DNA-induced spatial entrapment of general transcription machinery can stabilize gene expression in a nondividing cell

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An important characteristic of cell differentiation is its stability. Only rarely do cells or their stem cell progenitors change their differentiation pathway. If they do, it is often accompanied by a malfunction such as cancer. A mechanistic understanding of the stability of differentiated states would allow better prospects of alleviating the malfunctioning. However, such complete information is yet elusive. Earlier experiments performed in *Xenopus* oocytes to address this question suggest that a cell may maintain its gene expression by prolonged binding of cell type–specific transcription factors. Here, using DNA competition experiments, we show that the stability of gene expression in a nondividing cell could be caused by the local entrapment of part of the general transcription machinery in transcriptionally active regions. Strikingly, we found that transcriptionally active and silent forms of the same DNA template can stably coexist within the same nucleus. Both DNA templates are associated with the gene-specific transcription factor Ascl1, the core factor TBP2, and the polymerase II (Pol-II) ser5 C-terminal domain (CTD) phosphorylated form, while Pol-II ser2 CTD phosphorylation is restricted to the transcriptionally dominant template. We discover that the active and silent DNA forms are physically separated in the oocyte nucleus through partition into liquid–liquid phase-separated condensates. Altogether, our study proposes a mechanism of transcriptional regulation involving a spatial entrapment of general transcription machinery components to stabilize the active form of a gene in a nondividing cell.

transcriptional regulation | *Xenopus* oocyte | liquid–liquid phase separation | Ascl1 | stable gene expression

Nondividing cells including adult cells must establish and maintain a transcriptional identity necessary to support a specific phenotype for long periods of time (1). The acquisition of cell fate requires pioneer transcription factors (such as MyoD in muscle) to activate lineage-determining gene expression (2). Once established, their continued action is required in certain contexts to safeguard cellular identity, by preventing reversal to progenitor states (3) or transdifferentiating into alternative fates (4). However, the transcription factor binding dynamics that maintain the stability of a terminally differentiated cell remain elusive.

The *Xenopus* oocyte is an established suitable system for studying transcriptional regulation (5). Its ease of availability and large size enable experimental manipulation, such as injection of molecules at a precise location and concentration in a nondividing cell (6), whereas overexpression of transcription factors in mammalian nondividing cell models may disturb the nuclear environment (7). By contrast, injection of DNA into the germinal vesicle (GV) can form stable transcriptional complexes (8, 9) that can maintain expression for several days in culture, without disturbing the host cofactor pool. Therefore, the *Xenopus* oocyte can serve as an advantageous system to study the dynamics of protein complexes and cofactor interactions that regulate the early events of transcription factor binding and initiation.

We previously reported a DNA competition assay in the *Xenopus* oocyte, which can be used to model and study the stability of transcription in a nondividing cell (10). In this system, an injected transcription factor competes for binding at identical promoter sites (11) on two plasmids driving different reporter gene expression. We previously observed that when the two plasmids are sequentially injected, the second DNA fails to express. The mechanism governing resistance to competition by the second DNA remains unclear. It has widely been thought that pioneer transcription factors and the general transcription machinery form tight and stable complexes; however, it is equally plausible that stability is governed by local spatial entrapment of factors on DNA (12). Indeed, recent studies have highlighted liquid–liquid phase separation (LLPS) as a possible mechanism underlying gene activation. LLPS could therefore contribute to differential expression of sequentially injected plasmids.

Here, we demonstrate that the mechanism of resistance of expression of the second injected DNA in the *Xenopus* oocyte

Significance

How differentiated cells such as muscle or nerve maintain their gene expression for prolonged times is currently elusive. Here, using Xenopus oocyte, we have shown that the stability of gene expression in nondividing cells may arise due to the local entrapment of transcriptional machinery to specific gene transcription start sites. We found that within the same nucleus active versus inactive versions of the same gene are spatially segregated through liquid–liquid phase separation. We further observe that silent genes are associated with RNA-Pol-II phosphorylated on Ser5 but fails to attract RNA-Pol-II elongation factors. We propose that liquid–liquid phase separation mediated entrapment of limiting transcriptional machinery factors maintain stable expression of some genes in nondividing cells.

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is associated with the formation of subnuclear compartments. We conduct DNA competition assays, using Ascl-1 (a basic helix–loop–helix pioneer transcription factor for neuronal cell fate) (13). We elucidate the nature of promoter-specific regulation, by characterizing the chromatin binding dynamics of Ascl-1 and general transcriptional machinery. We find that competing DNA forms LLPS condensates that are semipermeable to some lineage-determining factors like Ascl-1 but actively sequester part of the general transcription machinery necessary for gene expression.

Results

Design of DNA Competition Experiment in *Xenopus* Oocytes. *Xenopus* oocytes that are sequentially injected with two DNA plasmids show DNA competition, in which only the first injected DNA is transcriptionally expressed (Fig. 1*A*). Previous studies have examined this phenomenon using constitutive cytomegalovirus (CMV) or simian virus 40 (SV40) promoters (14) or promoters of histone genes. However, a major limitation has been the inability to control gene expression from plasmid DNA after injection into the oocyte. Recently, Gurdon et al. (10) have used a transcription factor–mediated inducible expression system to study transcription dynamics on injected DNA templates in the *Xenopus* oocytes. In the plasmid design, the binding motif of Ascl-1 (taken from mouse Dll1 gene) (15) controlled the downstream expression of either *Firefly* (DNA-FF) or *Renilla* (DNA-Ren) reporter genes (Fig. 1*B*). Under the experimental setup, a limiting amount of 1 to 2 ng/oocyte mRNA encoding the Ascl-1 protein is injected on day 0, 210 to 350 pg/oocyte DNA-FF on day 1, and 2 to 3 ng/oocyte DNA-Ren on day 2, with overnight incubation between each step (Fig. 1*C*). Both injected DNA plasmids then compete in the GV for 24 h before the reporter gene expression is analyzed.

The Second Sequentially Injected DNA Fails to Express in *Xenopus* Oocytes. First, a series of DNA competition assays were performed to characterize our experimental setup. Ascl-1 mRNA

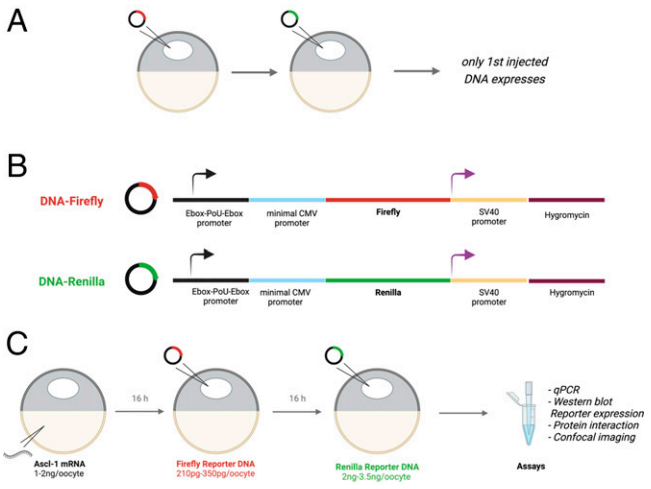

Fig. 1. Overall design of oocyte DNA competition assay. (*A*) Schematic diagram showing the sequential injection of DNA plasmids into the GV of the *Xenopus* oocytes. Only the first injected DNA plasmid is expressed despite a 10-fold excess of the second plasmid. (*B*) Regulatory sequences of the plasmids used in this study to investigate the resistance to DNA competition. An Ascl1 inducible promoter (minimal CMV promoter and Ebox-Pou-Ebox) controls the expression of a luciferase (DNA-FF) or DNA-Ren reporter gene. Both constructs contain a constitutive SV40 promoter that drives the expression of an hygromycin resistance gene. (*C*) Diagram indicating the quantity of mRNA and plasmids sequentially injected to study resistance to DNA competition in the oocytes. Time gap between sequential injection ranges from 16 to 24 h.

injection was followed by injection of DNA-FF or DNA-Ren alone, coinjection of both plasmids, or sequential injection, followed by an overnight incubation. Reporter gene expression was quantified by a luminescence assay as a readout of each plasmid's transcription (Fig. 2*A*). Ascl-1 could activate both plasmids injected individually and to a similar degree when they were coinjected; however, when there was sequential injection, the second injected plasmid failed to express (Fig. 2*B*). A similar result was obtained in qRT-PCR assays using green fluorescent protein and mCherry plasmid constructs which are driven by the constitutive CMV promoter (Fig. 2*C*). This led to the proposal that underexpression of the second sequentially injected DNA with an active promoter is a general phenomenon in *Xenopus* oocytes.

Next, we wondered whether DNA competition may depend on the concentration of the first injected plasmid. To investigate this, we injected variable amounts of the first plasmid (DNA-FF) and measured the second plasmid (DNA-Ren) expression (Fig. 2*D*). DNA-Ren expression was suppressed in a dose-dependent manner with increasing DNA-FF concentration; this could be partially rescued when DNA-FF had a mutated ΔSV40 promoter, demonstrating that the extent of resistance to competition is dependent on the number of transcriptionally active promoters (16).

Another important characteristic of DNA competition is the time duration separating two injections. While coinjected DNA-FF and DNA-Ren express equally, we sought to characterize the time interval required between template injection to form a stable resistant-to-competition state. To test this, we performed a time course DNA competition experiment in which we injected oocytes with a limiting amount of Ascl-1 mRNA (~1.4 ng of Ascl1 mRNA; *SI Appendix*, Fig. S1) on day 0. On the next day, we injected 210 pg of DNA-FF in the same oocytes, followed by 2 ng DNA-Ren injection at time 0 (time at which DNA-FF was injected), 10 min, 30 min, 60 min, 120 min, and 6 h. Surprisingly, DNA-Ren reporter expression was only detected in the 10-min gap setup, indicating that the resistance to competition starts as early as 30 min after the injection of the DNA-FF plasmid (Fig. 2*E*).

Xenopus oocytes have more than 10,000 actively transcribed extrachromosomal DNAs (17) in their GV. We asked whether DNA competition is observed specifically in extrachromosomal DNA or whether this phenomenon is seen more generally in the context of chromosomal DNA. To explore this, we sequentially injected mouse embryonic fibroblasts (18) and then normal human dermal fibroblast nuclei into the GV of the *Xenopus* oocytes (Fig. 2*F*). After 24 h of competition, we analyzed the expression of key pluripotency genes, namely, *Sox2* and *Oct4* as well as *c-Jun*, that are generally activated after nuclear transplantation. We observed that transplantation of mouse nuclei prior to human nuclei leads to a reduction in the expression of human *Sox2*, *Oct4*, and *c-Jun* compared to when the human nuclei are injected alone. The extent of competition observed with nuclei was significantly less than that with plasmid DNA; this may have arisen because far fewer nuclei can be transplanted into the GV than the number of plasmid molecules, with the latter having a significantly higher number of transcriptional sites. Nonetheless, this result demonstrates that DNA competition is observed both in extrachromosomal and nuclear DNA as a general phenomenon.

Transcriptional Activity of a First-Available DNA Template Is Required to Establish Dominance. We hypothesized that underexpression of the second injected template may be caused by tight binding of pioneer or basal transcription factors to the first DNA, such that they are unavailable to the second DNA. To evaluate this as the cause for DNA-FF dominance, we inverted the order of injections such that Ascl-1 mRNA was injected

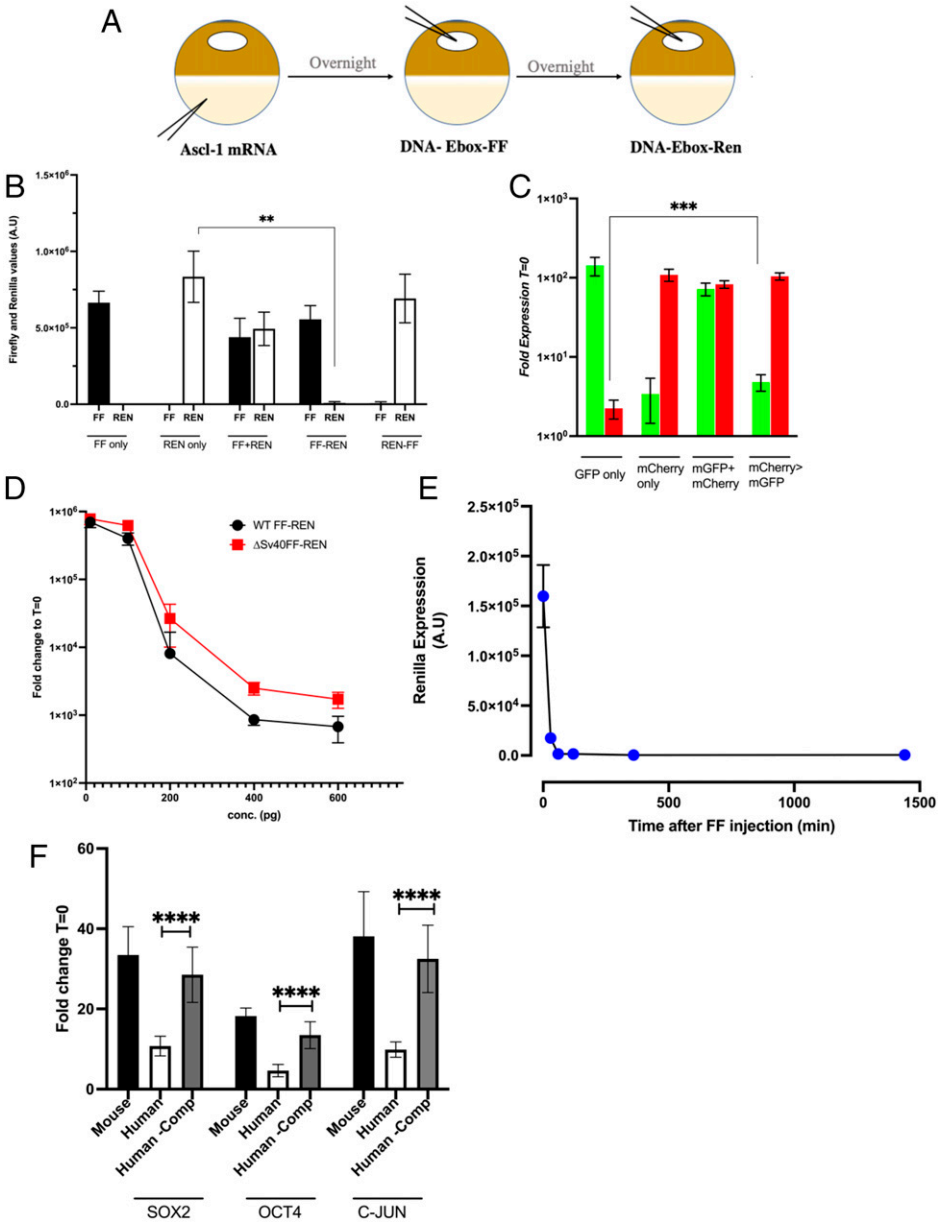

Fig. 2. Differential expression of sequentially injected templates. (*A*) Schematic of DNA and Ascl1 injection in the oocytes. Oocytes were injected with 1 ng of Ascl-1mRNA, 210 pg of DNA-FF, and 3 ng of DNA-Ren. (*B*) DNA-FF (black) and DNA-Ren (white) expression detected by luminescence assay. Oocytes were analyzed for reporter expression 24 h after the injection of competitor DNA-Ren plasmid. $n = 3$ independent experiments each with eight individual oocytes/experimental condition. (*C*) mCherry (red) and green fluorescent protein (GFP) (green) expression was analyzed by qRT-PCR following sequential injection of CMV-mCherry and CMV-GFP. (*D*) DNA-Ren expression was measured by luminescence assay in a sequential injection set up where increased dose (ranging from 10 pg/oocyte to 600 pg/oocyte) of DNA-FF (black) or DNA-FFΔSv40 (red) was injected prior to the injection of 3 ng of DNA-Ren. (*E*) DNA-Ren expression was measured by luminescence assay in a sequential injection set up where 3 ng DNA-Ren was injected 10, 30, 60, or 120 min or 6 h after the prior injection of 210 pg DNA-FF. All the samples were collected 24 h after last injection. (*F*) qRT-PCR analysis of gene expression from nuclei transplanted to *Xenopus* oocytes. Black bars show the expression of mouse genes of transplanted mouse fibroblast nuclei. White and gray bars show the expression of human genes 48 h after transplantation to oocytes of human nuclei only or human nuclei preceded by mouse nuclei, respectively. The expression data at 48 h are normalized to values detected in samples taken immediately after transplantation. Graph represents average of $n = 3$ or greater experiments. Error bars are SEM. $^{**}P < 0.001$, $^{***}P < 0.05$, $^{****}P < 0.05$ (Student *t* test and two-way ANOVA).

after both of the DNA templates. We injected DNA-FF on day 0 and DNA-Ren on day 1, followed by the injection of Ascl-1 mRNA on day 2 (Fig. 3*A*), reasoning that Ascl-1 should activate both DNA templates (which are already present in the GV of oocytes) equally. Surprisingly, Ascl-1 activated the expression of only the first template DNA-FF (Fig. 3*B*), despite having access to both Ebox-driven promoters, as shown by chromatin immunoprecipitation (ChIP)-qPCR (Fig. 3*C*). We detect a similar binding of TATA binding Protein like protein 2 (TBP2) to DNA-FF and DNA-Ren (*SI Appendix*, Fig. S2). Therefore, we conclude that the failure to activate transcription by the second DNA occurs at a stage downstream of Ascl1 and TBP2 binding.

The DNA-FF and DNA-Ren used in these injections also harbor, in addition to the Ascl1 responsive promoter, a highly active constitutive SV40 promoter (Fig. 1*B*). To evaluate the contribution of the constitutive promoter to the differential Ascl1-induced transcription elongation from reporter plasmids, we repeated the above experiment using either pUC19 (a

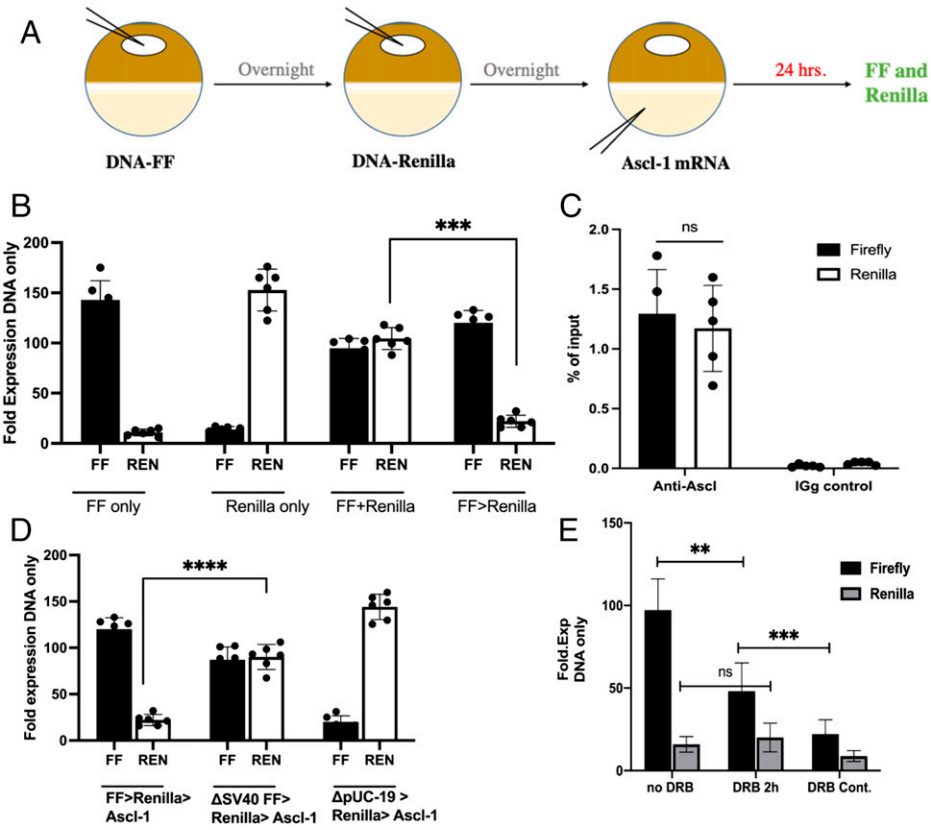

Fig. 3. A prior transcriptional activity is required to establish resistance to competition. (*A*) Schematic of an inverted injection setup. A total of 210 pg of DNA-FF was injected into the GV on day 0 followed on day 1 by injection of a 3 ng DNA-Ren. On day 2, 1.4 ng of Ascl-1 mRNA was injected into the oocytes and incubated a further 24 h before the assay for reporter expression. (*B*) Luminescence assay showing DNA-FF and DNA-Ren reporter expression in the inverted injection scheme. Reporter values are normalized to values detected without Ascl1 injection. (*C*) Ascl1 ChIP-qPCR showing the binding of Ascl-1 to DNA-FF and DNA-Ren when injected in inverted order. (*D*) Same setup as (*B*) except that the FF construct used in the first injection is either with or without (ΔSV40) promoter or replaced by a pUC19 constructs lacking eukaryotic a promoter. (*E*) Effect of DRB treatment on DNA-FF and DNA-Ren expression. A DNA competition assay is carried out with either no treatment or addition of 40 mM DRB either continuously or for a 2-h pulse at the time of DNA-Ren injection. Normalized reporter values are shown from samples collected 24 h after DNA-Ren injection. Error bars show the SEM in $n = 3$ and $n = 6$ in some experiments. Each experimental condition was measured from eight individual oocytes. $^{***}P < 0.05$, $^{**}P < 0.05$, $^{****}P < 0.02$ (Student paired *t* test and two-way ANOVA). ns, not significant.

plasmid devoid of any eukaryotic promoter) or a DNA-FF without the SV40 promoter, as the first injected DNA. When the transcriptionally silent plasmid (pUC-19) was injected as a first plasmid, Ascl-1 was able to drive the expression of the second injected template (DNA-Ren) (Fig. 3*D*). Similarly, when a mutated DNA-FF ΔSV40 was injected as the first DNA, resistance to competition was abolished and Ascl-1 activated expression of both DNA-FF and DNA-Ren templates (Fig. 3*D*). These results indicate that the resistance-to-competition state requires transcriptional elongation activity of the first injected DNA. It also suggests that the second DNA template fails to activate transcription downstream of Ascl1 binding.

Next, we investigated whether transcriptional complexes could be reconstituted from one plasmid to another chemically. We used 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole (DRB) (19), which blocks transcriptional elongation by interacting with a DRB sensitive factor in the Pol-II elongation complex. DRB removal from the medium reconstitutes DNA transcription (20). We optimized the time and concentration doses of the DRB so that we could transiently inhibit transcription from the first injected plasmid (*SI Appendix*, Fig. S3). We then designed an experiment in which we halted the transcription of DNA-FF by addition of DRB in the resistance-to-competition state, assuming that DRB would dislodge the Pol-II machinery from DNA-FF and then reload on both DNA-FF and DNA-Ren after DRB removal. Surprisingly, reconstitution of transcription only happened from DNA-FF (Fig. 3*E*). We conclude from this assay that transcriptional dominance of the first template does not require its continuous transcriptional activity.

RNA-Pol-II Complexes on DNA-Ren Are Not Phosphorylated at Ser-2. TBP2 binding to DNA-Ren in its underexpressed form may indicate the loading of transcriptional preinitiation complexes on DNA (21). To investigate the dynamics of whole transcriptional machinery on noncompeting DNAs, we performed a ChIP (22) experiment to evaluate the enrichment of Pol-II preinitiation and elongation complexes (23). We used antibodies specific to posttranslational modifications of the Pol-II C-terminal domain (CTD) (24) important in transcriptional initiation (Ser5 phosphorylation) (25) and transcriptional elongation (Ser2 phosphorylation). ChIP-qPCR experiments were performed on the Ebox and gene body (GB) regions of DNA-FF and DNA-Ren to investigate whether the second injected DNA (DNA-Ren) was deprived of factors required for transcriptional initiation or elongation (Fig. 4). When DNA-FF or DNA-Ren were injected alone, similar level of initiation (CTD-Ser5) and elongation (CTD-Ser2) were detected on Ebox sequence and GB (Fig. 4*B* and *C*), suggesting that the plasmids were transcriptionally active. When DNA-FF and DNA-Ren were coinjected, both injected DNAs again exhibit hallmarks of transcriptional activity, although

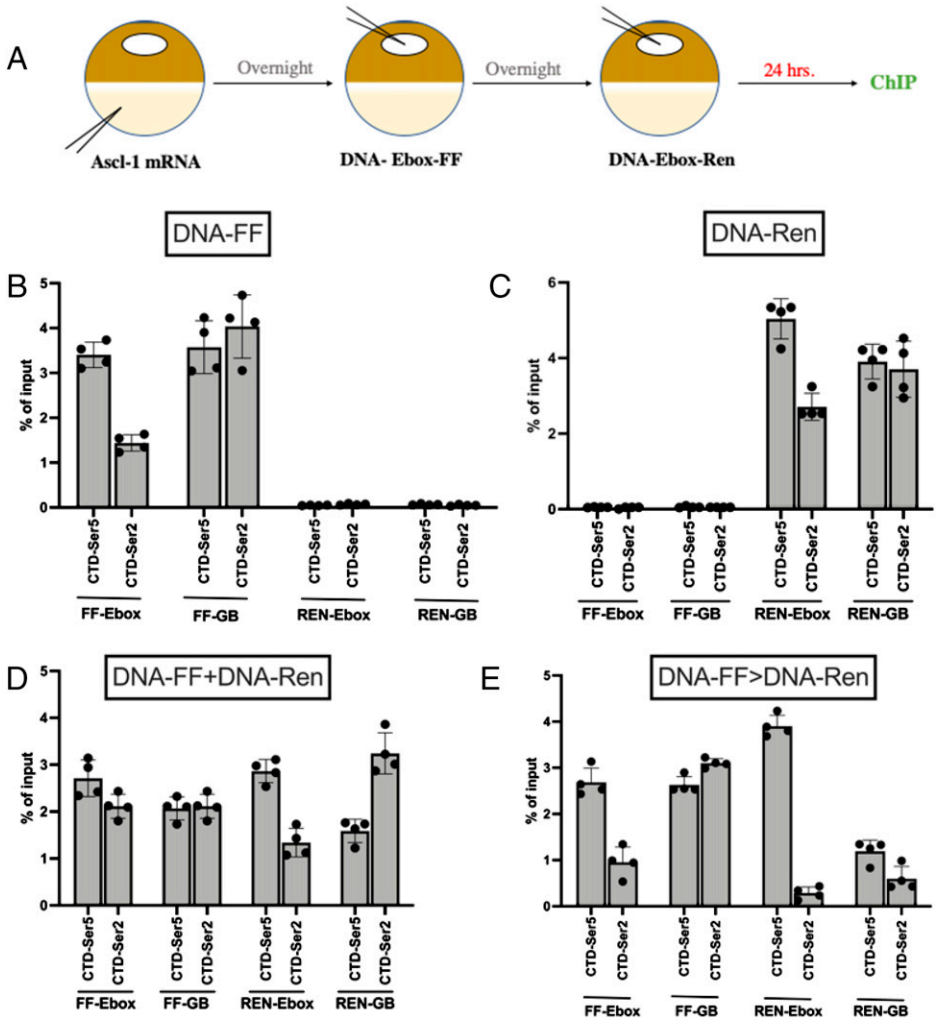

Fig. 4. Failure to transition from paused to elongating Pol-II characterizes the silent DNA template. (*A*) Schematic of DNA competition ChIP. ChIP was carried out using antibodies specific to Pol-II CTD phosphorylated on Ser2 or Ser5. qPCR assay was then used to assess ChIP signal on the EBOX (FF-Ebox) and *Firefly* gene body (FF-GB) of DNA-FF as well as EBOX (REN-Ebox) and *Renilla* gene body (REN-GB) of DNA-Ren. Results are shown as % of input signal for DNA-FF–only injection (*B*), DNA-Ren–only injection (*C*), DNA-FF and DNA-Ren coinjection (*D*), and DNA-FF followed 1 d later by DNA-Ren injection (*E*). Error bars show SD of $n = 4$ experiments with each experimental condition containing eight or more oocytes.

the strength of Pol-II Ser2 and Ser5 on each plasmid decreased (possibly due to distribution of transcription factors among two templates) (Fig. 4*D*). However, when DNA-FF and DNA-Ren were sequentially injected, while DNA-Ren had abundant CTD-Ser5 initiation complexes, the elongation complex (CTD-Ser2) was only seen on the GB of DNA-FF and was missing on that of DNA-Ren (Fig. 4*E*). Collectively, these results suggest that the major limiting factor(s) which contributes to the phenotype of second DNA underexpression could be one which regulates the transition of paused Pol-II to the elongation complex. Under competition ChIP conditions, we observed an abundance of CDK9 on the first injected plasmid (DNA-FF) both on the transcriptional start site (TSS) as well as on the DNA-FF reporter GB (*SI Appendix*, Fig. S4). Strikingly, CDK9 enrichment was extremely low on DNA-Ren, both on the TSS as well as on the DNA-Ren GB. These data, combined with TBPL2 and RNA-Pol-II Ser2 and Ser5 ChIP provide evidence that transcriptional elongation factors are the major responsible limiting factors driving underexpression of the second injected DNA template in *Xenopus* oocytes.

Rescue of Second DNA Expression by Nuclear Content Transfer in Competition State Oocytes. So far, our analysis indicates that, within the same nucleus, transcription factor–bound DNAs can coexist in active and inactive forms. It also suggests that part of the general transcription machinery, possibly factors associated with transition from paused to elongating Pol-II, are titrated away by the active DNA from the inactive competitor DNA template. If that is the case, we reasoned that the provision of such limiting cell cofactors should rescue the expression of the second plasmid. To that end, we developed a method to transfer the nuclear content of one oocyte to another that is undergoing DNA competition. Plasmid expression of sequentially injected DNA-FF and DNA-Ren was measured either under control conditions or with additional injection of *Xenopus* oocyte extract. Injecting oocyte factors could rescue DNA-Ren expression by 40%, an effect which was not further enhanced by coinjecting oocyte factors with Ascl-1 (Fig. 5*B*).

To further identify the nature of the oocyte factor(s) that could rescue DNA-Ren expression, injected DNAs were preincubated with oocyte extract (26) from either the cytosolic or GV compartments. When the second injected DNA-Ren was coinjected with oocyte cytoplasmic or GV extracts, the DNA-Ren reporter expression was rescued to approximately fourfold and sevenfold as compared with the unincubated controls, respectively (Fig. 5*C*). This suggested the presence of oocyte-specific cofactors that could be the limiting expression of the

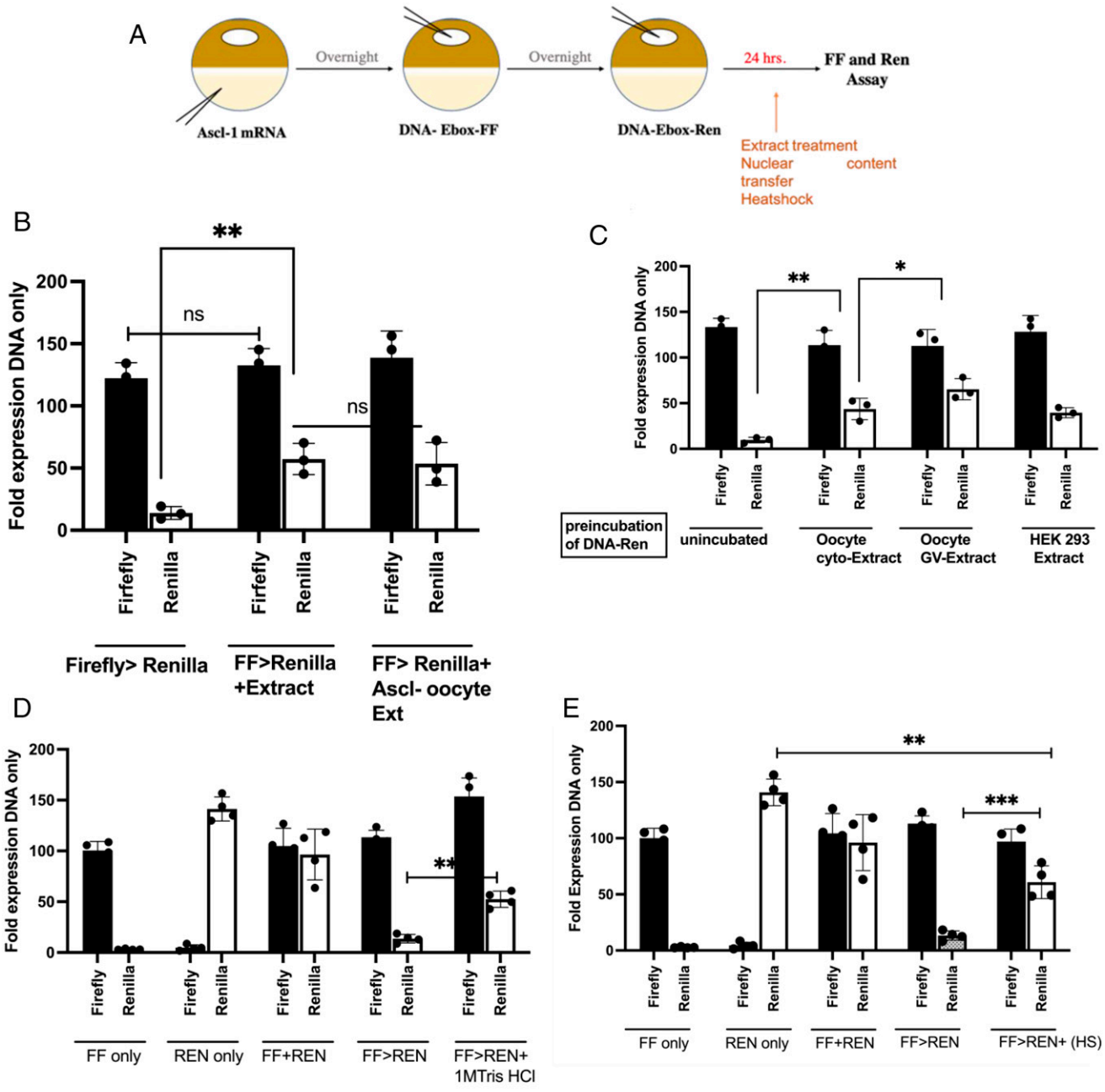

Fig. 5. DNA-Ren expression can be rescued by oocyte factor addition or disruption. (*A*) A general schematic diagram of the rescue experiments. Treatments were as follows: extract injections were 6 h after DNA-Ren injection, Tris injection was coinjected with DNA-Ren (18 nL of 1 M Tris [pH 8.00]), and heat shock (HS) was carried out for 30 min at 37 °C immediately after DNA-Ren injection. DNA was injected directly in the GVs of the oocytes. (*B*) DNA-FF (black) and DNA-Ren (white) reporter values in control situation, when the oocyte was injected with oocyte extract (+extract) or was injected with oocyte extract containing Ascl1 (+Ascl- oocyte extr). All data are from sequential plasmid injections. (*C*) DNA-FF (black) and DNA-Ren (white) reporter values in control situation (unincubated), when the oocyte was injected with oocyte cytoplasm extract (oocyte cyto extract), oocyte nuclear extract (oocyte GV extract), or 293 cells extract (HEK 293 extract). All data are from sequential plasmid injections. (*D*) DNA-FF (black) and DNA-Ren (white) reporter values following single plasmid injection, plasmid coinjection, and sequential plasmid injection with or without Tris injection. (*E*) DNA-FF (black) and DNA-Ren (white) reporter values following single plasmid injection, plasmid coinjection, and sequential plasmid injection with or without HS. Error bars show the SD of $n = 3$ and $n = 4$ experiments with each experimental condition with more than eight individual oocytes. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.05$ (Student *t* test and two-way ANOVA).

second DNA. To investigate whether the rescuing factors were oocyte specific or among the general transcriptional machinery present in other cell types, injected cells were further incubated with HEK293 cell extract. Surprisingly, HEK293 cell extract could rescue DNA-Ren expression by approximately fourfold. Collectively, these results suggest that the major limiting factor explaining the second plasmid underexpression is part of the general transcriptional machinery. This suggests that the dominance of the first injected DNA entails titration away of oocyte factors from competitor DNA.

We next wondered how, within the same nucleus, part of the general transcription machinery is available to a DNA template

while unable to act on another. We considered the possibility that sequentially injected plasmids may form phase-separated compartments similar to those associated with nucleoli and Cajal bodies (27). Such LLPS could trigger differential access to general transcription factors between two DNA templates within the same nucleus. To investigate this hypothesis, we sought to dissolve subnuclear compartments by altering salt concentrations, as previously described by others (28), to test whether this would rescue second DNA expression after sequential injection. When DNA-Ren was coinjected with 1 M Tris (pH 8.00), we observed an increased DNA-Ren expression by 3.5-fold (Fig. 5*D*). This suggests that a transient increase in nuclear salt concentration can redistribute general transcription factors between injected plasmids.

To further explore the partition in subnuclear compartments as a possible mechanism underlying resistance to DNA competition, we investigated whether heat shock could be used as a physiological method to rescue the expression of the second plasmid. Previous studies have indeed shown that the *Xenopus* oocyte responds to heat shock by forming mini Cajal bodies, which are usually part of the B snurposomes (29). Given this redistribution of transcriptional components, we wondered whether heat shock could redistribute transcription factors from DNA-FF to DNA-Ren and rescue the expression of the latter. *Xenopus* oocytes were heat shocked 4 h after injection of the second DNA, and an expression analysis was performed after a further 24 h. Heat shock treatment increased DNA-Ren expression by about fourfold and concurrently decreased DNA-FF expression, suggesting that transcription factors may have been displaced from DNA-FF to DNA-Ren (Fig. 5*E*). These results suggest that transcription machinery components can be redistributed between competing DNA templates under physiological conditions in the *Xenopus* oocytes.

Sequentially Injected DNA Templates in *Xenopus* Oocytes Become Physically Separated. Given that nuclear content transfer can rescue DNA-Ren expression to a considerable extent, we next considered the mechanism underlying the resistance-to-competition state in the first place. One possibility could be the differential location of the injected DNAs. To investigate this, we labeled the DNA-FF and DNA-Ren templates with Cy3 and Cy5 (30) dyes in a manner such that both of the plasmids retained their transcriptional activity (Fig. 6*B*), albeit it was reduced to ∼80% compared to wild-type DNA. We performed a DNA competition experiment to confirm that these labeled plasmids show the same resistance-to-competition effect as their unlabeled counterparts (Fig. 6*C*). Next, we asked about the localization of DNA-FF Cy3 and DNA-Ren Cy5 in the GV of the oocytes after they had been allowed to compete for 24 h. Fig. 6*D* shows confocal images of *Xenopus* GVs isolated into mineral oil under physiological conditions (31). By injecting the mRNA encoding RNA Pol-II ser2 mintbody (32), we confirmed the DNA aggregates are the sites of transcriptional assemblies (*SI Appendix*, Fig. S5). Coinjected DNA templates in the oocytes were physically colocalized (Fig. 6*D*, column 1); however, sequential injection of the DNAs led to physical separation (Fig. 6*D*, column 2). We estimated the correlation between the fluorescence intensities of DNA-FF and DNA-Ren at each spatial point along the x, y and z axes using the Pearson coefficient and created a Fiji-plugin called ESCop. When DNA-FF and DNA-Ren were coinjected, their correlation when offset by up to 10 μm along all axes remained above 0.8, implying strong spatial colocalization of large homogeneous regions (Fig. 6*E*). However, when the two templates were sequentially injected, their correlation with no offset was around 0.6 (and dropped to 0.3 with 2 μm offset), suggesting a slightly weaker colocalization of much smaller regions and therefore a greater degree of physical separation overall (Fig. 6*F*).

Previous work has shown that *Xenopus* oocytes can form physically separated compartments, such as the RNA Pol-III–related factors in the nucleolus (33). Upon addition of cytochalasin D, these compartments can be pulled down by gravity and fuse to form a giant nucleolus (34), confirming their liquid nature. We speculated that the administration of cytochalasin D may fuse the structures formed by DNA-FF and DNA-Ren in the oocytes. Incubation with cytochalasin D (Fig. 6*D*, columns 3 and 4) indeed results in an increase of the size of the structures containing the labeled plasmids (compare columns 1 and 2 to columns 3 and 4) and confirming their liquid characteristics. However, surprisingly, despite the fusion induced by cytochalasin D, DNA-FF and DNA-Ren fluorescence signals remained separated in the sequential injection scheme (Fig. 6*D*, column 4, *Bottom*). Additionally, GV injection with compounds targeting LLPS such as hexanediol (35) and 30 mM ATP (33) dissolve the plasmid compartments further suggesting that they correspond to phase-separated condensates.

These observations suggest that injected DNAs enter/assemble subnuclear compartments with LLPS characteristics. It also indicates that the active and inactive templates are associated with subnuclear compartments different in nature, as shown by their inability to fuse upon cytochalasin D treatment.

Discussion

The *Xenopus* oocyte is an ideal system to dissect mechanisms governing nuclear reprogramming (36), translation (37), and the dynamics of transcriptional complexes. We and others have previously observed that *Xenopus* oocytes exhibit an unusual property of resistance to DNA competition. Various models have been suggested to explain this phenomenon, including limiting the quantity of DNA, tight binding of transcriptional machinery (34), and limitation of unknown factors (38). However, conclusions from previous work have been limited by use of constitutive promoters, which are unable to achieve experimental control of injected plasmids. To address this, we previously developed a transcription factor–mediated inducible expression system. Here, we used this assay to show that there is turnover of a pioneer transcription factor between DNA and that resistance to expression from an injected plasmid is mediated by phase-separated condensates that limit access to the components of the of transcription machinery.

Our data suggest that the injected DNA plasmids in the *Xenopus* oocytes compete for free endogenous general transcription factors. Our data with Ascl-1 and TBP2 ChIP suggest that resistance to DNA competition is not caused by the long dwell time of either a pioneer or a transcription preinitiation complex in the nondividing *Xenopus* oocyte. This is consistent with binding kinetic studies that support very short dwell times of transcription factors including MyoD (39) and the glucocorticoid receptor (40) on their DNA binding sites, on the order of seconds to minutes. To determine whether resistance to competition is mediated by a limited pool of transcription factors, we tested whether transcription could be rescued by the injection of *Xenopus* GV extract. By performing a variety of rescue experiments, including *Xenopus* GV extract, *Xenopus* oocyte cytoplasmic extract, and nuclear extract of human cells, we have demonstrated that the resistance to competition is indeed a consequence of a limitation of transcription factor(s). By investigating how transcription factor(s) can be differentially available to two sequentially injected DNA templates, we propose a model of regulated spatial access in the *Xenopus* oocyte. Through direct observation of injected DNA in the GV of the oocytes, it was revealed that this resistance to competition is associated with the ability of DNAs to form LLPS condensates. It was further shown that DNAs that were injected into the same GV within 30 min of each other localize in distinct

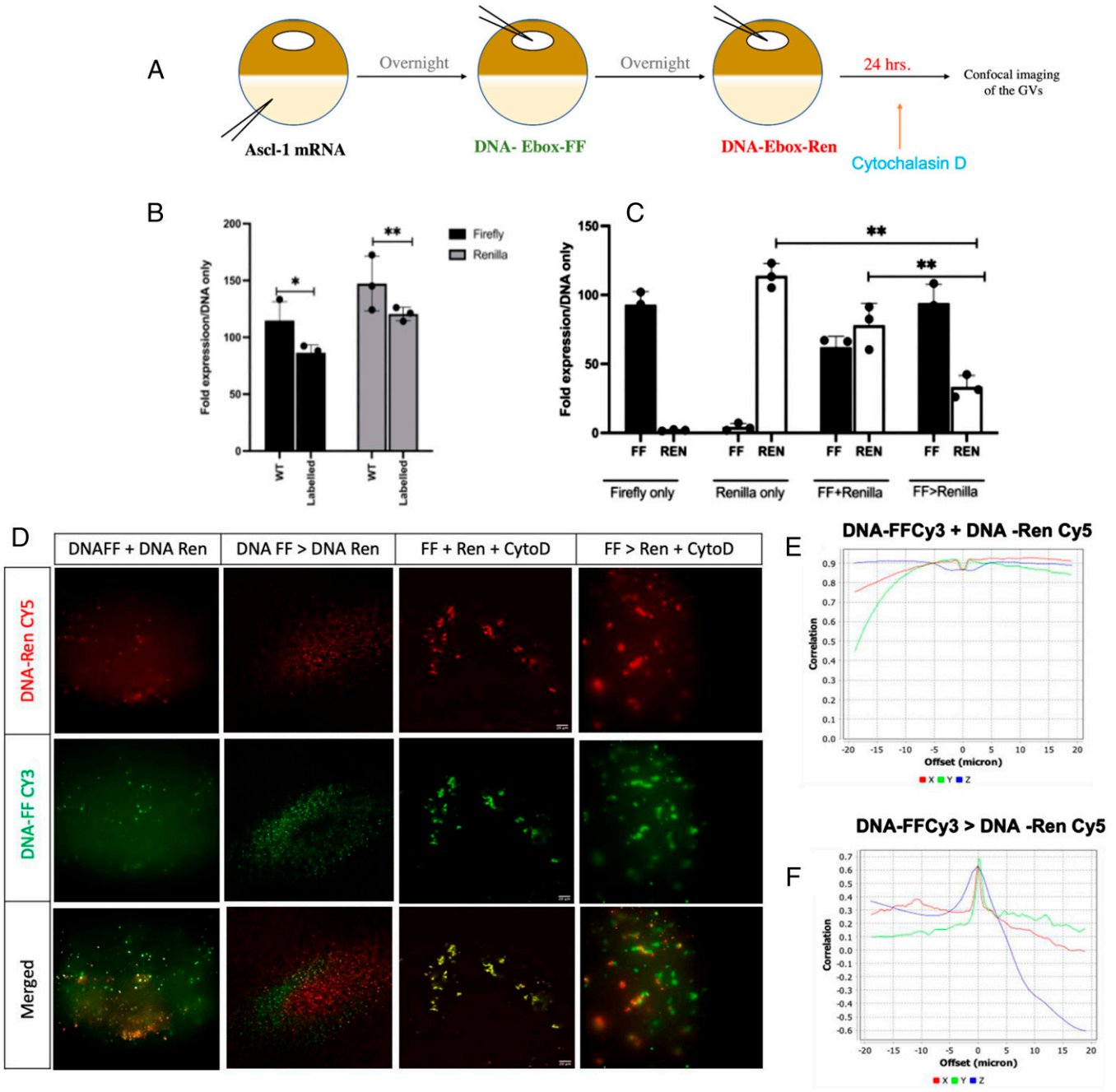

Fig. 6. Sequentially injected DNA is associated with distinct phase-separated condensates in *Xenopus* oocytes. (*A*) Schematic diagram showing the injection of DNA-FF and DNA-Ren into the GV of the oocytes. Cy3-labeled DNA-FF (DNA-FF Cy3) is injected first on day 1 while Cy5-labeled DNA-Ren (DNA-Ren Cy5) was injected 24 h after. The DNAs were injected on the similar needle prick site to ensure the maximum location control. (*B*) Reporter expression of DNA-FF Cy3 and Cy5-Ren compared to that of their unlabeled counterpart. (*C*) Reporter expression following single plasmid injection, plasmid coinjection, and sequential plasmid injection using Cy3-DNA-FF and Cy5-DNA-Ren. (*D*) Confocal images of DNA-FF CY3 and DNA-Ren Cy5 when coinjected (DNA-FF+DNA-Ren) or sequentially injected (DNA-FF > DNA-Ren) and when coinjected (FF+Ren+CytoD) or sequentially injected (FF > Ren+CytoD). (*E* and *F*) Pearson correlation between Cy3 and Cy5 signals across z-stack images from coinjected (*E*) or sequentially injected plasmids (*F*). Pearson coefficient of 1 means absolute colocalization of the signals, and at a higher value, i.e., close to 1 means, the signals are more likely to come from same area. The analysis was performed in $n = 6$ samples, and representatives are shown from each condition. Cross-correlation based on Van Steensel's method values of the Pearson coefficient range from -1, indicating complete inverse correlation, to 1, indicating complete correlation of Cy3 and Cy5 signal. A higher correlation demonstrates colocalization of signal in regions with sizes shown by the offset required for the correlation to drop to a lower level. Error bars show the SD of $n = 3$ experiment, with each containing more than eight oocytes. For the confocal experiment, the images represent $n = 9$ per condition $^*P < 0.05$, $^{**}P < 0.05$.

condensates. The liquid nature of DNA aggregates was confirmed by the addition of cytochalasin D, which induced the formation of larger structures. Our finding regarding DNA–protein complexes confirms the previous literature (41–43) as well as adds an important insight by explaining the possible mechanism of their operation in a nondividing germ cell. We show that these LLPS assemblies can act as a physical barrier between active and inactive transcriptional sites in the same cell. By overexpressing some general transcription factors like TBP2 and TBP, we have shown that this phenomenon is not

dependent on a single factor but may consist of multiple factors that enable the transition of Pol-II into its elongating form.

Altogether, our combination of DNA competition assay, transcription factor ChIP, and confocal imaging have indicated that the nondividing cell can stabilize its gene expression by physically entrapping the transcription machinery of the cell in certain loci. It will be interesting to evaluate if this mechanism applies to other cells where a limited number of Pol-II factories is associated with expression of more than 10,000 genes (44).

Despite presenting a model for transcriptional regulation in the *Xenopus* oocyte, we have not yet identified a single factor which can explain the resistance to DNA competition in the GV by the formation of phase-separated complexes. We are now designing proteomics tools such as proximity labeling (45) and super-resolution imaging techniques to better delineate the composition of these complexes in the oocytes.

Data Availability. All study data and EScoP code are included in the article and/or *SI Appendix*.

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