2% glycerol, and protease inhibitor cocktail (P3100; Gendepot) 4 times. Protein samples were obtained from the precipitates and analyzed by immunoblotting as described above.
**Immunofluorescence staining**

For immunofluorescence staining of liver tissues, frozen tissues were cut to a thickness of 5 µm using a cryocut microtome (Leica).

PROM1 immunofluorescence in the liver section using a rat monoclonal antibody (13A4) was performed as previously described\(^2^6\). In detail, for PROM1 double immunofluorescence with HNF4α or CK19, the sections were incubated with proteinase K (0.06 U/mg) for 5 min, followed by blocking with 2.5% normal horse serum for 30 min at room temperature. Then, the sections were incubated with mouse anti-HNF4α (ab41898; Abcam, UK) or rabbit anti-CK19 (ab52625; Abcam) overnight at 4 °C. Next, for double immunofluorescence with PROM1, the sections were incubated with 4% paraformaldehyde in 0.1 M
phosphate buffer for 30 min at 37 °C and then incubated with rat anti-PROM1 (Thermo Fisher Scientific, eilosciences, clone 13A4) overnight at 4 °C. Then, the sections were incubated with fluorescence-conjugated secondary antibody (Thermo Fisher Scientific, USA) for 1 h at room temperature. The information of fluorescence-conjugated secondary antibodies was provided in Supplementary Table 3.

For tdTom or Ki-67 double immunofluorescence with HNF4α or CK19, heat-mediated antigen retrieval using a pressure cooker in citrate buffer (pH 6.0) was performed on frozen sections. After antigen retrieval, the sections were blocked with 2.5% normal horse serum for 30 min at room temperature. Then, the sections were incubated with rabbit (600-401-379; Rockland) or rat (TA180009; Thermo Fisher Scientific) primary antibodies in Tris-HCl buffer (pH 8.0, 50 mM EDTA) for 5 min at room temperature. The slides were incubated with 20 μg/ml proteinase K in 4% paraformaldehyde in PBS 5 min at room temperature. The slides were labeled with DNA labeling solution containing biotinylated dUTP and observed with confocal microscopy (Zeiss, LSM700). The images were captured using an LSM800 confocal microscope with ZEN 2009 software (Zeiss, Germany) and analyzed by ZEN 3.5 blue edition (Zeiss).

For immunofluorescence staining of cells, cells were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 15 min at room temperature. After fixation, the cells were permeabilized with 0.1% Triton X-100 for 10 min at room temperature and blocked with 5% bovine serum albumin for 30 min at room temperature. The cells were then incubated with primary antibody at their working concentrations for 1 h at room temperature followed by incubation with fluorescence-conjugated secondary antibody (Thermo Fisher Scientific) for 1 h at room temperature. The information of fluorescence-conjugated secondary antibodies was provided in Supplementary Table 3.

Correlative light and electron microscopy
Correlative light and electron microscopy (CLEM) was performed as previously described. The liver tissues from Phx PromFlox and PromFlox male mice were fixed with 4% paraformaldehyde, cryoprotected with 2.3 M sucrose (0.1 M phosphate buffer) and frozen in liquid nitrogen. The frozen tissues were cut to 1-µm-thick at −100 °C with Leica EM UC7 ultramicrotome. The sections were labeled at 4 °C overnight using an anti-PROM1 rat monoclonal antibody (13A4, 1:200) and visualized using an Alexa Fluor 488-Fluoro Nanogold (Nanoprobes, 1:100). Cover slipped sections were detected with a confocal microscope (Zeiss, LSM700) with a differential interference contrast setting to find specific areas for later examination by electron microscopy. After cover slips had been floated off, silver enhancement was performed using HQ silver enhancement kit (Nanoprobes). After prepared for electron microscopy, areas of interest were excised and cut into 70–90 nm thick. The samples were observed in an electron microscope (JEM 1010; JEOL, Tokyo, Japan).

Giant plasma membrane vesicles isolation
The isolation of giant plasma membrane vesicles (GPMVs) was performed according to previous reports (Levental et al.)

Wheat germ agglutinin (WGA) labelling
For WGA labelling, cells were washed with phosphate-buffered saline (PBS), pH 7.4 and stained with 5 μg/ml wheat germ agglutinin Alexa Fluor 488 conjugate (W11261; Thermo Fisher Scientific) in PBS for 10 min. After labelling, the cells were washed with PBS and fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 15 min at room temperature.

Immunohistochemistry
For hematoxylin-eosin staining of liver tissues, paraffin-embedded tissues were cut to a thickness of 5 µm using a microtome (Leica). The sections were stained with hematoxylin-eosin according to a standard protocol. After mounting with synthetic mountant (Thermo Fisher Scientific), the images were captured using a light microscope (Leica).

TUNEL assay
To analyze apoptosis in liver, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was performed according to the manufacturer’s protocol (ab6110; Abcam). Briefly, frozen liver sections were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The slides were incubated with 20 μg/ml proteinase K in Tris-HCl buffer (pH 8.0, 50 mM EDTA) for 5 min at room temperature and refixed with 4% paraformaldehyde in PBS 5 min at room temperature. The slides were labeled with DNA labeling solution containing TdT enzyme and Br-dUTP for 1 h at 37 °C. The slides were then incubated with anti-BrdU-Red antibody for 30 min at room temperature. After labelling, the slides were counter-stained with DAPI and detected by LSM700 confocal microscope (Zeiss).

Serum IL-6 ELISA
Serum IL-6 levels were quantified using a commercial mouse IL-6 ELISA kit (RAB0308; Sigma) according to the manufacturer’s instructions.
Fig. 8 | PROM1 promotes hepatocyte proliferation through facilitating IL-6-GP130 signaling pathway in lipid rafts during liver regeneration. After 2/3 partial hepatectomy or CCl4 injection, PROM1 expression increases in hepatocytes. Upregulated PROM1 recruits GP130 into lipid rafts by interacting with GP130. PROM1/GP130 complex in lipid rafts facilitates the IL-6-GP130-STAT3 signaling pathway. Therefore, PROM1 promotes hepatocyte proliferation during liver regeneration.
and analyzed using spectra-iMAX with SoftMaz Pro V6 software (Molecular Devices, USA).

**Detergent-resistant lipid raft isolation**
The detergent-resistant lipid raft isolation was performed as previously described. To obtain detergent-resistant lipid rafts, homogenized liver tissues were lysed with buffer containing 1% Brij-35, 25 mM HEPES pH 6.5, 150 mM NaCl, 1 mM EDTA, and protease inhibitor cocktail (P3100; Gendepot) on ice for 30 min. Then, the lysates were subjected to discontinuous sucrose gradient (40, 35, and 5%) ultracentrifugation using SW55Ti rotor or SW41Ti rotor (28,700 x g) for 18 hours at 4 °C. After ultracentrifugation, the sucrose solutions were fractionated into 10-12 fractions. A cloudy band corresponding to the lipid rafts was collected at the interface between the 35 and 5% sucrose solutions and confirmed by immunoblotting for Flotillin-1 as a lipid raft marker.

**Plasmid construction and transient transfection**
Deletion mutants of FLAG-tagged human PROM1 transcript variant 2 (PROM1-FLAG) were generated by reverse PCR as previously described. FLAG-tagged GPI-anchored PROM1-EX1 was generated by the DNA assembly method (#E2621, NEB, Ipswich, MA, USA). The GPI-anchor signal sequence from pCAG-GPI-GFP (#32601, Addgene) was added at the C-terminus of PROM1-EX1 (1-99)-FLAG. His-tagged GP130 CDS obtained from the cDNA library of HEK293 cells.

DNA transfection was performed using Lipofectamine 3000 reagent (Invitrogen, USA) according to the manufacturer’s instructions.

**Statistics and reproducibility**
The number of mice used in each experiment was determined based on preliminary experiments in the same model. Immunofluorescence images and immunoblotting band intensities were quantified using ImageJ 1.52i (NIH) or Photoshop CD5 (Adobe) software. The images used for statistics contained more than ~250 cells per field and were taken from a minimum of 3-5 fields per sample. Data are expressed as the mean ± SEM with individual values using Graphpad Prism 6 software. Sample numbers are indicated in the figure legends. A two-tailed Student’s t test was used to calculate the p values. Significance levels were *p < 0.05; **p < 0.01; ***p < 0.001; and n.s, nonsignificant. A p value <0.05 was considered statistically significant. Each experiment was repeated independently three times with similar results.

**Reporting summary**
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
Source data containing uncropped blots and raw data for all plots are provided with this paper. All other data supporting this study are available within the paper and its supplementary information. Source data are provided with this paper.

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Author contributions
M.-S.B., D.-M.Y., M.L., S.-J.J., J.-W.L., H.-C.K., H.L., H.L.K., A.K., and J.S.K. performed the experiments; J.-H.H., S.-H.K., J.-S.L. and Y.-G.K. designed the experiments and analyzed the data; and M.-S.B., D.-M.Y. and Y.-G.K. wrote the manuscript.

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