Liver cell hydration and integrin signaling

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Summary

Liver cell hydration (cell volume) is dynamic and can change within minutes under the influence of hormones, nutrients, and oxidative stress. Such volume changes were identified as a novel and important modulator of cell function. It provides an early example for the interaction between a physical parameter (cell volume) on the one hand and metabolism, transport, and gene expression on the other. Such events involve mechanotransduction (osmosensing) which triggers signaling cascades towards liver function (osmosignaling). This article reviews our own work on this topic with emphasis on the role of β_1 integrins as (osmo-)mechanosensors in the liver, but also on their role in bile acid signaling.

Introduction and general considerations

Liver cell volume can change within minutes under the influence of nutrients, hormones, and toxins due to the creation or dissipation of osmotic gradients and corresponding water fluxes across the plasma membrane (for reviews, see (Graf and Häussinger, 1996; Häussinger, 1996a, b)). Although hepatocytes, like virtually every cell type, possess powerful volume-regulatory mechanisms, these mechanisms only prevent excessive volume changes but allow variations of cell volume within narrow limits. Most importantly, such small changes in liver cell volume have been identified as an important and until then not recognized regulator of diverse hepatocyte functions, including metabolism, membrane transport, cell fate, and gene expression (Figure 1; for reviews, see (Häussinger and Lang, 1991); (Häussinger, 1996a, b)). Many effects of amino acids and hormones can, at least in part, be attributed to cell hydration changes, which correspond on a short-term time scale to cell volume changes.

Indeed, cell volume changes were identified as important mediators of proteolysis control by amino acids and hormones in the liver (Hallbrucker et al., 1991; Häussinger et al., 1991; vom Dahl et al., 1991), and the cellular hydration state, in general, was suggested to be an important determinant of protein catabolism in health and disease (Häussinger et al., 1993).

The effectors depicted in Figure 1 can change within minutes the hepatocyte water content by up to $\pm$ 15%. Such volume changes can also be experimentally induced by changing the extracellular osmolarity by $\pm$ 80 mosmol/l. Thus, hyper- or hypoosmotic exposure of isolated hepatocytes or perfused rat liver was frequently used to mimic hepatocyte hydration changes and investigate the functional consequences of hepatocyte swelling and shrinkage. Several long-known, but mechanistically unclear effects of amino acids, which could not be related to their metabolism, include the stimulation of glycogen synthesis (Katz et al., 1976; Lavoinne et al., 1987) or inhibition of proteolysis (Mortimore and Pösö, 1987). Such effects can quantitatively be mimicked by hypoosmotic hepatocyte swelling to an extent as these amino acids do (Baquet et al., 1990; Hallbrucker et al., 1991; Häussinger et al., 1991). Against this background, it has been suggested that Na^+-dependent amino acid transport systems in the plasma membrane should not merely be viewed as simple amino acid translocators but also as transmembrane signaling systems, which alter cell hydration and, accordingly, cell function in response to amino acid supply (Häussinger, 1996b). A cell type- and development-specific expression of concentrative vs. simply equilibrating amino acid transporters may add to the complexity of such signaling processes. Roughly spoken, hepatocyte swelling provides a protein anabolic, choleretic, proliferative, and anti-apoptotic signal, whereas hepatocyte shrinkage is catabolic, cholestatic, and proapoptotic.

Much effort has been devoted to the question of how hepatocyte volume changes are sensed („osmosensing“) and how this information signals towards hepatocyte function („osmosignaling“). Several candidates for osmosensing have been discussed in the past, including macromolecular crowding (Minton et al., 1992; Parker and Colclasure, 1992), stretch-activated ion channels (for review see (Naruse, 2018)), and in yeast, histidine kinases were identified that might act as putative osmosensors (Maeda et al., 1994). In the liver, however, we identified $\alpha_5\beta_1$-integrins as volume(osmo)sensors in response to hepatocyte swelling (Häussinger et al., 2003; Schliess et al., 2004; vom Dahl et al., 2003), whereas early endosomes were identified as chloride-governed osmosensors that are activated in response to hyperosmotic hepatocyte shrinkage (Reinehr et al., 2006) (for review see (Reinehr et al., 2013)). This review summarizes some aspects of our work on osmosensing and osmosignaling in the liver. For further aspects and topics on other mammalian cell types, yeast, bacteria, and plants, the reader is referred to (Häussinger and Sies, 2007).

In addition to a volume-regulatory uptake and synthesis of organic osmolytes in response to cell shrinkage and their release in response to cell swelling, an early evolutionary mechanism for the maintenance of cell volume homeostasis is the polymerization of small osmotically active molecules. This polymerization occurs in response to cell swelling in hypoosmotic environments and, conversely, the depolymerization of such macromolecules in response to hyperosmotic cell shrinkage. Such a polymerization/depolymerization strategy is also found in some algae and primitive organisms (Chamberlin and Strange, 1989): here, the cellular metabolism is at the service of cell volume maintenance in order to compensate for osmolarity changes in the surrounding watery environment. It seems that this archaic mechanism is still present in higher organisms, such as mammals, whose cells, however, are mostly in an osmotically stable environment. In line with this, experimental hepatocyte swelling favors the “polymerization” of amino acids and glucose to proteins and glycogen, respectively, whereas “depolymerization” (increased proteolysis and glycogenolysis and inhibition of glycogen and protein synthesis) occurs in response to hyperosmotic hepatocyte shrinkage. A cell volume-stabilizing effect of such maneuvres, however, is doubtful in view of the osmotically stable environment in higher organisms. Instead, it seems that these organisms use the archaic polymerization/depolymerization strategy in an opposite way: changes in cell volume are artificially created by hormones or cumulative substrate uptake in order to regulate metabolism (Häussinger, 1996a).

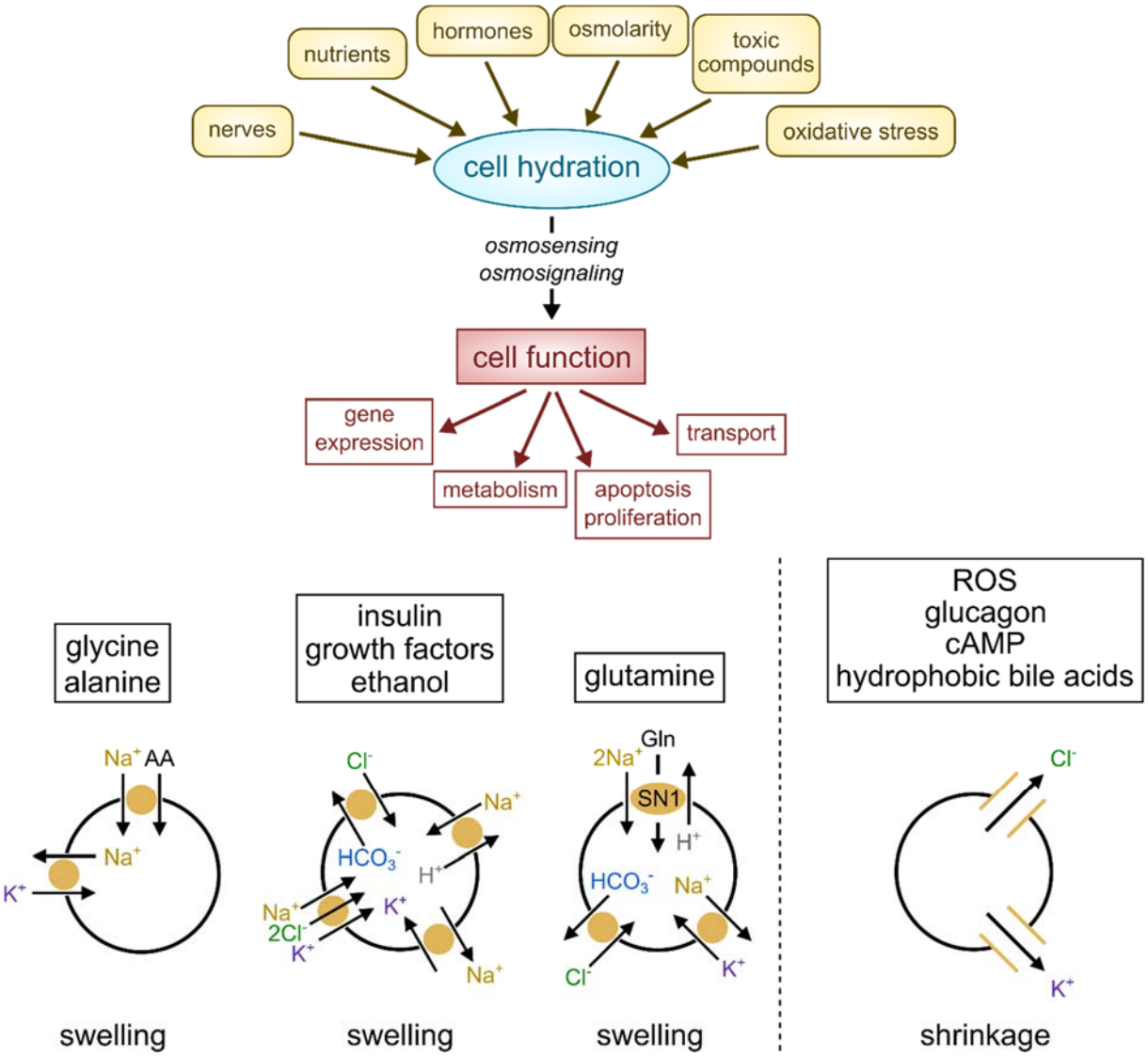

Figure 1: Liver cell volume and hepatocyte function. Liver cell volume, i.e., hepatocyte hydration, is dynamic and under the influence of hormones, nutrients, nerves, ambient osmolarity either due to cumulative substrate transport or generation or dissipation of ion gradients across the plasma membrane. Osmosensing and osmosignaling triggered by such cell volume changes are potent regulators of hepatocyte function. SN1: system N isoform 1 glutamine transporter (Na^+- and H^+-dependent).

Osmosensing and osmosignalling in response to hyperosmotic hepatocyte shrinkage

Hyperosmotic exposure of hepatocytes leads to hepatocyte shrinkage and an accompanying increase of the intracellular chloride concentration due to water efflux and a volume-regulatory uptake of K^+, Na^+, and Cl^- into the hepatocyte (Graf and Häussinger, 1996; Häussinger et al., 1990). Early endosomes were identified as chloride-governed osmosensors, which are activated in response to hyperosmotic hepatocyte shrinkage (Reinehr et al., 2006) (for review, see (Reinehr et al., 2013)). The increase of the intracellular chloride concentration directly activates the vacuolar H^+-ATPase (for review, see (Faundez and Hartzell, 2004)) and triggers a DIDS- and bafilomycin-sensitive endosomal acidification following hyperosmotic exposure, as shown

in studies using endocytosed FITC-dextran (Schreiber et al., 1994). Endosomal acidification activates acidic sphingomyelinase, and the accompanying ceramide formation leads to an activation of NADPH oxidase and the formation of reactive oxygen species (ROS) (Reinehr et al., 2006), for review, see (Reinehr et al., 2013)). The hyperosmotic ROS response was shown to trigger the activation not only of c-Jun N-terminal kinase (JNK) but also of the Src family kinase members Fyn and Yes (Cantore et al., 2011; Reinehr et al., 2004a; Sommerfeld et al., 2015a) and to induce proapoptotic miRNA 15/107 (Santosa et al., 2015). Hyperosmotic Fyn activation triggers cholestasis due to retrieval of Bsep and Mrp2 from the canalicular membrane (Cantore et al., 2011) and Ntcp from the sinusoidal membrane (Sommerfeld et al., 2015a) (Sommerfeld et al., 2015a) of the hepatocyte. The activation of Yes and JNK triggers a proapoptotic state of the hepatocyte (Reinehr et al., 2004a; Reinehr et al., 2002; Reinehr et al., 2003), for review, see (Reinehr et al., 2013)). It should be noted that the hyperosmolarity-induced signaling pathway is also activated by the hydrophobic bile acid glycochenodeoxycholic acid (GCDC) (Becker et al., 2007a; Becker et al., 2007b; Mayer et al., 2019; Reinehr et al., 2004b).

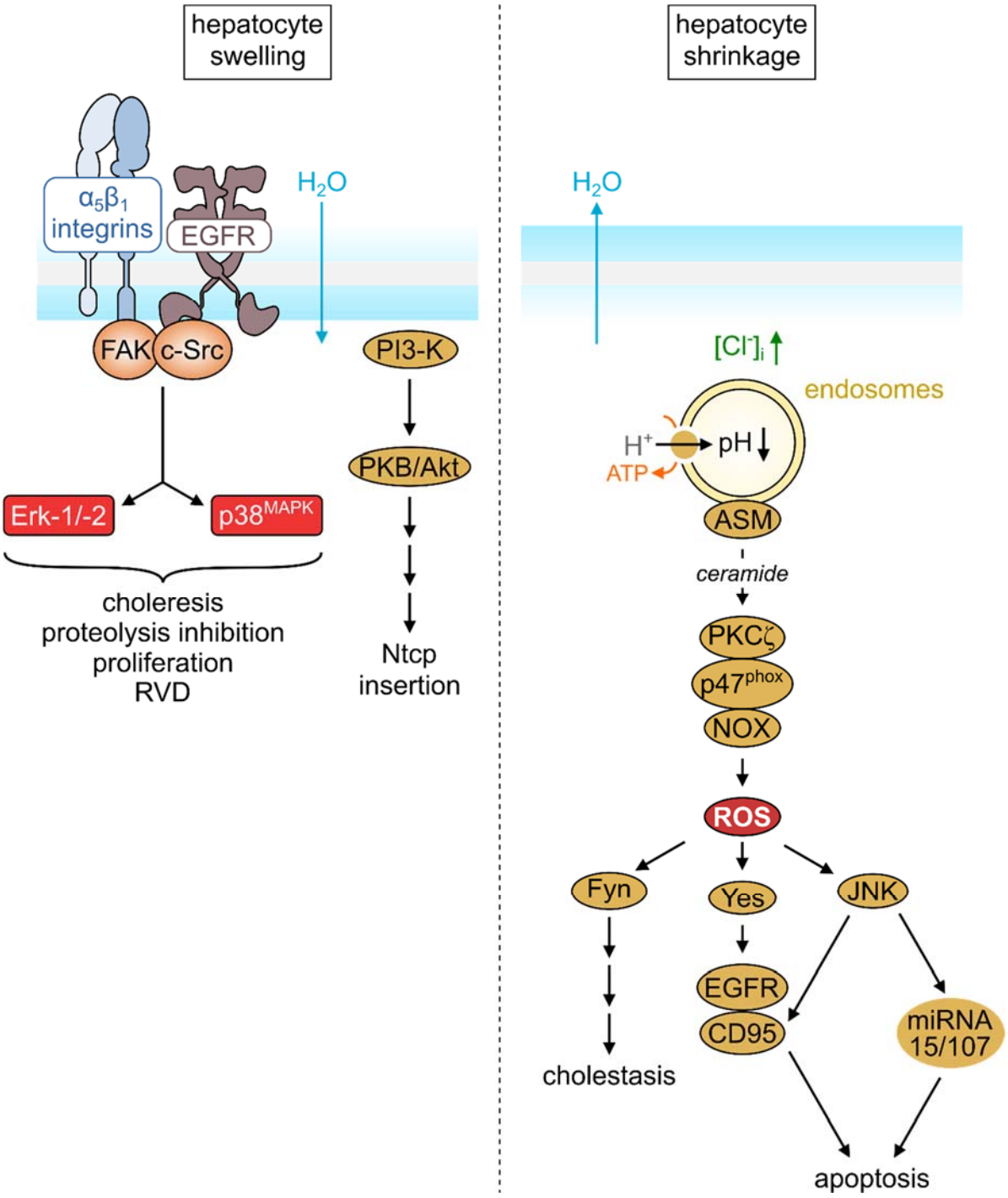

Figure 2: Osmosensing and osmosignaling in response to hypoosmotic hepatocyte swelling and hyperosmotic hepatocyte shrinkage. For details, see text.

Osmosensing and osmosignaling in response to hypoosmotic hepatocyte swelling

Hypoosmotic hepatocyte swelling inhibits autophagic proteolysis and glycogenolysis but stimulates protein and glycogen synthesis and bile acid excretion and triggers a volume-regulatory decrease (RVD) (for review see (Häussinger, 1996b)). Hepatocyte swelling may also

inhibit viral replication, as shown for the duck hepatitis B virus replication (Offensperger et al., 1994), although the underlying mechanisms remained unclear. Concerning the stimulation of glycogen synthesis and acetyl-CoA carboxylase by hepatocyte swelling, a decrease of the intracellular chloride concentration was suggested to be an important trigger (Baquet et al., 1993; Meijer et al., 1992), for review, see (Hue, 1995)). However, concerning hypoosmotic proteolysis inhibition, RVD, and stimulation of bile acid excretion, $\alpha_5\beta_1$-integrins were identified as osmosensors (Häussinger et al., 2003; Schliess et al., 2004; vom Dahl et al., 2003), and the downstream osmosignaling events involve activation of focal adhesion kinase, c-Src, the epidermal growth factor receptor (EGFR), and the mitogen-activated protein kinases Erks and p38 (Figure 2). The latter mediates RVD and proteolysis inhibition, whereas dual MAP kinase activation is required for the insertion of Bsep and Mrp2 into the canalicular membrane and choleresis (Häussinger et al., 2003; Kubitz et al., 1997; Noé et al., 1996; Schmitt et al., 2001).

Swelling-induced activation of $\alpha_5\beta_1$-integrins is also an integral part of insulin action in the liver (Reinehr et al., 2010b; Schliess et al., 2004), for review see (Schliess and Häussinger, 2003)). Activation of the insulin receptor triggers, among many other events, also the activation of PI3 kinase, which activates the $Na^+/K^+/2Cl^-$ cotransporter (NKCC1) leading to hepatocyte swelling (Häussinger and Lang, 1992), subsequent integrin-dependent osmosensing, and signaling towards proteolysis and EGFR activation (Reinehr et al., 2010b; Schliess et al., 2004), as schematically depicted in Figure 3. The involvement of osmosensing and osmosignaling events can well explain the prodiabetic action of loop diuretics, hypernatremia, oxidative stress, dehydration, glucagon, and gain of function mutations of KCNQ1 (Boini et al., 2009; Schliess and Häussinger, 2003; Schliess et al., 2001; Unoki et al., 2008; Yasuda et al., 2008).

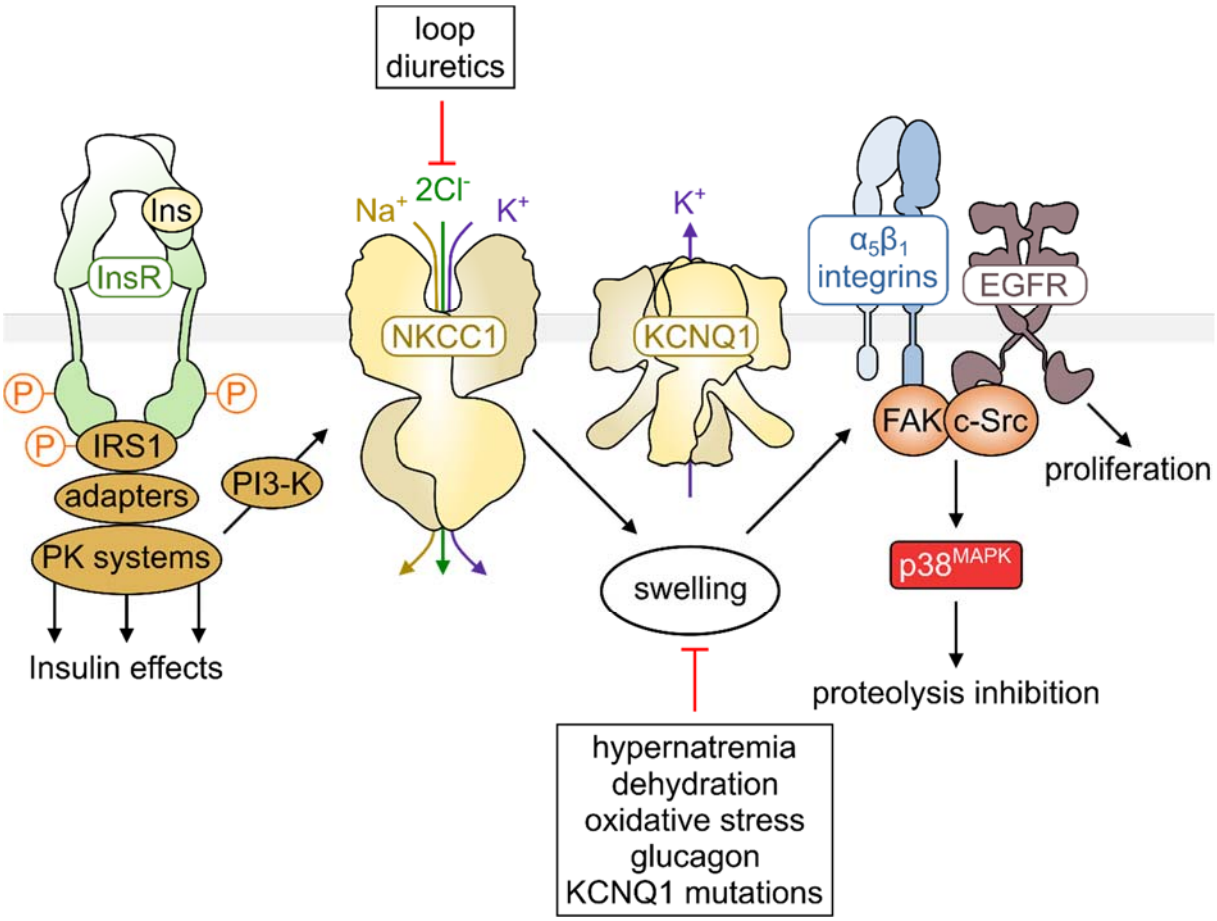

Figure 3: Hepatocyte hydration, insulin action, and resistance. Insulin triggers a PI3 kinase-driven phosphorylation and activation of NKCC1, which triggers cell swelling and activates integrin-dependent osmosensing and -signaling. This results in proteolysis inhibition and EGFR activation. Factors preventing or counteracting insulin-induced hepatocyte swelling include furosemide, oxidative stress, hypernatremia, or KCNQ1 gain of function mutations and are known to be prodiabetic (Boini et al., 2009; Schliess et al., 2001; Unoki et al., 2008; Yasuda et al., 2008).

The $\alpha_5\beta_1$-integrins can also be activated non-osmotically/non-mechanically by urea in high concentrations and tauroursodeoxycholate (TUDC), and signaling events similar to those found after hypoosmotic exposure are initiated (Bonus et al., 2020; Gohlke et al., 2013; Reinehr et al., 2010a), which are mechanistically discussed below. The signaling events triggered by TUDC can explain the choleretic and hepatoprotective action of this bile acid (Häussinger et al., 2003; Kurz et al., 2001), which is also a mainstay in the treatment of cholestatic diseases. Interestingly, TUDC also triggers the formation of cyclic AMP (cAMP) in an integrin-dependent manner (Sommerfeld et al., 2015b). Whether cAMP formation also occurs in response to hypoosmotic hepatocyte swelling remains to be investigated.

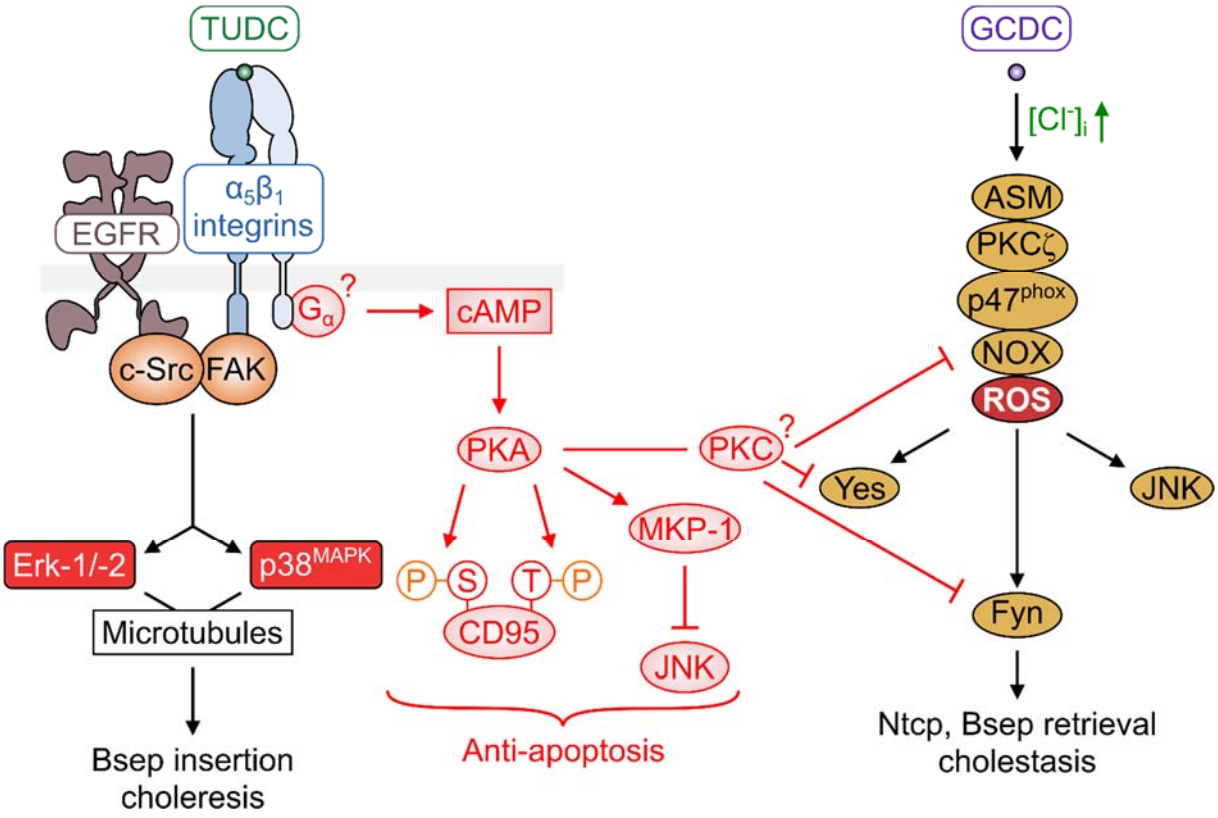

Figure 4: TUDC and GCDC signaling. GCDC triggers signaling events such as hyperosmolarity, which results in cholestasis and a proapoptotic state. This is counteracted by TUDC, which triggers via cAMP an inhibition of GCDC signaling towards cholestasis and apoptosis.

The TUDC-triggered cAMP formation strongly counteracts the proapoptotic action of the hydrophobic bile acid GCDC by preventing Yes, Fyn, and JNK activation (Sommerfeld et al., 2015b) and triggering an inactivating Ser/Thr-phosphorylation of (Reinehr and Häussinger, 2004). In addition, TUDC prevented GCDC-induced changes in gene transcription (Paluschinski et al., 2019).

Early indications of TUDC-mediated integrin activation

Early studies demonstrated that TUDC activates the mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinase (Erk)-1 and Erk-2 in isolated, cultured hepatocytes and perfused rat liver (Schliess et al., 1997). These TUDC-mediated signal transduction events were similar to those triggered by cell swelling (Noé et al., 1996; Schliess et al., 1995) but, conversely, independent from G protein-mediated pathways and amenable to inhibition by high concentrations of glucagon and CPT-cAMP, i.e., conditions that allow for a rapid induction of MAP kinase phosphatase 1 (MKP 1). For reasons not yet fully understood, this is in contrast to the low levels of TUDC-induced cAMP formation that mediate the hepatoprotective effects of TUDC ((Sommerfeld et al., 2015a); see Figure 4). Since Erks can phosphorylate proteins that

control microtubule dynamics and in this way regulate vesicular trafficking of canalicular transporters, they were considered early on as a central node for TUDC- and hypoosmolarity-mediated choleresis. Soon after PI3-K and Ras activation were confirmed as upstream events of the TUDC-mediated, choleretic Erk signal (Kurz et al., 2000), $p38^{MAPK}$ was identified as the second major downstream component to convert a hypoosmolarity- (Häussinger et al., 1999) or TUDC-induced (Kurz et al., 2001) signal into a cellular response. While hypoosmotic cell swelling induced inhibition of autophagic proteolysis via $p38^{MAPK}$ without any involvement of Erks (Häussinger et al., 1999), swelling and liver perfusion with TUDC triggered choleresis only when Erk-1/2 and $p38^{MAPK}$ were both activated. Such dual activation was found essential for vesicular trafficking of the bile salt export pump (BSEP) and multidrug resistance-associated protein 2 (MRP2) (Kubitz et al., 1997; Kurz et al., 2001) to the canalicular membrane.

Considering that the described TUDC-induced signal transduction pathways occurred without any involvement of G proteins (Schliess et al., 1997) and the potency of TUDC toward the farnesoid X receptor (FXR) is only modest (Liu et al., 2003), GPCR and nuclear receptors were ruled out as the primary TUDC sensors (Häussinger and Kordes, 2017). A subsequent study (Häussinger et al., 2003) revealed an altered phosphorylation pattern of other kinases associated with the Erk pathway (c-Src and FAK), which turned the focus to integrin $\alpha_5\beta_1$, the most abundant integrin in hepatocytes (Volpes et al., 1993). The same study (Häussinger et al., 2003) demonstrated abrogation of the complete TUDC-induced signal transduction in the presence of the hexapeptide G*RGD*SP, which inhibits RGD-integrins such as $\alpha_5\beta_1$ (Figure 5A). Swelling-induced signal transduction was likewise abolished through inhibition of integrin activation (vom Dahl et al., 2003), leading to the hypothesis that $\alpha_5\beta_1$ is a sensor for TUDC, besides it mediating changes in the hepatocellular hydration state. Likewise, TUDC-induced cAMP formation was inhibited by the integrin inhibitory hexapeptide G*RGD*SP (Sommerfeld et al., 2015b), and β_1-integrin-dependent cAMP formation has also been described in tumor cell lines (O'Connor and Mercurio, 2001; Whittard and Akiyama, 2001).

Structural aspects of integrin activation

Structures of integrin ectodomains (Xiong et al., 2001; Xiong et al., 2002) and an understanding of how conformational changes are associated with integrin activation opened up the opportunity to complement the *in vitro* and *in vivo* findings with a mechanistic understanding on the structural level. Humans express 18 α and 8 β subunits, which can arrange into 24 distinct integrin heterodimers (Figure 5A). The global architecture of these heterodimers is conserved

across all multicellular organisms (Kadry and Calderwood, 2020). A large extracellular region (ectodomain) that consists of several domains is connected to two generally short cytoplasmic tails via two single-span transmembrane regions (Figure 5B). α and β subunits are unrelated with respect to their sequence, but the subunits themselves are globally conserved across almost all species. An exception to this is a group of vertebrate α subunits with an additional inserted domain. This insertion, the αI or αA domain, forms the binding site in collagen-binding integrins and some laminin-binding integrins. A divalent cation (usually Mg^{2+}) within the "metal ion-dependent adhesion site" (MIDAS) of this binding site mediates binding of extracellular ligands either via an aspartate or glutamate residue. Regardless of whether an αI domain is present or absent, the ectodomains of all integrin α subunits carry a β-propeller domain, two calf domains, and a thigh domain with an IgG-like structure. Ectodomains of β subunits all bear a βI (or βA) domain homologous to the αI domain, a hybrid domain, a plexin-semaphorin-integrin (PSI) domain, four epidermal growth factor (EGF)-like domains, and a β-tail domain (Xiong et al., 2001). In integrins without an αI domain, the βI domain takes over ligand engagement; however, the βI domain carries three divalent cations instead of one. While the βI MIDAS carries a Mg^{2+} ion, as it does in αI domains, the structural information available to this date suggests that the site "adjacent to MIDAS" (ADMIDAS) and the synergistic metal ion-binding site (SyMBS, formerly referred to as LIMBS for "ligand-associated metal-binding site") both carry a Ca^{2+} ion.

According to the currently accepted model, derived from the first crystal structures of a complete integrin ectodomain (Xiong et al., 2001; Xiong et al., 2002) and electron microscopy studies (Nishida et al., 2006; Takagi et al., 2002), integrins adopt at least three stable conformational states during their activation cycle (Figure 5C). The "bent-closed" (Figure 5C, left) conformation with low affinity for extracellular matrix (ECM) ligands represents a resting state of integrins. In contrast, the "extended-closed" conformation (Figure 5C, center) with medium affinity and the "extended-open" conformation (Figure 5C, right) with high affinity are binding-competent (Arnaout et al., 2005; Kadry and Calderwood, 2020). Integrin-dependent ligand binding was thought to always follow the same basic multi-step procedure (Bachmann et al., 2019; Kadry and Calderwood, 2020; Kechagia et al., 2019; Sun et al., 2019). First, intracellular adaptor proteins such as talin and kindlin are recruited. The binding of these adaptor proteins to the integrin cytoplasmic tails induces the long-range conformational changes described in Figure 5C, which increase the affinity for ECM ligands. Now the integrins engage their respective ECM components with high affinity and simultaneously bind to the actin cytoskeleton. This bilateral connection results in a clustering of integrins and the

formation of anchoring cell-matrix connections known as focal adhesions. Numerous intracellular binding partners can subsequently be recruited, which may initiate various downstream signaling pathways (Kim et al., 2011; Morse et al., 2014).

Note that this model neither includes nor excludes the possibility of ligand binding to the low-affinity “bent-closed” conformation (Figure 5C, left). Omitting this scenario is reasonable in the context of binding to voluminous ECM ligands, which are unlikely to reach the binding pocket in the “bent-closed” state for steric reasons. However, the model may imply that the modulation of integrin activity and signaling by extracellular binding partners of lower molecular weight is not possible either. Moreover, the model limits the concept of bidirectionality and reciprocity (Hynes, 2002) of integrin signaling to the focal adhesion phase. By contrast, a large body of experimental evidence suggests that the “bent-closed” conformation may very well bind small- and macromolecular ligands (Adair et al., 2005; Zhu et al., 2013). Furthermore, ligand binding to an isolated headpiece induces a conformational change that exactly matches the conformational change following adaptor protein-induced integrin activation (Springer et al., 2008; Zhu et al., 2013). Therefore, ligand binding to the “bent-closed” ectodomain likely influences the integrin conformational equilibrium similar to the binding of intracellular adaptor proteins to the cytoplasmic tail.

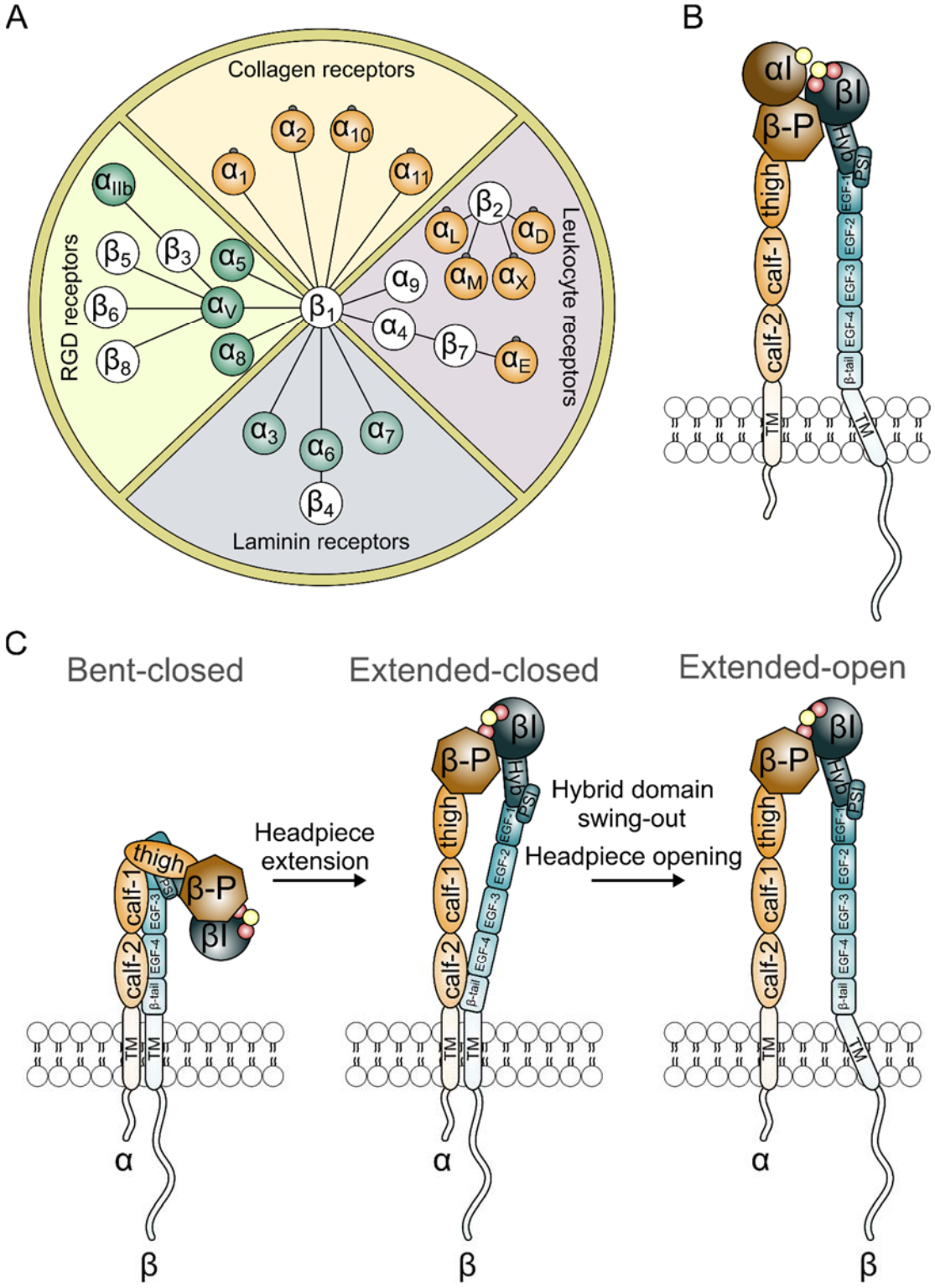

Figure 5: Integrin subunit assemblies and structural aspects of integrin activation. (**A**) Representation of known integrin heterodimer pairs in vertebrates and their grouping by ECM ligand. Integrin α subunits are colored according to their evolutionary relationship; α subunits with an αI domain carry a gray attachment (Figure modified from (Hynes, 2002) and (Barczyk et al., 2010)). (**B**) Domain architecture of an integrin with an αI domain. The MIDAS is depicted as a yellow sphere, the ADMIDAS (distal) and SyMBS (proximal) are depicted as red spheres. Membrane lipids indicate the membrane region in the background of the transmembrane regions. β-P: β-propeller domain; Hyb: hybrid domain; TM: transmembrane domain. (**C**) Integrins undergo a large conformational transition from a "bent-closed" (left) to an "extended-closed" conformation (center) by extending their ectodomain headpiece via the "joints" between thigh and calf-1 domains and EGF-1 and EGF-2 domains. The transition between "extended-closed" and "extended-open" conformation (right) is achieved by a swing-out of the hybrid domain and dissociation of the transmembrane helices. The latter has a contributing role in the subunit-specific sensitivity of integrin activation (Pagani and Gohlke, 2018). The conformational equilibrium is depicted for an integrin lacking an αI domain (Figure modified from (Kadry and Calderwood, 2020)).

Activation of integrin $\alpha_5\beta_1$ by urea

Early studies revealed that high urea concentrations induce a paradoxical regulatory volume decrease with an opening of K^+ channels followed by hepatocyte shrinkage, although the hepatocyte membrane is expected to be freely permeable to urea (Hallbrucker et al., 1994). Cryosections of urea-perfused rat liver immunostained for the active β_1 integrin subunit showed that high urea concentrations (100 μM) cause activation of the hepatocyte β_1 integrin system and trigger the same signal transduction mechanisms as hypoosmolarity and TUDC (Reinehr et al., 2010a). Molecular dynamics (MD) simulations of a model of $\alpha_5\beta_1$ integrin in aqueous urea solution revealed that urea disrupts stabilizing hydrogen bonds between the leg regions of both integrin subunits and between the hybrid domain and the leg region of the α-subunit. Breaking these interactions led to a pronounced integrin unbending, which is part of the conformational transition towards the "extended-closed" state, corresponding to the first step in the activation cycle of integrins (Figure 5C). Hence, urea-mediated $\alpha_5\beta_1$ integrin activation in hepatocytes is suggested to occur through direct urea-integrin interactions, although not at the extracellular ligand binding site of $\alpha_5\beta_1$.

Integrin $\alpha_5\beta_1$ acts as a sensor for TUDC in hepatocytes

Abrogation of the complete TUDC-induced signal transduction in the presence of the hexapeptide G*RGD*SP (Häussinger et al., 2003), the lack of TUDC-induced kinase activation after β_1 integrin knockdown in isolated rat hepatocytes (Gohlke et al., 2013), and the pharmacophoric similarity of TUDC with tirofiban, a small molecule inhibitor binding to the extracellular binding site of integrin $\alpha_{IIb}\beta_3$, led to the suggestion that TUDC can activate integrin $\alpha_5\beta_1$ by specifically binding to its extracellular site. Immunofluorescence staining experiments revealed that only TUDC, but neither taurocholic acid (TC), glycochenodeoxycholic acid (GCDC), taurochenodeoxycholic acid (TCDC), or taurolithocholic acid 3-sulfate (TLCS), activated β_1 integrins (Gohlke et al., 2013). These bile acids primarily differ from TUDC with respect to substitutions at or configurations of substituents of the cholane scaffold. Furthermore, integrin β_1 was activated predominantly inside hepatocytes and required TUDC uptake through the Na^+/taurocholate cotransporting polypeptide (NTCP) (Gohlke et al., 2013). This mode of activation was in stark contrast to integrin activation by cell swelling, predominantly involving membrane-bound integrins (Gohlke et al., 2013; Reinehr et al., 2010a). Intriguingly, TUDC-mediated integrin activation was also suppressed in the presence of higher TC concentrations, suggesting that both bile acids bind to the RGD binding site but with opposite effects (Gohlke et al., 2013). In line with this data, MD simulations of $\alpha_5\beta_1$ integrin in complex with TUDC, TC, or G*RGD*SP, docked to the RGD binding site, respectively, demonstrated that only TUDC

induces conformational changes between the propeller and the βA domains (Figure 6A) and in helices α1 and α7 (Figure 6B) that are consistent with integrin activation (Bonus et al., 2020; Gohlke et al., 2013). Hence, we concluded that TUDC can directly activate intrahepatocytic β_1 integrins, which trigger signal transduction pathways toward choleresis.

Functional selectivity of TUDC and *nor*UDCA for integrin-mediated signaling pathways

Chemical modifications to the ursodeoxycholane scaffold of TUDC led either to a complete loss of activity or, in the case of taurocholic acid (TC), to an antagonist of TUDC-induced $\alpha_5\beta_1$ integrin activation (Gohlke et al., 2013). Whether chemical modifications of the bile acid's side chain –preserving the ursodeoxycholane scaffold – retain or even increase activity towards $\alpha_5\beta_1$ integrin was investigated in a subsequent study (Bonus et al., 2020). Here, MD simulations were conducted first to compare the ability of *nor*UDCA, T*nor*UDCA, GUDC, and UDCA to evoke activation-related conformational changes in $\alpha_5\beta_1$ integrin (Figure 6A, B) with that of TUDC and TC. The extent of these conformational changes, described using three geometric descriptors, was subsequently correlated with an activity ranking of these bile acids (Figure 6C) derived from the amount of immunostained, active β_1 integrin determined at fixed time points (Figure 6D). Based on this data, TUDC and *nor*UDCA were classified as highly active, T*nor*UDCA and GUDC as weakly active, and UDCA and TC as inactive or inhibitory ligands of $\alpha_5\beta_1$ integrin, respectively. A competitive ELISA-based solid-phase assay (Bochen et al., 2013) confirmed direct binding of TUDC and *nor*UDCA to the MIDAS in $\alpha_5\beta_1$ integrins and revealed similar binding affinities of both compounds (Bonus et al., 2020). In view of this, the different extent of $\alpha_5\beta_1$ integrin activation by TUDC and *nor*UDCA could not have been a consequence of different binding site occupancy.

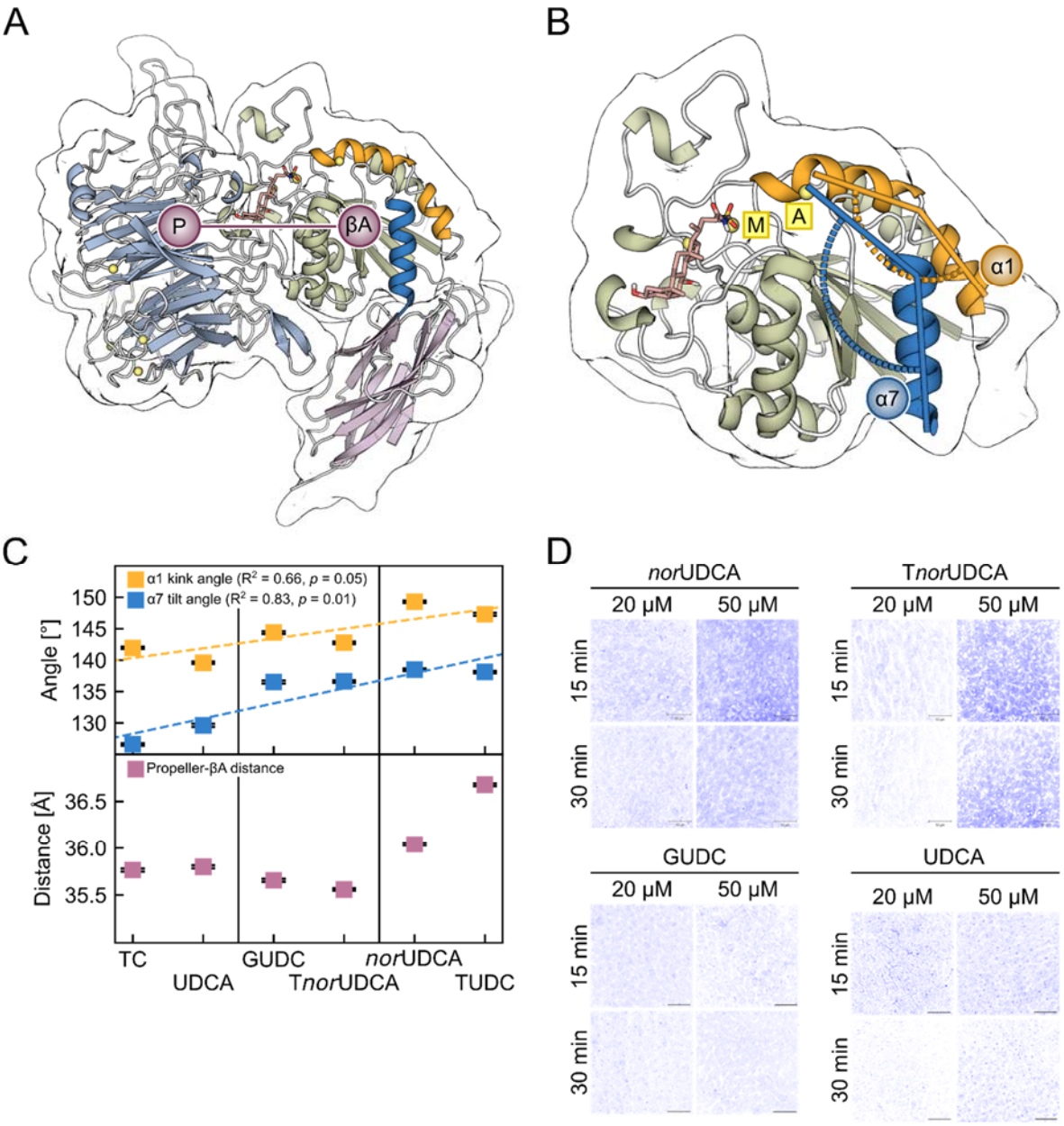

Figure 6: Geometric descriptors and activation of $\alpha_5\beta_1$ integrin in MD simulations and immunofluorescence staining experiments. (**A**) Part of the $\alpha_5\beta_1$ integrin headpiece in cartoon representation. Helices α1 and α7 are highlighted in orange and blue, respectively. The propeller-βA distance was measured between the respective centers of mass (violet circles). The propeller domain is colored in light blue, the βA domain in pale green, and the hybrid domain in violet. (**B**) Close-up view of the βA domain with the docked TUDC structure (stick representation) (Gohlke et al., 2013). This complex structure was used to generate the other starting structures by modifying the bile acid side chain. Angles measured during the MD simulations: orange: α1 kink angle; blue: α7 tilt angle. Mg^{2+} ions are depicted as yellow spheres; the metal ion at the MIDAS is labeled *M*, the metal ion at the ADMIDAS is labeled *A*. (**C**) Average ± SEM values of the α1 kink angle (orange), α7 tilt angle (blue), and propeller-βA distance (violet) over three replicate MD simulations versus the rank of the bile acids according to their agonistic activity towards $\alpha_5\beta_1$ integrin as observed in immunofluorescence staining experiments (panel D). Dashed lines depict linear regressions; fit parameters are given in the figures. Vertical lines separate the dataset into inactive (left), weakly active (middle), and highly active (right) bile acids. (**D**) Effect of *nor*UDCA, T*nor*UDCA, GUDC, and UDCA on β_1 integrin activation. Rat livers were perfused with the respective bile acids for up to 60 min with the concentrations indicated. Liver samples were immunostained for the active conformation of β_1 integrin (blue, false-color images; for original images cf. Figure 3 in (Bonus et al., 2020)). (Figure and caption were modified from (Bonus et al., 2020).)

As described before (Kurz et al., 2001; Schliess et al., 1997), TUDC (20 µM)-induced $\alpha_5\beta_1$ integrin activation led to *sustained* activation of Erk-1/-2 and $p38^{MAPK}$ (Figure 7A-C), the

critical downstream signal towards choleresis. Perfusion with lower (10 μM) and higher (50 μM) TUDC concentrations did not change this kinetic profile, ruling out a concentration effect (Bonus et al., 2020). In contrast, *nor*UDCA-induced $\alpha_5\beta_1$ integrin activation only led to *transient* activation of these MAPKs (Figure 7C), which may have been a consequence of weaker $\alpha_5\beta_1$ integrin activation. Since TUDC, but not *nor*UDCA, significantly enhanced the EGFR/c-Src association, a c-Src-dependent trans-activation of EGFR is likely a determining factor for prolonged MAPK activation (Bonus et al., 2020). Inhibiting PI3-K abolished *nor*UDCA-induced phosphorylation of only Erk-1/-2, but inhibiting c-Src abolished phosphorylation of both Erk-1/-2 and $p38^{MAPK}$, indicating that c-Src activation occurs upstream of PI3-K activation. In an earlier study (Häussinger et al., 2003), which investigated TUDC-induced signaling towards choleresis, inhibition of c-Src did not prevent Erk-1/-2 phosphorylation but only delayed it by ~8 min. Hence, inhibition of c-Src activity only prevents Erk-1/-2 phosphorylation after *nor*UDCA-induced but not after TUDC-induced $\alpha_5\beta_1$ integrin activation.

Together with further experiments on FAK phosphorylation patterns (Bonus et al., 2020), these observations suggested a ligand-dependent selectivity for signaling pathways induced by $\alpha_5\beta_1$-integrin (Figure 7D, E), a property widely known for G-protein coupled receptors (Violin and Lefkowitz, 2007), but that had not yet been described for integrins without an αI domain (Simon, 2011). The insights gained from this study also provide a rationale for the different therapeutic applications of UDCA and *nor*UDCA in primary biliary cholangitis and primary sclerosing cholangitis, respectively (Trauner et al., 2015).

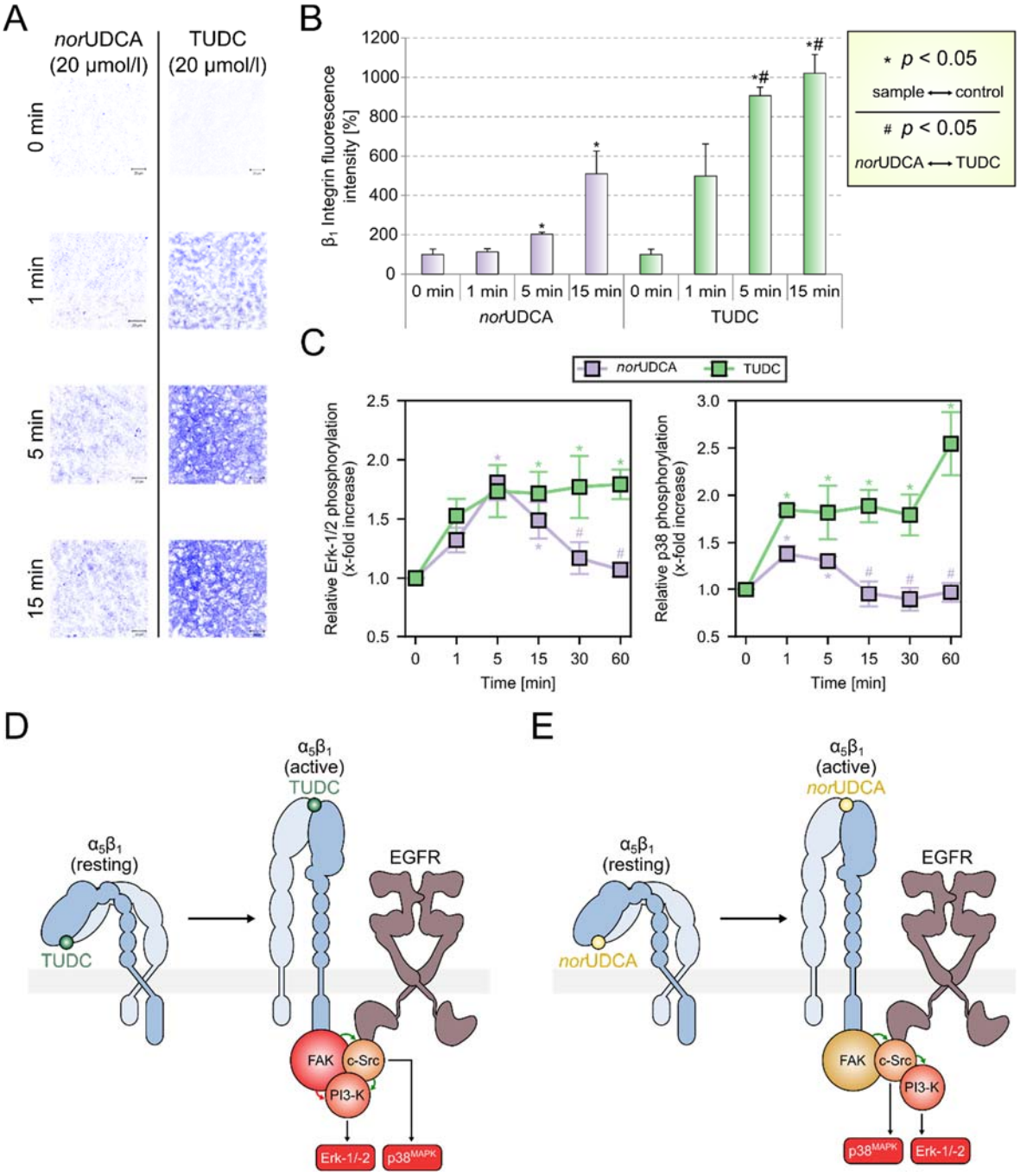

Figure 7: Comparison between TUDC- and *nor*UDCA-induced $\alpha_5\beta_1$ integrin activation and MAPK signaling. (**A**) Immunofluorescence staining of active β_1 integrin after liver perfusion with either 20 µM *nor*UDCA (left) or TUDC (right) at timepoints t = 0, 1, 5, and 15 min. The scale bar corresponds to 20 µm. Representative images of three independent experiments are depicted. (**B**) Quantification of β_1 integrin fluorescence. (**C**) Densitometric quantification of Erk-1/2 (left) and $p38^{MAPK}$ (right) phosphorylation after liver perfusion with either 20 µM *nor*UDCA (violet) or TUDC (green). Total Erk-1/2 and total $p38^{MAPK}$ served as respective loading controls. Phosphorylation at t = 0 min was set to 1. Data represents the mean ± SEM of at least three independent experiments. (**D**) Activation of $\alpha_5\beta_1$ integrin by TUDC leads to high $FAK^{Y397\text{-}P}$ levels, which trigger both a slow, c-Src and EGFR-independent PI3-K activation (red arrow) and a fast, c-Src and EGFR-dependent PI3-K activation (green arrows). The subsequent PI3-K-mediated Erk-1/2 activation is sustained. (**E**) Activation of $\alpha_5\beta_1$ integrin by *nor*UDCA leads to lower $FAK^{Y397\text{-}P}$ levels, which trigger the fast, c-Src and EGFR-dependent pathway of PI3-K activation (green arrows), but abolish the slower FAK-mediated PI3-K activation. The subsequent PI3-K mediated Erk-1/2 activation is only transient. (Figure and caption were modified from ref. (Bonus et al., 2020).)

Concluding remarks

Hormones, nutrients, and oxidative stress can lead to changes of liver cell hydration (cell volume) within minutes. The changes act as important modulators of cell function and couple the physical parameter cell volume to metabolism, transport, and gene expression. For this to occur, mechanotransduction (osmosensing) is required, which triggers signaling cascades towards liver function (osmosignaling). β_1 integrins have a central role as (osmo-)mechanosensors in the liver, but also are involved in bile acid signaling.

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