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Dynamic Expression of *Groucho*-Related Genes *Grg1* and *Grg3* in Foregut Endoderm and Antagonism of Differentiation

Pilar Santisteban[1,2], **Pablo Recacha**[1,2], **David E. Metzger**[2], and **Kenneth S. Zaret**[2]

[1] Instituto de Investigaciones Biomédicas “Alberto Sols” Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, 28029 Madrid, Spain

[2] Cell and Developmental Biology Department, University of Pennsylvania School of Medicine, 421 Curie Blvd., Philadelphia, PA 19104

Abstract

While much is known about *Groucho* corepressors in Drosophila development, less is known about *Grg* homologs in mammalian embryogenesis. The transcription factors FoxA1 and FoxA2 are redundantly necessary for liver-inductive competence of the endoderm, and recently we found that FoxA factors bind Grg3, recruit the corepressor to FoxA target genes, and cause transcriptional repression, when Grg3 is ectopically expressed in adult liver cell lines that express little or no endogenous Grg. Unexpectedly, we now find that *Grg1* and *Grg3* mRNAs are co-expressed with FoxA factors in the foregut endoderm, prior to liver differentiation; though only Grg3 protein is expressed there. *Grg3* mRNA and protein are extinguished at the onset of liver differentiation. Lentiviral delivery of *Grg3* to explants of foregut endoderm suppresses liver gene induction. We suggest that *Grg* expression in the endoderm helps suppress the liver program and find that endodermal competence involves a balance between activators and corepressors.

Keywords

Grg; groucho; endoderm; FoxA; competence; corepressor

INTRODUCTION

In mammals, the endoderm germ layer arises during gastrulation and generates diverse tissues, including the thyroid, lung, liver, and pancreas. In the foregut endoderm of the mouse embryo, genes specific to these latter tissues, such as *alb1* for liver, become activated by 8.5 days of gestation (E8.5), corresponding to the 7–9 somite pair stage (7–9S) (Cascio and Zaret, 1991; Lazzaro et al., 1991; Jonsson et al., 1994; Gualdi et al., 1996; Zannini et al., 1997). Different inductive signals impinging upon different domains of foregut endoderm activate the tissue programs, leading to the view that endoderm differentiation involves positive regulation (Zaret and Grompe, 2008). Indeed, the FoxA1, FoxA2, GATA4, GATA6, and Sox17 transcriptional activators are expressed in the endoderm and are important for endoderm differentiation (Ang et al., 1993; Sasaki and Hogan, 1993; Bossard and Zaret, 1998; Kanai-Azuma et al., 2002; Zhao and Duncan, 2005; Watt et al., 2007). Chromatin sites for FoxA at *alb1* are occupied prior to liver induction (Gualdi et al., 1996), Fox factors open local chromatin structure (Cirillo et al., 2002; Yan et al., 2006; Cuesta et al., 2007; Hatta and Cirillo, 2007), and FoxA1 and FoxA2 are redundantly required for hepatic gene induction (Lee et al., 2005). Together, this has led to the proposal that FoxA

[2]Corresponding author: Dr. Ken Zaret, tel. 215-573-5813, fax 215-746-8791, zaret@upenn.edu.

proteins are competence or pioneer factors for the endoderm (Zaret, 1999). Yet as described in the present study, a deeper analysis of factors functioning with FoxA in endoderm is needed to understand how their target genes are kept competent for expression.

Groucho in Drosophila, *Groucho-related genes (Grgs)* in mouse, and *Transducin-like Enhancer of Split* genes (*Tle*s) in humans encode a family of evolutionary conserved corepressors family that play critical roles in diverse developmental processes (Fisher and Caudy, 1998; Chen and Courey, 2000; Courey and Jia, 2001). Part of the transcriptional repressor activity of the Grgs arises from their recruitment of histone deacetylases (HDACs) (Chen et al., 1999), and hence Grgs can indirectly suppress gene expression (Flores-Saaib and Courey, 2000). However, Grgs also have the intrinsic capacity to compact chromatin structure (Sekiya and Zaret, 2007). Furthermore, Grgs bind to FoxA proteins (Wang et al., 2000) and thereby can become recruited to genes FoxA would otherwise activate, such as the *alb1* gene when Grg is ectopically expressed in liver cells (Sekiya and Zaret, 2007). This can lead to local nucleosome structures that exclude other transcription factors from binding, resulting in transcriptional repression.

Based on the interactions of Grgs with FoxA in adult cells, herein we explored the expression profile and function of Grgs during the period when FoxA enables endoderm development and liver gene activation. The results suggest a new model for the FoxA-mediated state of endodermal competence, whereby Grgs restrain FoxA target genes from expression until the developmental time when the target genes are normally activated.

MATERIALS AND METHODS

In situ hybridization

In situ hybridization with 200 ng/ml of DIG-labeled Grg1 and Grg3 cRNA probes was performed as described (Ang and Rossant, 1993; Jung et al., 1999). Grg1 and Grg3 riboprobes, from nucleotides 1091–1617 and 1175–1728, respectively, were cloned from embryonic mRNA into TOPO-TA (Invitrogen).

Foregut endoderm tissue explants, lentivirus infections

Under a dissecting microscope, using electrolytically etched tungsten needles, the presumptive ventral endoderm and its associated cardiogenic mesoderm and septum transversum mesenchyme was separated from the embryo and either used for RNA or cultured in microwells as described (Gualdi et al., 1996).

Grg3-Flag and Grg3-ΔN-Flag lentiviruses were prepared as described (Sekiya and Zaret, 2007). Two hours after starting a foregut explant culture, each explant was infected with 8×10^5 (Grg3-Flag) or 2.4×10^5 (Grg3-ΔN-Flag) viruses. Twenty four hours later, the medium was exchanged for fresh medium without virus. Cultures were maintained for 72 hours, after which RNA was extracted.

RT-PCR

RNA was isolated with the RNeasy Micro Kit (Qiagen), including a DNAase treatment, and eluted in 10 μl of water. For RT-PCR cycle titration analysis, RNA was reverse transcribed with oligo-dT primers and subjected to PCR as described (Gualdi et al., 1996). The primers used were *alb*1 sense 5′-AGCACACAAGAGTGAGATCGCC-3 ′ , anti sense 5 ′-TGGCATGCTCATCGTATGAGC-3′; *β-actin* sense, 5 '-AAAGACCTGTACGCCAACACAGTC-3'; antisense, 5'-GTCATACTCCTGCTTGCTGATCCA-3'; Grg 1 sense 5 ′-AAATCGCCAAGAGATTGAAC-3 ′ , anti sense 5 ′-GGGTCCTCGTTAGACACATC-3′;

Grg3 sense 5 ′-TGGAGGTCCTGCACCACACTAA-3 ′ , antisense 5 ′-GCTCACAAACCACTTGCCACA-3′; *FoxA2* sense 5 ′-TCTCCGTGTCAGGAGCACAA -3 ′ , anti sense 5 ′-TGCTCGGAGGACATGAGGTT -3′. Each point shown in Fig. 3 depicts data from 4–6 separate explants.

For qRT-PCR, approximately 100 ng of RNA was used with the Reverse-It^{TM} 1st strand Synthesis Kit (*Abgene*). Quantitative PCR was performed using SYBR® Green PCR Kit (*Stratagene*). Samples were subjected to 40 cycles of PCR amplification in a Stratagene Mx3000P System for 10 min at 95°C, 30 sec at 95oC, 1 min at 59°C, and 25 sec at 72°C. qPCR were normalized using *β-actin* as Standard with ROX as Reference dye. The primers were: *β-actin* sense 5′-ACCCACACTGTGCCCATCTA , antisense 5′-CGCTCAGGAGGAGCAATGAT; *alb1* sense 5 ′-AACAAGGAGTGCTGCCATGGT, antisense 5 ′-GCTGGAGATAGTCGCCTGGT; *Grg1* sense 5′-*AAATCGCCAAGAGATTGAAC*′, antisense 5 ′-GGGTCCTCGTTAGACACATC; *Grg3* sense 5 ′-TGGAGGTCCTGCACCACACTAA, antisense 5 ′-GCTCACAAACCACTTGCCACA; *FoxA2* sense 5 ′-TCTCCGTGTCAGGAGCACAA; antisense 5′-TGCTCGGAGGACATGAGGTT; *TTR* sense 5′-TTCCATGAATTCGCGGATGT, antisense 5′-AGCCGTGGTGCTGTAGGAGTAT.

Antibodies and Immunofluorescence

Flag-tag antibodies were used as described (Sekiya and Zaret, 2007). The αGrg1 and αGrg3 antibodies were raised in rabbits immunized with peptides containing residues specific to Grg1, LAIKDDKKHHDAERHRDRE, or to Grg3, YSMEAKKRKAEEKDSLSR (ProSci, Inc.; Poway, CA). Immunofluorescence was performed on 8 micron-thick cyrosectioned embryos using αGrg1 (rabbit, 1:2500), αGrg3 (rabbit, 1:3000), and FoxA2 (Santa Cruz Biotech, SC6554, goat, 1:100) primary antibodies. Immunofluorescence development was achieved using tyramide signal amplification (Hunyady et al., 1996) with a TSA Kit (Invitrogen). To assess the specificity of αGrg1 and αGrg3, the antibodies were incubated with immunizing peptide for 1 hour prior to application on sections ("peptide block").

RESULTS AND DISCUSSSION

Grg expression in undifferentiated endoderm becomes repressed at the time of tissue specification

In situ hybridization studies indicated that *Grg1* and *Grg3* are expressed in various tissues of the mouse embryo at 8.25 days gestation (E8.25; 4–5 somite pair stage (4-5S)) (Fig. 1A, E), corresponding to the time at which the undifferentiated endoderm is receiving patterning signals for organogenesis. Sense strand control probes gave no signal (Fig. 1C, D). *Grg1* was expressed prominently in the head folds and dorsal endoderm (Fig. 1A, black and red arrowheads; and data not shown). Shortly later, at E8.75 (12S), *Grg1* was expressed in the head and other embryonic tissues, but not the heart and liver/midgut region (Fig. 1B). *Grg3* was expressed more pervasively in the E8.25 embryo (Fig. 1E). Detailed section analysis revealed that *Grg3* at 4-5S is expressed throughout the foregut endoderm, specifically including the dorsal, ventral-medial, and ventral-lateral domains that contain liver and ventral pancreatic progenitors (Fig. 1G-J, H’, I’) (Tremblay and Zaret, 2005). Several hours later, at 7-8S, the upper part of the dorsal foregut, containing pharyngeal progenitors, still expressed *Grg3* (Fig. 1K-M; M’, orange arrowheads), but the ventral foregut began to extinguish expression (Fig. 1K-M; M’, green arrowhead). By 16-18S, *Grg3* was not expressed in the heart and liver bud region (Fig. 1N-P, P’, brown arrowhead). In summary, *Grgs* are expressed in the foregut endoderm in the period preceding tissue specification (4-5S) and their expression diminishes shortly afterward.

To more precisely characterize the dynamics in *Grg1* and *Grg3* expression, we dissected foregut tissues from embryos pooled from carefully timed somite pair stages, isolated RNA, and performed RT-PCR cycle titration analysis (Fig. 2A) and qRT-PCR (Fig. 2B). As seen in Fig. 2, *Grg1* and *Grg3* mRNAs are present in the foregut until the 6-7S stage, agreeing well with the in situ hybridization data in Fig. 1. However, the abundance of those mRNAs drops strikingly several hours later, by the 9-10S stage, and remains low or absent in the midgut at 18-20S (Fig. 2); agreeing with the in situ data. By contrast, mRNAs for *FoxA2*, an endoderm marker, and *actin*, a ubiquitously expressed gene, remain constant. More strikingly, the *Grg* expression patterns are the exact reciprocal of that for *alb1*, a definitive marker for the induction of the hepatic program (Gualdi et al., 1996). Notably, the *alb1* gene is induced within the hours in which the *Grgs* are shut off in the endoderm (Fig. 2). This also corresponds to the time when other early liver genes, including *Ttr*, are induced (Jung et al., 1999) .

To assess whether Grg1 and Grg3 proteins follow a similar course of expression, we raised antibodies to several peptides specific to each. We obtained one antibody each that specifically recognizes the expected Grg in Western blots and is of much higher affinity to each protein than a commercially available "pan-Grg" antibody (Supp. Figure 1). The Grg1 antibody gave an immunoflourescence pattern that was spotty throughout much of the embryo at the 3-4S, 7-8S, and 23S stages, but mostly non-nuclear and not blocked by the specific peptide (Supp. Fig. 2 for 7-8S, 23S; data not shown for 3-4S). However, we observed nuclear staining in the head of 7-8S embryos and in the neural tube, dorsal mesenchyme, and lateral endothelium of 23S embryos, and this staining was blocked by the peptide immunogen (Supp. Fig. 2). Notably, no endodermal or liver bud nuclear staining was observed for Grg1 at any stage. We conclude that Grg1 protein expression is much more limited than its mRNA and that it is not expressed in the endoderm, or that the antigen is blocked in endoderm nuclei, in a cell-specific fashion.

The Grg3 antibody, by contrast, gave more interesting results. First, nuclear Grg3 staining was detected in 6-7S (E8.5) ventral foregut endoderm cells in a pattern that is virtually superimposable over that for the in situ hybridization (Fig. 3A-C). Such staining was blocked by peptide immunogen (Fig. 3D, E). The staining was also detected at the earlier 3-4S stage and was superimposable on the staining elicited by FoxA2, a definitive endoderm marker (Ang et al., 1993; Sasaki and Hogan, 1993; Bossard and Zaret, 1998; Kanai-Azuma et al., 2002; Zhao and Duncan, 2005; Watt et al., 2007) (Fig. 3F-I; see yellow, double-labeled cells depicted by arrows in the merged, magnified panel I). By 8-9S, within a few hours of hepatic specification (Gualdi et al., 1996; Jung et al., 1999) Grg3 protein persisted in the ventral foregut endoderm (Fig. 3J-M). These studies show that Grg3 protein persists for several hours after the time its mRNA disappears (Fig. 2)

Notably, several hours later, by 13S, Grg3 protein disappeared from most cells of the nascent liver bud; the latter being marked by FoxA2 expression (Fig. 4A-D; see mostly green, not yellow, cells in the liver bud in the merged, magnified panel D). Interestingly, Grg3 was also expressed with FoxA2 in the neural tube floor plate at 13S (Fig. 4B-D; n.t.f.p.), though Grg3 expression there diminished at later stages (Fig. 4G and data not shown). By 20S and 22-23S, the liver bud cells were completely devoid of Grg3, as seen by the green and not yellow cells in the merged images (Fig. 4E-L). However, Grg3 was robustly expressed in the stromal cells that surround the liver bud and in the midgut epithelium. At E12.5, Grg3 was not expressed in the liver, though the mesothelium (liver capsule) was positive (Fig. 4M, N). The absence of Grg3 protein in the E12.5 liver parenchyma concurs with the absence of Grg3 mRNA there at E14.5, seen in a previous study (Dehni et al., 1995). The presence of Grg3 protein in the liver mesothelium at E12.5 concurs with Grg3 mRNA expression seen there in another previous study (Leon and Lobe,

1997). We conclude that Grg3 protein is markedly down-regulated during the earliest stages of hepatic differentiation, in the cells that express FoxA2. We note that such cells also express FoxA1 (Ang et al., 1993), and that Grg3 interacts with both FoxA1 and FoxA2 (Sekiya and Zaret, 2007).

Ectopic expression of Grg3 suppresses liver gene induction in endoderm

Given our previous studies showing that FoxA1 recruitment of Grg3 to liver genes suppresses expression (Sekiya and Zaret, 2007), we speculated that *Grg* expression in the undifferentiated endoderm prevents liver gene induction. To test this hypothesis, we sought to extend the time over which *Grg3* is expressed in the endoderm and assess the consequences for *alb1* and *Ttr*. We employed lentiviruses encoding a wild type, Flag-tagged Grg3 protein or Flag-tagged Grg3-ΔN, the latter containing an N-terminal deletion of Grg3 that is not recruited by FoxA1 (Sekiya and Zaret, 2007), as a control. We developed infection conditions whereby Flag antigen could be detected in most epithelial cells of an infected ventral foregut explant (Supp. Fig. 3), when the explant was generated at the 5-6S or 7-8S stages and cultivated for 3 days. Uninfected controls were negative for Flag antigen (Supp. Fig. 3).

As expected from the data in Fig. 2, qRT-PCR analysis showed that the levels of *Grg3* mRNA were diminishing in the initial dissected tissues isolated from 7-8S embryos, compared to 5-6S embryos (Fig. 5A, "initial explant"). Endogenous *Grg3* mRNA levels declined in cultured explants from both conditions, showing that the down-regulation of *Grg3* observed in vivo was recapitulated, at least in part, after three days culture in vitro (Fig. 5A, "cultured explant"). The *Grg3*-Flag lentivirus infected cultures expressed the Flag sequence (Supp. Fig. 3) and caused the persistence of *Grg3* expression in both the 5-6S and 7-8S explants (Fig. 5A). Notably, persistent *Grg3* expression caused a failure to induce *alb1* and *Ttr* mRNA expression (Fig. 5B, C), paradigm markers for the induction of the hepatic program (Gualdi et al., 1996;Jung et al., 1999). This effect was not a nonspecific effect of lentiviral infection, because infection with the Grg3-ΔN virus did not prevent *alb1* and *Ttr* induction (Fig. 5B, C). Furthermore, the effect of ectopic expression of full-length *Grg3* was specific to the liver genes because wild type *Grg3* virus infection had no effect on the expression of *FoxA2* (Fig. 5D). This also demonstrated the integrity of the endoderm tissue in the *Grg3*-infected explant.

New model for endodermal competence

Taken together, the data show that Grg3 is expressed in the undifferentiated endoderm, it is rapidly down-regulated during the time of foregut tissue induction, and if *Grg3* expression is maintained during this period, it prevents the induction of *alb1*, a paradigm sentinel of mammalian liver specification. These findings, along with our previous discovery of FoxA factors being able to recruit Grg3 to liver genes and suppress their expression (Sekiya and Zaret, 2007), lead to a revised model for the basis of hepatic competence imparted by FoxA factors. That is, given the potent ability of FoxA factors to enable transcription (see Introduction) (Fig. 5E, panel I), we suggest that FoxA binding of Grg3 may create a closed chromatin structure local to the regulatory sequence (Fig. 5E, panel II), as was seen in our previous study (Sekiya and Zaret, 2007). This would disallow recruitment of other factors or target gene activation until the appropriate time in development, when down-regulation of Grg3 would relieve repression and allow FoxA target genes to be fully induced (Fig. 5E, panel III). Our findings reveal a dynamic balance between activators and repressors during the earliest stages of hepatic programming and add an additional parameter to consider for understanding how endoderm cells can be deliberately programmed to foregut tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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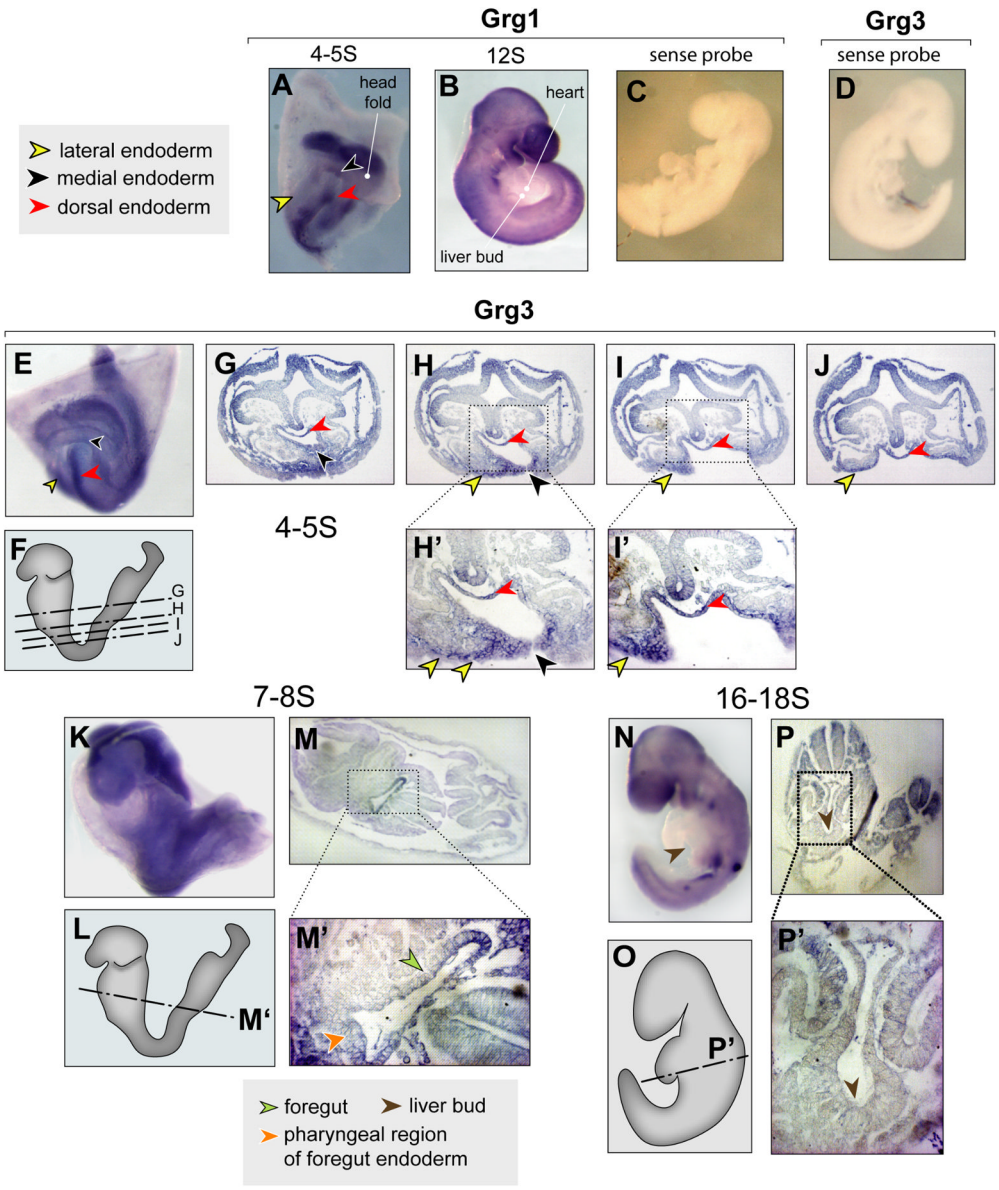

Fig. 1. Dynamic expression of *Grgs* in endoderm development

A-D, E, K, N. Whole mount in situ hybridization with the designated antisense (not denoted) and sense (denoted) probes, at the somite pair stages shown in the Figure. G-J, M, P. Sections as shown in panels F, L, and O, respectively. H', I', M', P', magnifications as shown. Arrowheads indicate tissue domains. *Grg1* and *Grg3* are expressed in the endoderm at the 4-6S stage but then are suppressed in expression in the nascent liver bud (12-18S).

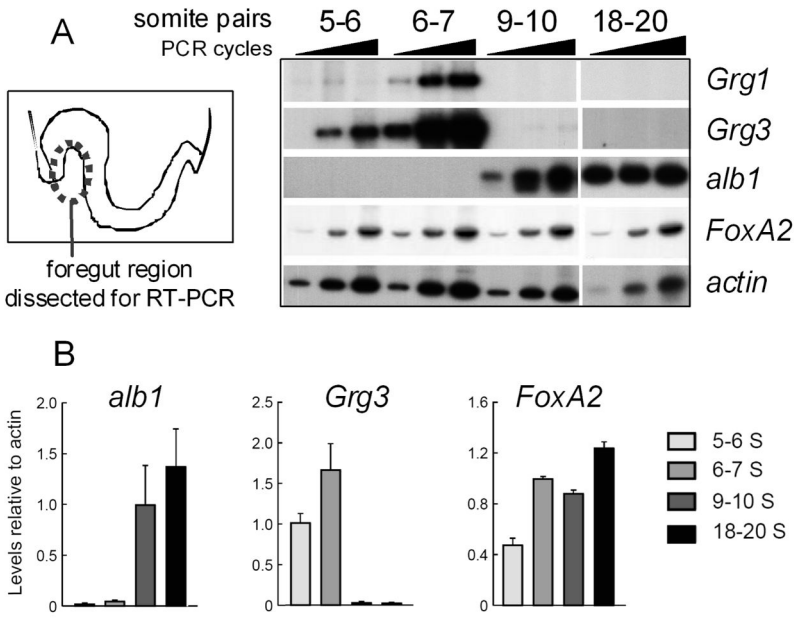

Fig. 2. Abrupt down-regulation of *Grg1* and *Grg*3 mRNA at the time of hepatic gene induction in the endoderm

A, left. Diagram of embryonic region dissected for RT-PCR analysis. A, right. Autoradiographs of RT-PCR products at different cycle steps. B. qRT-PCR analysis normalized to fewer cycles of β-actin. Bars depict average of four independent experiments ± S.E.

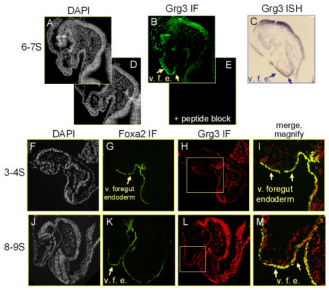

Fig. 3. Expression of Grg3 protein in ventral foregut endoderm

A-E. DAPI, Grg3 immunofluorescence (IF), and in situ hybridization (ISH) as shown of sagital sections of 6-7S mouse embryos. The signal in panel E was absent due to the presence of the Grg3 immunogenic peptide as a block. Yellow arrows, ventral foregut endoderm (v. f. e.). F-M, similar DAPI and IF images as above. The boxed regions in H and L are merged with the same domain in G and K and magnified in I and M. The yellow cells co-express FoxA2, a definitive endoderm marker, and Grg3.

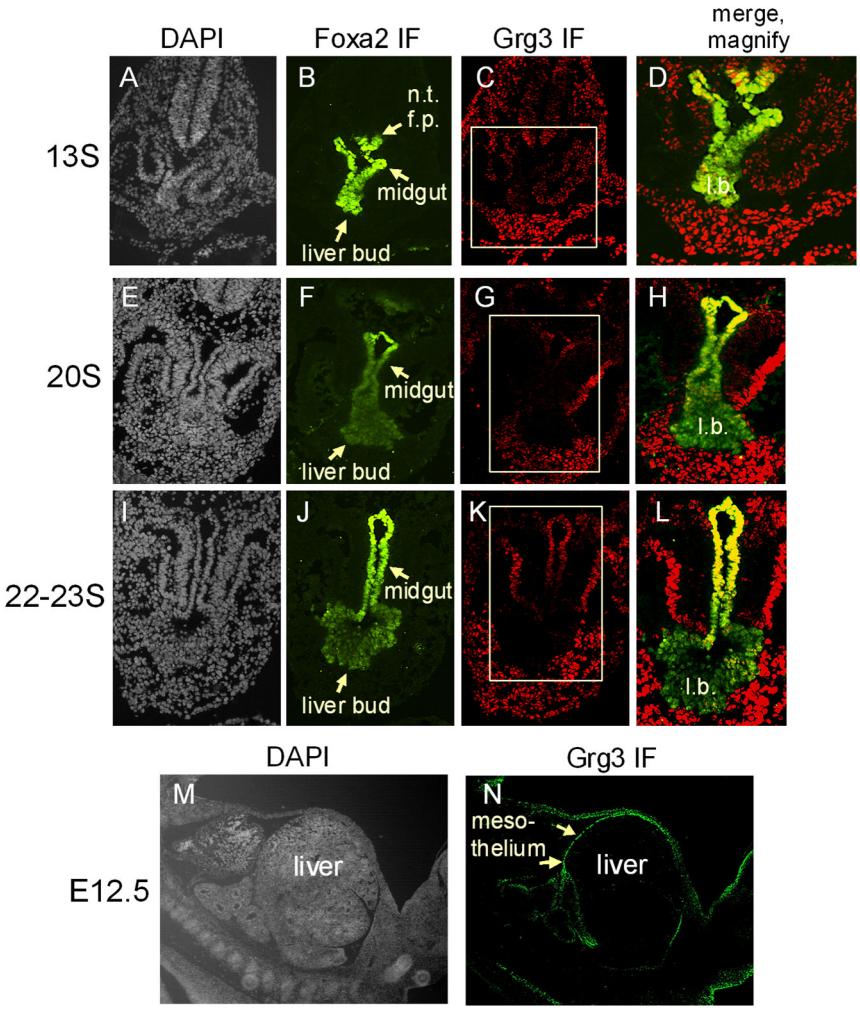

Fig. 4. Down-regulation of Grg3 protein in the liver bud and fetal liver

A-L. DAPI, immunoflorescence (IF), and merged and magnified images as in Fig. 3. Note absence of red (panels C, G, K) or yellow (panels D, H, L) Grg3-positive cells in the liver bud, but Grg3-positive cells in the nearby midgut and surrounding stroma. M, N. Sagital section of E12.5 embryo, showing the absence of Grg3 in the liver but its presence in the mesothelium surrounding the liver.

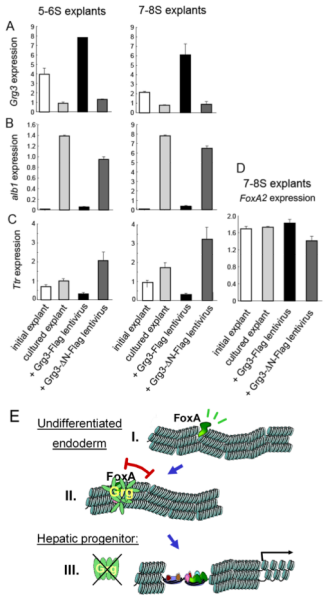

Fig. 5. Persistent Grg3 expression suppresses alb1 induction in ventral foregut explants

A-D. qRT-PCR analysis of designated genes in dissected ventral foregut tissues and cultured explants, infected or not with lentivirus constructs encoding *Grg3* or *Grg3-ΔN*. Bars represent expression levels relative to that for β-actin in the same samples as average of 4-6 independent experiments ± S.E. The PCR primers for Grg3 were specific to the N-terminal coding region of the mRNA and did not reveal elevated signals in the *Grg3-ΔN*-infected cultures; see Supp. Fig. 3 for evidence of *Grg3-ΔN* expression. E. New model for endoderm competence. The figure depicts liver gene chromatin to which FoxA factors are bound in the endoderm. I. In undifferentiated endoderm, FoxA binding opens the local chromatin, providing entry (green lines) for factors that could bind flanking sequences. II. Grg binding closes the FoxA-bound chromatin and prevents other factors from binding. III. During tissue specification, Grg factors are down-regulated, relieving repression and allowing other factors to enter the chromatin and activate target gene expression.