Gene regulatory networks in differentiation and direct reprogramming of hepatic cells

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Footnote

Abbreviations: BMP, Bone Morphogenic Protein; Cdx2, caudal type homeobox 2; C/EBP, CCAAT/Enhancer Binding Protein; ES, embryonic stem; FGF, Fibroblast Growth Factor; FoxA ,Forkhead box factor A; GRN, gene regulatory network; Grg3, groucho-related gene 3; HNF, Hepatocyte Nuclear Factor; OC2, Onecut2; Prox1, Prospero homeobox 1; Sox, SRY-related high mobility group box transcription factor; TβRII, TGFβ type II receptor; Tbx3,T-box 3; TGF, Transforming Growth Factor; TF, transcription factor; YAP; Wnt, wingless-type MMTV integration site; Yes-Associated Protein.

Abstract

Liver development proceeds by sequential steps during which gene regulatory networks (GRNs) determine differentiation and maturation of hepatic cells. Characterizing the architecture and dynamics of these networks is essential for understanding how cell fate decisions are made during development, and for recapitulating these processes during *in vitro* production of liver cells for toxicology studies, disease modelling and regenerative therapy. Here we review the GRNs that control key steps of liver development and lead to differentiation of hepatocytes and cholangiocytes in mammals. We focus on GRNs determining cell fate decisions and analyse subcircuitry motifs that may confer specific dynamic properties to the networks. Finally, we put our analysis in the perspective of recent attempts to directly reprogram cells to hepatocytes by forced expression of transcription factors.

Keywords

Liver development, hepatic cell differentiation, hepatoblast, hepatocyte, cholangiocyte, network dynamics

Contents

1. Liver development: selection of gene regulatory network representation

The sequential steps of liver development are coordinated by intercellular signaling effectors that modulate the activity of intracellular transcription factor (TF) networks [1]. The combination of cell-extrinsic and cell-intrinsic cues constitutes gene regulatory networks (GRN) in which TFs determine spatial and temporal expression of genes and eventually drive hepatic cell differentiation and liver morphogenesis. GRNs acting in liver development can be reconstructed through the analysis of genome-wide studies and by assembling subnetworks identified in experiments addressing the function of small sets of genes. Therefore the multiple sources of data and the inherent complexity of the GRNs led to various modes of network representations. Particularly convenient for concise representations of GRNs in liver development are activity flow diagrams representing epistatic relationships. While not providing detailed mechanistic insight, such maps are easily transposable when designing cell culture protocols for production of hepatic cells for regenerative therapy. They also convey essential information and provide a strong framework for qualitative and quantitative dynamic modelling.

Here we review the key cell fate decisions made during liver development. At each step, we attempt to define GRNs that are represented as directed, sequential but non-mechanistic activity flow diagrams. To avoid designing GRNs that inappropriately integrate components from distinct species, our analysis focuses on mammalian systems. We then discuss the subcircuitry motifs and the implementation of GRNs for TF-mediated reprogramming of cells to hepatocytes.

2. Gene regulatory network operating during liver specification

The liver precursor cells are located in a midline domain and in two lateral and more posterior domains of the ventral foregut endoderm [2, 3]. Liver specification, *i.e.* the initiation of hepatic gene expression, occurs when these domains merge at the ventral midline. The pioneer TFs Forkhead box (Fox)A1/A2 and GATA4/6 initially open the chromatin of liver genes which become primed ("competent") for subsequent occupancy by additional transcriptional regulators, eventually leading to transcriptional activation [4]. FoxA1 and FoxA2 function redundantly and are required for initiation of liver development [5]. Similar functional redundancy was suggested for GATA4 and GATA6 [6], but recent studies identified unique functions for each factor [7].

These observations raised questions on the mechanisms that trigger transition from competency to specification. Fibroblast Growth Factors (FGFs), Bone Morphogenic Proteins (BMPs) and Wingless-type MMTV integration site (Wnt) proteins are secreted by mesodermal tissue adjacent to prehepatic endoderm and promote early hepatogenesis [8-10]. The FGF, BMP and Wnt signaling pathways are conserved across species during liver specification [11, 12].

In mammals, the involvement of Wnt is not demonstrated *in vivo* but is suggested from the presence of non-canonical Wnt signaling components in liver progenitors, and from the need to repress canonical Wnt signaling when specifying stem cell-derived endoderm to a hepatic fate [13-16]. Which FGF ligand is responsible for hepatogenesis in mice remains unclear, but the ERK1/2 pathway was shown to be necessary for hepatic gene induction downstream of FGFR1/2/4 [17]. FGFs also cooperate with BMP4; downstream of FGFR1/2/4 they activate the RAS-RAF-ERK and PI3K-Akt pathways, and the Wnt signaling inhibitor NKD1, as well as several TFs [15, 17]. BMP activity is mediated by SMAD1/5/8 which forms a complex with SMAD4.

The latter recruits the histone acetyltransferase P300 to hepatic genes and stimulates GATA4 expression, indicating that BMP has direct effects on liver genes via SMAD4 and indirect effects via enhanced expression of GATA4 [10, 18]. Therefore, hepatic specification is controlled by feedforward loops: a first loop is formed within the BMP cascade by the direct and indirect effects of SMAD4, and a second loop is constituted within the FGF pathway by the ERK/Hepatocyte Nuclear Factor (HNF) 4 and NKD1/β-catenin cascades.

Transcriptional components of the GRN driving hepatic specification further include Klf6, which enhances the expression of GATA4 and FoxA2 in cultured embryonic stem (ES) cells [19]. FoxA2 is inhibited by groucho-related gene 3 (Grg3), a co-repressor that silences FoxA-bound hepatic genes in undifferentiated endoderm, and which becomes extinguished during specification to enable FoxA factors to stimulate transcription [20]. At the specification stage HNF4 is required for expression of several other liver-specific TFs [21]. Hhex, whose expression is only marginally controlled by HNF4, indirectly regulates the response to extracellular signals by determining the position of the endoderm with respect to adjacent sources of FGF and BMP [22]. Finally, HNF1β has little or no effect on endodermal competence as evidenced by near normal expression of *FoxA2* at the 6-8 somite stage in HNF1β-deficient endoderm. However it is critically required for FGF-induced specification of the endoderm in mammals [23].

Fig. 1A proposes an epistasis-based GRN for hepatic specification. This GRN does not take quantitative, spatial and dynamic aspects of signaling into consideration [24-26], despite that induction of liver genes requires well-defined levels of FGFs, and that FGFs are not required for all liver precursor domains: specification of the ventral midline precursor domain is indeed dependent on FGF signaling, whereas the lateral

precursor domains develop normally in the presence of FGFR inhibitors [27]. In addition, the midline and lateral precursor domains are subjected to distinct temporal responses to BMPs and FGFs, with induction of BMP signaling preceding FGF signaling in the midline precursors and *vice versa* in the lateral precursors [26]. Together these data indicate that distinct thresholds of FGF signaling determine organ specification along the antero-posterior axis of the endoderm, and also that the requirements and dynamics of FGF- and BMP-mediated induction of hepatic gene expression differ among subsets of liver precursor cells.

3. Liver bud outgrowth and hepatoblast migration

The specified ventral endoderm forms a multilayered pseudostratified epithelium composed of hepatoblasts, which then proliferate, delaminate from the endoderm, and invade the septum transversum. Endothelial cells are dispensable for endoderm specification *in vivo*, but *in vitro* they promote hepatic specification of cultured ES cell-derived endoderm by inhibiting Wnt and Notch signaling [14]. Beyond the stage of specification, endothelial cells are essential for liver bud outgrowth [28].

Liver budding follows shortly after specification, and regulators of specification, like FGFs and BMPs, continue to play a role at the budding stage. Specific functions at the budding stage were identified for a number of TFs: pseudostratification of endoderm cells requires Hhex, and migration of the hepatoblasts into the septum transversum is coordinately controlled by T-box 3 (Tbx3), Prospero homeobox 1 (Prox1), HNF6 and Onecut2 (OC2) [29-32]. The analysis of mice knockout for these TFs revealed that Prox1, HNF6 and OC2 repress E-cadherin and so allow the hepatoblasts to dissociate from each other during migration in the surrounding mesenchyme. Epistatic relations between TFs are shown in Fig. 1B.

4. Proliferation *versus* growth arrest: role in hepatobiliary lineage segregation

Hepatoblasts proliferate and are protected against apoptosis to enable liver growth. Secreted factors controlling proliferation and apoptosis have been reviewed elsewhere [1, 33]. Here we focus on mechanisms that control the balance between maintenance of an immature hepatoblast phenotype and differentiation towards the hepatocyte or cholangiocyte lineages in relation with the cells' proliferative state (Fig. 2A).

Hepatoblasts express hepatocyte-specific genes and proliferate while differentiating to hepatocytes. Instead, cells differentiating to cholangiocytes repress hepatocyte genes and undergo growth arrest: cholangiocytes organised as a ductal plate around the branches of the portal vein do not proliferate until they have formed bile ducts [34]. In this context Tbx3-induced proliferation functions by preventing growth arrest via repression of $p19^{ARF}$ (Cdkn2a, $p14^{ARF}$ in humans), an effect which is likely mediated by Prox1: Prox1 indeed prevents growth arrest by repressing $p16^{INK4a}$ which belongs to the same locus as $p14^{ARF}$/$p19^{ARF}$, and it stimulates expression of cyclins D2, E1 and E2 [32, 35, 36]. Prox1 represses LRH1 activity by means of a protein-protein interaction, and so alleviates the inhibition exerted by LRH1 on Prox1's pro-proliferative effects, thereby constituting a feedforward loop [36].

Growth arrest resulting from the lack of Prox1 or Tbx3 in knockout mice is associated with increased commitment of hepatoblasts to the biliary lineage [32, 35, 37]. Growth arrest can function as a trigger for cholangiocyte differentiation, since overexpression of $p19^{ARF}$ in hepatoblasts suppresses growth but also induces biliary gene expression [35]. Still, growth arrest is not sufficient to induce biliary gene expression in all circumstances: Lin28b stimulates hepatoblast proliferation and maintains the

cells in an immature state (Fig. 2A), but inhibition of Lin28b does not lead to cholangiocyte gene expression despite that the cells are growth arrested [38]. Similarly, the absence of the polycomb protein Ezh2 is associated with an increase of p14ARF/cdkn2A/p19ARF, but without induction of cholangiocyte genes [39]. Therefore, the balance between proliferation and growth arrest is a determinant of cholangiocyte differentiation in developing liver, but context-dependency must be considered to fully appreciate the role of cell cycling in hepatoblast fate allocation.

5. TGFβ-dependent network driving cholangiocyte differentiation

Since hepatoblasts express a number of hepatocyte-specific functions and TFs, biliary differentiation must combine activation of cholangiocyte genes and repression of hepatocyte functions. Biliary differentiation is spatially restricted and occurs in the vicinity of the branches of the portal vein where hepatoblasts form a ductal plate.

Several signaling pathways cooperate to induce cholangiocyte differentiation (Fig. 2B). Hepatoblasts are exposed to a gradient of Transforming Growth Factor (TGF) β signaling which peaks around the portal vein mesenchyme - the predominant source of TGFβ - and fades off at a distance of the vein [40, 41]. At the onset of cholangiocyte differentiation only one layer of cholangiocytes surrounds the portal vein. This indicates that the hepatoblasts' response to TGFβ is tigthly controlled by yet unkown mechanisms which ensure that only the cells adjacent to the portal mesenchyme and exposed to the highest TGFβ concentration differentiate to cholangiocytes. Perturbation of the TGFβ signaling gradient, as occurs in mouse livers deficient in HNF6/OC2 or Prox1, is associated with induction of cholangiocyte genes at a distance from the portal vein [37, 40].

The concentration of TGFβ type II receptor (TβRII) at the surface of hepatoblasts is critical to determine the fate of hepatoblasts since overexpression of TβRII stimulates biliary gene expression while repressing hepatocyte genes [42]. As a consequence, TβRII concentration is tightly controlled. In line with its hepatocyte-promoting function CCAAT/Enhancer Binding Protein (C/EBP) α, represses TβRII [42, 43], but its paralog C/EBPβ stimulates TβRII expression and promotes cholangiocyte gene expression. This suggests that hepatoblast fate decision is determined by the C/EBPα:C/EBPβ ratio, and is consistent with the observation that depletion of C/EBPα in mouse livers is associated with increased development of biliary-like structures [44]. How the C/EBPα:C/EBPβ ratio is determined was recently found to depend on the expression levels of microRNA miR-92b which directly represses C/EBPβ in fetal liver cells. Overexpression of miR-92b further induces proliferation and promotes maintenance of an immature state characterized by low levels of hepatocyte functions [45]. Interestingly, there is also evidence that TGFβ signaling represses C/EBPα thereby creating a positive feedback loop that supports TβRII expression and signaling [46] (Fig. 2B).

HNF6 and OC2 are expressed in differentiating hepatocytes and cholangiocytes. Since the levels of HNF6 and OC2 are higher in the developing cholangiocytes, we speculate that their expression is stimulated by cholangiocyte-inducing signals. TGFβ is an obvious candidate: the absence of C/EBPα induces TβRII and is associated with increased expression of HNF6 [44], suggesting the existence of a C/EBPα -| TβRII → HNF6 cascade. Considering that HNF6 is in turn a repressor of TβRII (indirectly, and possibly via HNF1β [42, 47, 48]), expression of this receptor would be kept within tight limits by a negative feedback loop involving HNF6 and by a double

negative - and therefore positive - feedback loop involving C/EBPα, which together would correctly shape the TGFβ signaling response gradient.

Hippo signaling is yet another regulator of TGFβ, but which acts on ligand secretion. Following up on earlier work revealing that Yes-Associated Protein (YAP) inactivation inhibits cholangiocyte development [49], Lim and coworkers showed that YAP activation commits hepatoblasts to the biliary lineage by directly stimulating TGFβ2 expression [50]. Simultaneously, YAP binds to and represses the *Hnf4* locus, thereby inhibiting hepatocyte differentiation and contributing, together with its paralog TAZ, to the proper hepatocyte/cholangiocyte balance during hepatoblast differentiation. The targets of YAP/TAZ are not restricted to TGFβ2 and HNF4, since extracellular matrix-related proteins such as laminins and collagen IV are also induced following YAP activation. This might lead to a stiffening of the mesenchyme along the ductal plate cells, which might positively feedback on YAP, and eventually on TGFβ [50]. Among laminins, the α5 isoform is the best candidate to commit hepatoblasts to the biliary lineage [51].

An additional layer of TGFβ regulation targeting SMAD3/4/5 was identified. SMAD5 is activated by BMP signaling and is found predominantly in hepatoblasts located near the portal vein, that is where the ductal plate forms. Therefore BMP, whose function might be spatially restricted by chordin, can both modulate the effects of TGFβ and contribute to spatial location of differentiating cholangiocytes [52]. At a distance of the portal vein, microRNAs from the miR-23b cluster downregulate the SMADS in developing hepatocytes, resulting in inhibition of TGFβ signaling and repression of cholangiocyte gene expression, yet with limited impact on hepatocyte gene expression [53].

TGFβ signaling appears very dynamic during cholangiocyte differentiation. At the onset of ductal plate formation, when developing cholangiocytes organise as a single cell layer around the portal mesenchyme, TβRII is detected in all cholangiocytes. Later, when luminal ducts start to form, TβRII is rapidly repressed. This repression is dependent on SRY-related high mobility group box transcription factor (Sox) 9 and Sox4, and likely also by HNF6 and HNF1β, which together constitute a regulatory cascade [41, 47, 48, 54]. We therefore underline that the GRN depicted in Fig. 2B represents the initiation of cholangiocyte differentiation, but cannot apply to maturing cholangiocytes.

Finally, how and if TGFβ promotes growth arrest in hepatoblasts, which we described above as a cholangiocyte-promoting mechanism, remains undetermined. We can only speculate, as suggested by Rogler and coworkers, that biliary cells like many cell types become growth arrested as a result of TGFβ-induced repression of Myc [53].

6. Notch pathway: connections with TGFβ signaling for cholangiocyte differentiation

The initial view shared by several authors was that Jagged1 in the portal mesenchyme induces Notch2-mediated signaling in hepatoblasts located near the portal vein, eventually resulting in cholangiocyte gene induction [55-58]. Several experiments showed that overexpression of the Notch intracellular domain can stimulate biliary-enriched TFs and repress hepatocyte-specific TFs [59]. One could then conclude that Notch signaling primes hepatoblasts for differentiation to cholangiocytes, and spatially restricts differentiation around the portal mesenchyme. But Notch signaling in biliary development was then shown to be more complex than

anticipated. The expression of Jagged 1 is not only detected in the mesenchyme but also in differentiating cholangiocytes, suggesting that cell-intrinsic activation of Notch is active as well [56]. More recent work with hepatoblast lines provided evidence that the Delta-like 1 ligand promotes biliary differentiation by inhibiting hepatocyte genes, in a way that involves both cell-extrinsic and cell-intrinsic Delta-like 1activity [46]. Using an elegant array approach the authors also uncovered that both cell-extrinsic and cell-intrinsic Jagged1 affect cholangiocyte gene expression. Yet, these experiments must be interpreted with care since we do not know whether biliary gene induction in cultured cells reflects the onset of cholangiocyte differentiation *in vivo*, *i.e.* development of the first layer of the ductal plate apposed to the portal mesenchyme, or whether they also recapitulate the induction of biliary genes in hepatoblasts that participate to duct morphogenesis but which are not in contact with the portal mesenchyme.

The concept that Notch might prime hepatoblasts for spatially-restricted biliary differentiation stimulated a search for other signaling pathways that might cooperate with Notch. TGFβ was a good candidate, since overexpression of TβRII *in vitro* stimulates Sox9, a direct target of Notch [42, 56]. Moreover, in distinct *in vitro* experiments with cultured hepatoblasts, TGFβ induced expression of Notch ligands, mediators and targets, and the cholangiocyte-inducing effects of TGFβ were blocked by γ-secretase inhibitors, demonstrating that at least part of the function of TGFβ in biliary differentiation is Notch-dependent [46]. YAP and TAZ, as stated above, act upstream in the cascade by stimulating TGFβ2 production (Fig. 2B), yet they do not affect the expression of genes that are downstream of the Notch receptor, like Notch2, Hes1, Hey1, or Sox9 and HNF1β. Inhibition of Notch signaling by RBPJk inactivation does not affect the cholangiocyte-promoting effects of YAP either [50].

Finally, Notch activity is controlled and mediated by a set of TFs. Expression of Jagged1 and Notch2 is stimulated by Sall4 [60]. More downstream, Sox9 is a direct target of Notch and functions redundantly with Sox4. While most effects of the Sox factors relate to the later stages of cholangiocyte maturation and bile duct morphogenesis, Sox4 and Sox9 redundantly stimulate the initiation of cholangiocyte differentiation, as evidenced by reduced expression of HNF6 and HNF1β in the first layer of the ductal plate in livers doubly deficient for Sox4 and Sox9 [54]. In addition, there is a reciprocal stimulation of Sox4/Sox9 and HNF6 [54, 61], and Hhex is required for normal biliary development, at least in part by stimulating HNF1β [62]. At later stages of development, Notch signaling participates to bile duct morphogenesis. These aspects will be reviewed elsewhere (Ober and Lemaigre, *in preparation*).

7. Wnt and FGF signaling in hepatoblast differentiation: knowns and unknowns

Investigating Wnt signaling resulted in conflicting results regarding β-catenin function. A recent paper which addressed stage-specific effects revealed that accurate β-catenin levels are required for normal liver development, suggesting that earlier conflicting data might result from dose-dependent effects, or from the mode of β-catenin activation [63]. The current lack of knowledge about β-catenin targets prevents us from determining its position in the hepatoblast GRN. This holds true also for FGF effectors which are epistatically upstream of β-catenin in growth regulation [64]. However, FGF-2 was shown in chicken hepatoblasts to cooperate with BMP4 [65], suggesting that it contributes to cholangiocyte differentiation by impacting on the TGFβ/Notch axis (Fig. 2B). Wnt signaling also controls liver development via the non-canonical pathway where Wnt5a shifts the balance of

hepatoblast differentiation in favor of hepatocytes, by activating a calcium calmodulin kinase II-dependent pathway [66].

8. Hepatocyte differentiation: increased robustness of a hepatocyte-specific transcriptional network

Since hepatocyte-specific TFs are already expressed at the hepatoblast stage, entering the hepatocyte lineage program is a matter of stabilization of the TF network and induction of its target genes (Fig. 2C). A landmark paper from Talianidis' group showed that hepatocyte-specific TFs auto- and cross-regulate each other during mouse development, and that the number of TF cross-interactions increases during progression to a mature hepatocyte stage [67]. Within the GRN a core group of six TFs bind to the regulatory regions of each other and of peripheral members of the network. The stability of the hepatocyte-specific TF network arises from the redundant functions shared by multiple TFs. Positive feedback loops between TFs and microRNAs, such as the mutual stimulation of miR-122 and liver-specific TFs further contribute to the rise in TF expression during differentiation [68-71].

The rise in TF expression enables reaching threshold levels necessary for interaction with co-factors, such as PGC-1α, in order to activate hepatocyte-specific genes in a temporally-controlled manner [72]. In that context, some enhancers controlling hepatic gene expression are bound by HNF4α and FoxA2 in a temporal-dependent way. Indeed, a subset of enhancers regulated by HNF4α and FoxA2 are co-bound by YAP and its cofactor TEAD2 in embryonic liver but not in adult liver [73]. Another example of temporal-specific function of TFs is provided by the observation that FoxA1 and FoxA2 are redundant in development but progressively diverge in their target gene selection during maturation [74]. These data indicate that the activity of

GRN members is temporally regulated, including by co-factors and hippo signaling, the latter likely reflecting the influence of changing cell-cell and cell-matrix interactions during hepatocyte differentiation.

9. Subcircuitry motifs in liver gene regulatory networks: potential determinants of cell fate decisions

Topological analysis of GRNs revealed the presence of repeatedly occuring subcircuitry motifs, which confer specific dynamic properties to gene expression [75]. How the dynamical properties of the GRNs influence hepatic cell fate decisions has not been addressed experimentally, but here we speculate about how network motifs may impact on differentiation.

Negative feedback loops may be critical to stabilize a signal response. Fig. 3A illustrates that the expression of genes A and B as a function of the input signal S can saturate when transitioning from differentiation state Y to Z, thereby stabilizing phenotype Z in a wide range of S concentrations. C/EBPβ and HNF6/OC2-mediated control of TβRII constitutes an example negative feedback loop that may potentially stimulate differentiation of hepatoblasts to cholangiocytes and stabilize the biliary phenotype until environmental changes modulate the GRN to promote duct maturation. Positive feedback loops are also found in the liver GRNs. Such loops are defined by a double positive regulation or a double negative regulation, and are exemplified by the mutual activation between HNF1α and HNF4α, or the mutual inhibition between miR-125 and Lin28b (Fig. 3B-C). Thresholds or bistable switches with hysteresis are two dynamical properties that can emerge from positive feedback loops. While the expression of the components is positively correlated in a mutual activation loop (Fig. 3B), they are negatively correlated in a mutual inhibition motif

(Fig. 3C). Bistability promotes transition from differentiation state Y to Z when signal S reaches the activation threshold (green arrows in Fig. 3). However, cells that are in phenotypic state Z only revert to state Y when S decreases to the inhibition threshold (red arrow). Bistability can ensure robust phenotypic switches since the existence of two distinct thresholds impedes the reverse transition that might be caused by small variations in signal intensity.

Feed-forward loops also occur frequently in GRNs. An example of coherent feed-forward loop is given by the stimulation of hepatic genes by SMAD4 and GATA4 during liver specification (Fig. 3D). Such motifs could protect against small stochastic fluctuations of SMAD4, in particular if SMAD4 and GATA4 are simultaneously required for hepatic gene induction [76] (Fig. 3D). Finally, an incoherent feedforward loop is illustrated by Tbx3-mediated activation and repression of E-cadherin via HNF6/OC2 and Prox1 (Figs. 1B and 3B). Such motifs may elicit a biphasic output response indicating that well-defined concentrations of the signal can determine the cells' phenotypic response [77].

Whether the motifs identified in the hepatic GRNs confer the proposed properties remains speculative. Yet their analysis points toward the necessity to investigate GRNs by resorting to more quantitative methods such as mathematical modelling to capture their system-level dynamical properties.

10. Turning gene regulatory networks into tools for direct differentiation of cells to hepatocytes

Knowledge about GRNs constitutes a framework for reprogramming cells to a hepatic fate by forced expression of liver-specific TFs. Several cell types have been used as starting material, but embryonic or skin fibroblasts were the most commonly

selected. The cells were directly reprogrammed to a hepatocyte state or to an intermediate hepatoblast stage that was subsequently differentiated to a more mature phenotype. In a number of cases, forced expression of TFs was complemented by growing the tranduced cells in the presence of growth factors or epigenome modifying factors (Table 1) [78-88].

Most attempts to reprogram cells resorted to the use of FoxA and GATA factors, the selection of which being in part justified by their pioneer properties. Also, since forced expression of liver-specific TFs can lead to premature growth arrest, the inactivation of the $p19^{ARF}$ locus, which is shown above to promote cholangiocyte differentiation of hepatoblasts, was implemented in some protocols, yet with limited succes [80, 82]. An alternative approach consisted in inhibiting p53 [88].

Meanwhile, the study of hepatic GRNs could not identify the perfect combination of reprogramming TFs, prompting several groups to resort to a trial-and-error strategy. Moreover, despite the extensive cross-species conservation of hepatocyte-specific TFs, their binding sites within orthologous target promoters differ significantly between mouse and human [89]. It was therefore no surprise that transdifferentiation of murine and human fibroblasts required different TF combinations [80] [82].

Direct reprogramming of cells met with promising succes: HNF1β/FoxA3-induced hepatoblasts are bipotential and able to differentiate toward the cholangiocyte or hepatocyte lineages [78], and induced hepatocytes display hepatocyte-like morphology and function [80] [81, 82, 88]. However, they remain immature [79-84]; and gene expression profiling performed with help of the computational platform CellNet revealed that HNF4α/FoxA1-induced cells display intestinal cell properties, including expression of caudal type homeobox 2 (Cdx2), a master regulator of intestinal differentiation [90]. Similar observations were made by others [81, 91].

Unexpectedly, fibroblasts with inactivated alleles of Cdx2 failed to generate hepatocyte-like cells following HNF4α/FoxA1 induction, suggesting that Cdx2 must be expressed at some stage to enable reprogramming of fibroblasts to a hepatic fate [90]. Quantitative issues were also raised regarding HNF4α and FoxA1 expression: low HNF4α and FoxA1 levels, supplemented with Cdx2, promoted hepatocyte differentiation whereas high levels favored intestinal differentiation [90].

Finally, other attempts to improve the phenotype of induced hepatocytes consisted in adding epigenetic modifiers such as the histone demethylase Kdm2b, which likely destabilizes the starting cell genome and facilitate the action of pioneer transcription factors [85-87]. In a different perspective, adding ATF5, PROX1 and CEBPα to the cocktail enabled to improve maturation as evidenced by reduced fetal gene expression and production of phase I and II drug-metabolizing enzymes together with phase III drug transporters [88]. These data suggest that efficient direct reprogramming might only be achieved when developing and mature GRNs will faithfully be recapitulated by the appropriate selection and quantitative induction of TFs.

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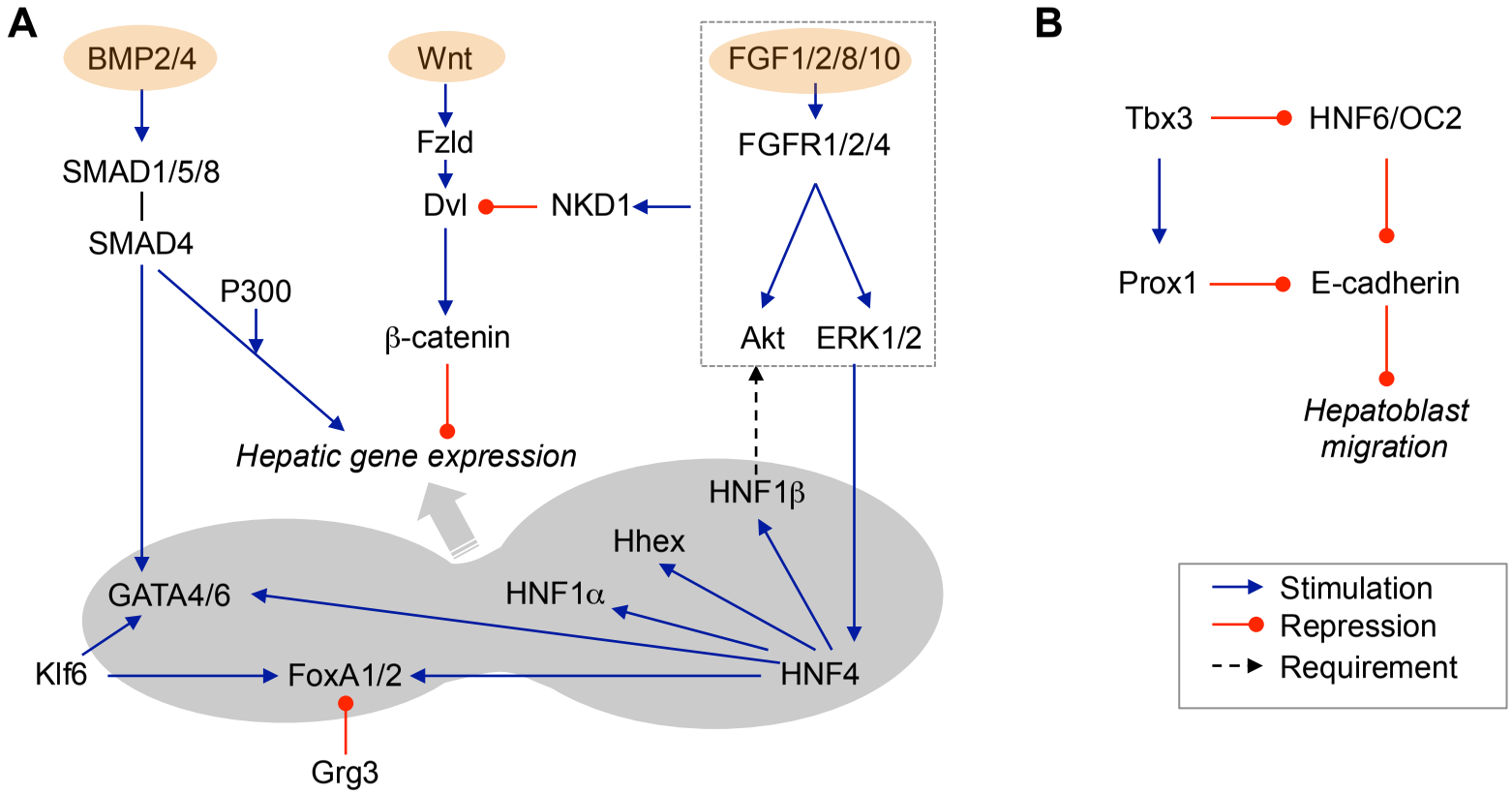
Figure Legends

Fig. 1. GRNs driving mammalian liver specification and hepatoblast migration. (A) Signaling pathways and TFs control the initiation of liver-specific gene expression. TFs in the grey shaded area collectively stimulate hepatic gene expression in prehepatic endoderm. (B) Network regulating migration of hepatoblasts through the septum transversum mesenchyme.

Fig. 2. GRNs controlling hepatoblast fate decisions. (A) GRN showing how TFs, microRNAs and chromatin modifiers regulate growth arrest and proliferation, which are associated with cholangiocyte gene induction and hepatocyte gene expression, respectively. (B) Network driving cholangiocyte differentiation from hepatoblasts. Jagged1$_m$ and Jagged1$_h$ refer to ligands expressed in the portal mesenchyme and in hepatoblasts, respectively. (C) GRN driving hepatocyte differentiation in mouse embryos. The GRN refers to embryonic day 18.5; the gray shaded area corresponds to the core network [67].

Fig. 3. Potential dynamic properties confered to hepatic GRNs by subcircuitry motifs. Negative (A) and positive (B-C) feedback loops. Coherent (D) and incoherent (E) feedforward loops.

Figure 1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β-catenin
Akt
ERK1/2
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Hhex
GATA4/6
HNF1α
Klf6
FoxA1/2
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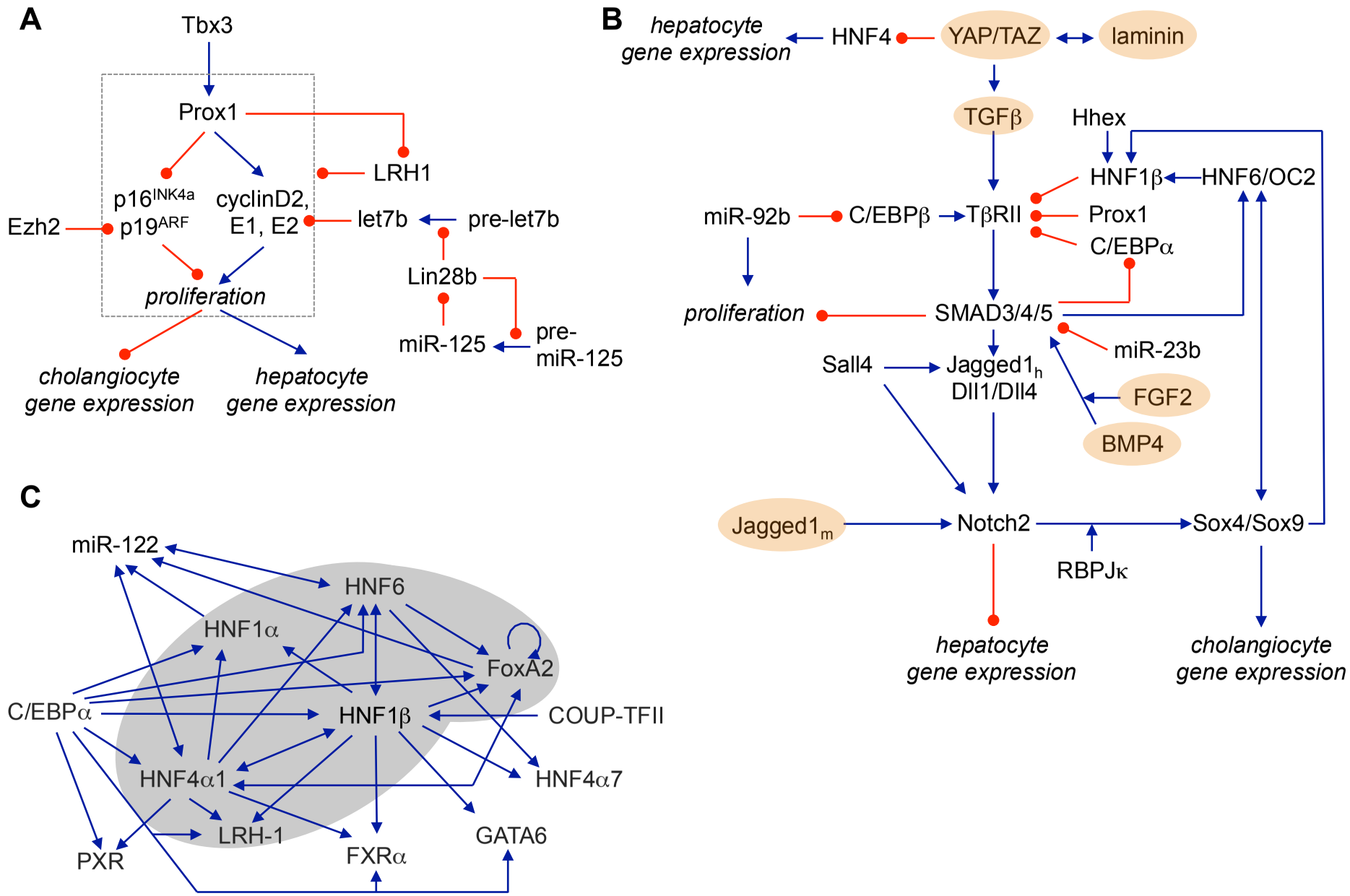
Prox1
E-cadherin
Hepatoblast migration
Stimulation
Repression
Requirement

Figure 2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pre-
miR-125
proliferation
cholangiocyte
gene expression
hepatocyte
gene expression
B
hepatocyte
gene expression
HNF4
YAP/TAZ
laminin
TGFβ
Hhex
HNF1β
HNF6/OC2
miR-92b
C/EBPβ
TβRII
Prox1
C/EBPα
proliferation
SMAD3/4/5
miR-23b
Sall4
Jagged1h
Dll1/Dll4
FGF2
BMP4
Jagged1m
Notch2
Sox4/Sox9
RBPJκ
hepatocyte
gene expression
cholangiocyte
gene expression
C
miR-122
HNF6
HNF1α
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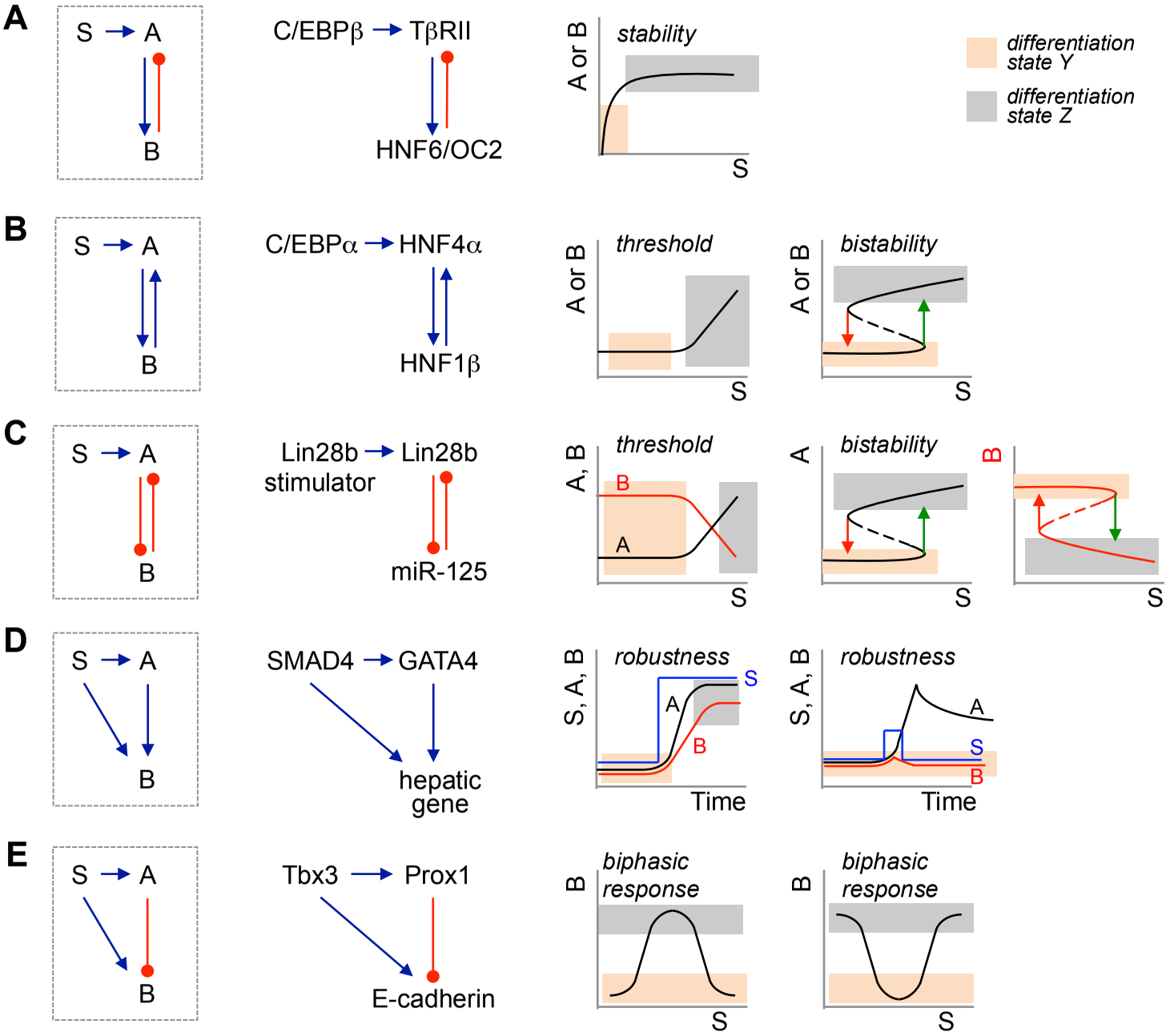
HNF4α7
LRH-1
GATA6
PXR
FXRα

Figure 3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C/EBPα
HNF4α
HNF1β
A or B
threshold
S
A or B
bistability
S
C
S
A
B
Lin28b stimulator
Lin28b
miR-125
A, B
threshold
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