

Dynamics of Germ Cell Specific Stable Transcriptional Complexes in Xenopus Oocytes



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Preface

The Work described in this Thesis has been conducted at the Gurdon Institute, University of Cambridge, Cambridge United Kingdom, under the Supervision of Prof. John B Gurdon, Dr Jerome Jullien and Dr Torsten Krude.

I certify that, this dissertation is a result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text.

The Work described here is novel and has not been submitted to any other University

Khayam Javed

Statement of Length

This Thesis does not exceed the prescribed word limit (i.e., it is below 60,000 words) excluding bibliography, figures and Appendices.

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Summary

Early animal development depends largely on the activation/expression of genes, and this often depends on the binding of transcription factors to genomic DNA sequences on which they act. This is particularly true of genes that determine cell fate; for example, *Asc1-1* leads to neural differentiation and *MyoD* for muscle. In early development, different genes need to be activated in sequence by appropriate transcription factors. It is commonly believed that a transcription factor has only a very short dwell time of seconds or minutes on its appropriate DNA sequence. On the other hand, differentiated cell strains retain their differentiated state for many years. Does this mean that a transcription factor molecule must be exchanged for another molecule of the same kind, or could it be that a transcription factor dwell time might be significantly prolonged in stably differentiated cells, as are most non-dividing adult cell types?

I have investigated the dwell time of the neurogenic transcription factor *Asc1-1* by injecting mRNAs or DNAs into the non-dividing oocyte of *Xenopus*. Transcription factor proteins can be introduced by mRNA injection and DNA sequences to which they bind, when required; induced gene expression is monitored by expression of fluorescent reporter genes.

I have used competition experiments with differently marked transcription factor proteins and DNA binding sequences to measure dwell time in living oocytes. I find that the dwell time of the factor proteins on the DNA or chromatin to which they specifically bind is much longer than is generally believed. This unexpected stability may contribute to the strength of cell differentiation in normal development. Several aspects of the regulation of transcription in non-dividing cells have been studied. *Xenopus* oocyte can form stable transcriptional complexes on an injected DNA template. Once formed, these transcriptional complexes stay stable for several rounds of transcription, which can last for days without letting any other DNA compete for the mRNA expression. The nature of these complexes has been thought to be tightly bound to their template DNA. However, a new mechanism governing this phenomenon of stabilised transcription in *Xenopus* oocyte has been proposed. According to this phenomenon, stabilised mRNA expression by a DNA template in *Xenopus* oocyte is established by local transcription factors' entrapment. RNA polymerase II factors have been shown to enable the formation of stabilised nuclear compartments. This finding proposes a new way of how the non-dividing cells can maintain their gene expression for a prolonged time.

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Introduction:

During embryonic development, cells divide and acquire the specialised functions which are essential for embryonic patterning (Edgar & O'Farrell, 1989) and for the survival of an organism (Burrue, Klooster, Barker, Pera, & Meyers, 2014). The earliest cell fate decision happens at the mammalian late blastula stage when the cells (Mihajlović & Bruce, 2017) divide into three groups, namely Trophectoderm, Inner Cell Mass (ICM), and Epiblast tissue. Each of these special cell types has a distinct role in development, such as the formation of extra-embryonic tissue in the case of trophectoderm (Bratt-Leal et al., 2009), while the specification of all other body cell types arises from the (ICM). The process of lineage specification is governed by complex interactions between chromatin-associated proteins, i.e., transcription factors, chromatin modifiers, and long-distance DNA interactions themselves. Morphogen gradients, cell signalling, and position of cells in embryonic tissue are other vital determinants of cell fate. During the course of mammalian development, ICM goes through a series of divisions to acquire specialised cell states like neurons and cardiomyocytes. The process of cell specialisation is divided into two stages, each one of which has its special role (Bergsmedh, Donohoe, Hughes, & Hadjantonakis, 2011) in development. First, embryonic stem cells with a diverse developmental potential differentiate into multiple or unipotent cell state, which is an organ's reserve in response to an injury (Esrefoglu, 2013) or infection. An example of this process is the differentiation of mesenchymal stem cells to replenish the loss of hepatocytes in response to an injury (Sarugaser, Hanoun, Keating, Stanford, & Davies, 2009). To do so, cells with more developmental potential undergo terminal differentiation and turn themselves into specialised cells for the rest of their life. Terminal differentiation is governed by the additional changes in chromatin architecture (Larson & Yuan, 2012), cell cycle exit,(Buttitta & Edgar, 2007) exchange of General Transcription Factors (GTFs) as well as recruitment of distinct

chromatin modifier complexes. Often, the cells which undergo specialization do not re-enter the proliferative state again in their life span (Buttitta & Edgar, 2007). However, in some cases, cells can reactivate their proliferative state e.g., expression of E1A in skeletal muscle cells reactivate the cell cycle and suppresses the tissue-specific genes (Tiainen et al., 1996).

However, Technologies like Somatic Cell Nuclear Transfer (SCNT) (Fischberg, Gurdon, & Elsdale, 1958), (Wilmut, Bai, & Taylor, 2015), Cell fusion and over-expression of four transcription factors Oct3/4, Sox2, Klf4 and c-Myc (OSKM) (Takahashi & Yamanaka, 2006) have enabled the conversion of mature cells into pluripotent or to a totipotent stage. Similarly, Trans-differentiation causes somatic cell interconversion, e.g., Mouse Embryonic Fibroblasts (MEFs) can be converted into muscle cell fate by over-expression of a Basic Helix loop Helix transcription factor (bHLH) MyoD (Bichsel et al., 2013). Interestingly, over-expression of MyoD mRNA into *Xenopus laevis* embryos led to the conversion of ectoderm cells to muscles like cells for a defined period (Hopwood, Pluck, & Gurdon, 1989). Another example of trans-differentiation is the Ascl-1 mediated conversion of MEFs into induced Neurons (iN) within 22 days (Wapinski et al., 2013a). However, the efficiency of reprogramming by these methods is relatively low <5% (Takahashi & Yamanaka, 2006). (Whitworth & Prather, 2010) describe that SCNT efficiency decreases as the donor nucleus is taken from more mature cells. Furthermore, a similar trend has been observed in iPSCs conversion when immature B cell and Mature B cells were converted into a pluripotent state (Hanna et al., 2008). The conversion of mature B cells to iPSCs needs an additional step of either expression of EBP α or knockdown of Pax5 (Hanna et al., 2008). Similarly, the induction of cardiomyocytes from MEF cells by overexpression of Gata4, Mef2c, and TBX5 yields cells with a limited activation of the cardiac program upon transplantation to mouse heart (Chen et al., 2012). This reflects that, as cells mature, they acquire some resistance to be reprogrammed by the ectopic expression of transcription factors. A mechanistic understanding of resistance

to reprogramming and stable cell fate requires a deep understanding of the mechanism of reprogramming and that of terminal differentiation.

1.1.Mechanism of reprogramming and cell fate change

A complete definition of cellular reprogramming consists of multiple criteria, e.g., first, the activation of genes of desired cell types such as Nanog (Ling, Chen, Wang, & Zhang, 2012) in induced pluripotent stem cells and Tuj1(Menezes & Luskin, 1994) in transdifferentiated neurons. Second, inhibition or inactivation of host cell characteristic genes. Third, relative gene expression and functionality compared to *in vivo* cells or cells in developing embryos. The following are the hallmarks of the mechanism of nuclear reprogramming both by nuclear transplantation and iPSCs.

1.2.Decondensation of the genome

As a cell progresses towards the terminal fate, its plasticity decreases by permanent shut-down of transcription of irrelevant genes, sometimes even the cell cycle genes. The region of DNA which contains mostly turned off genes is enriched in histone modification, called H3K9me3. The presence of this modification leads to the compaction of the chromatin (Arya & Schlick, 2009), which becomes inaccessible to local transcription factors. The success of reprogramming depends on the decompaction of chromatin (Apostolou & Hochedlinger, 2013) and activation of a new genetic program which has been dictated by the cell fate specific transcription factors. An example of such a process is the shutting down of fibroblast specific genes and induction of neural cell fate by expressing neural markers under the transcriptional program of Ascl-1(Wapinski et al., 2013a). The other example of chromatin decompaction and its impact on the success of Somatic Cell Nuclear transfer (SCNT) is presented as the nuclei taken from earlier stages of development, such as blastula, transferred to an amphibian egg support the development of swimming tadpoles, among those 32% of the embryos reached

adulthood (Gurdon, 1962). However, using the cells taken from skin, a similar potential of adulthood is negligible. Most of the embryos made from skin cell nuclei do not reach the tadpoles stage, hence adulthood (Gurdon, Laskey, & Reeves, 1975). This suggests that the repression of irrelevant genes during the course of development might be governed by the deposition of Histone marks on DNA (H3K4me9), which results in the compaction of the chromatin (Chandra et al., 2012). Surprisingly, when the cells from the skin are injected into the egg followed by another cycle of transplantation, about 43% (Gurdon et al., 1975) of those embryos gave rise to swimming tadpoles. This implies that the serial transplantation of the same nuclei can be more decondensed and then become more permissive to the process of reprogramming.

1.3.Reconstitution of chromatin marks:

The division of cells during development restricts their fate towards a specialized function. This restriction comes from the accessibility of chromatin (Popova, Claxton, Lukasova, Bird, & Grigoryev, 2006) for the local transcription factors. Modification of Histone H3 variants and certain DNA modifications such as CpG islands (Jeziorska et al., 2017) near the promoter/enhancer regions are crucial for regulating the gene expression. For a cell to undergo an efficient nuclear reprogramming, most, if not all, of chromatin marks need to be reversed and incorporated as commanded by the transcriptional regulators. Inefficient reversal of chromatin and DNA marks results in abnormal embryos. One example is the overexpression of a muscle-specific transcription factor MyoD in endoderm cells where it would not normally express. The overexpression of MyoD resulted in transient induction of muscle cells in the *Xenopus* ectoderm cells. The degradation of mRNA encoding for MyoD caused the reversal of the generated muscle cells back to ectoderm stage (Hopwood et al., 1989). Similarly, nuclear transfer in mouse (nuclei were taken from female Donor) an aberrant reprogramming of inactive X chromosome resulted in the expression of *Xist* in differentiating epiblast cells (Nolen

et al., 2005). Aberrance in epigenetic reprogramming can arise during the process of reprogramming itself e.g., inactivation of *Igf2* locus during the formation of iPSCs (Stattfeld et al., 2010) leads to the iPSCs colonies with limited developmental potential.

Similarly, in trans-differentiation of Normal Human Dermal Fibroblasts (NHDF), and Normal Human Epidermal keratinocytes (NHEK), the pioneer transcription factor *Ascl-1* can induce neurons from NHDF but not NHEK. Chromatin analysis reveals a primed permissive tri- histone H3 mark (H3K4me3, H3K27Ac, and H3K4me9) (Wapinski et al., 2013a) which exists in NHDF but not in NHEK. The relevance of this chromatin mark has been considered important for the induction of the cells toward neural lineage. Furthermore, the presence of trivalent and bivalent chromatin marks in the cells are linked to the low-level methylation, which results in the low level gene activation (Voigt et al., 2013.). Most of the genes under the control of bivalent signatures are developmentally regulated and activated upon induction by a specific signal or inducer. The examples given above show that reconstitution of chromatin marks is an important indicator of success of reprogramming. Additionally, in some cases presence of specific chromatin marks is a determinant of the outcome of reprogramming.

1.4. Role of Co-factors and chromatin architecture during reprogramming

The other important determinant of successful reprogramming is the pool of host-cell co-factors. Co-factors interaction with a pioneer transcription factor or with a lineage specifying transcription factor (Aksoy et al., 2013) can alter its binding on chromatin and on the RNA in the case of *Sox2* (Z. Yang, Augustin, Hu, & Jiang, 2015). For instance, the binding of *FoxA2* and *Gata4* readily changes when it is expressed +/- *Oct4* in multiple cell types. This could be a result of host cell co-factors, which, through protein-protein interaction, change the *FOXA2* interaction with the chromatin binding site (Donaghey et al., 2018). Similarly, the co-operative binding of transcription factors OSKM leads to a global switch-on of the transcription factors that activate the pluripotency genes in the MEFs. Mechanistically, *Oct4* and *Sox2* interact

together at the enhancer regions of pluripotency genes; their binding then results in the recruitment of Hdac1, which in return, turns off the host cell-specific genes and promotes the pluripotent program (Chronis et al., 2017). Generally, the interaction of a transcription factor with its chromatin binding site recruits general transcription machinery and chromatin modifiers for the regulation of transcription from that particular locus. Similarly, the interaction of pioneer or lineage-specific transcription factors with the host cell-specific co-factors can dramatically change the outcome of a cascade. The analysis shows 50% of the binding sites on ES cell chromatin is shared by MyoD and Ascl-1, as they both bind to a canonical Ebox sequence (CANNTG); this is surprising that within the same co-factor pool and with a similar chromatin architecture, Ascl-1 and MyoD start a very distinctive gene expression program with one leading to neural fate and other to muscle cell fate (Casey, Kollipara, Pozo, & Johnson, 2018). ChIP-Seq, followed by Motif analysis, reveals that the binding of MyoD and Ascl-1 is not because of the interaction or existence of the adjacent co-factor motifs but may arise because of the protein-protein interaction of co-factors. In support of this argument, a recent study shows that swapping the N and C terminal of Ascl-1 with MyoD can change the outcome of cell type generated (Lee et al., 2020). This perhaps occurs because of the local protein-protein interaction of distinct domains present on both Ascl-1 and MyoD.

The above mentioned studies lead to the conclusion that changing a cell's fate requires a variety of processes to occur simultaneously in a combinatorial way. Any hindrance in these several intricate pathways results in incomplete reprogramming or a complete failure of cell fate change.

The other vital determinant of successful cellular differentiation is the fact that a cell undergoing nuclear reprogramming acquires a stable fate, which shows the relevance to its target cell's gene expression and to its function. Studies show the earlier process of cell fate change in remarkable detail; however, how a newly acquired cell state is established is not

completely known. In some systems, a detailed study of terminal differentiation has been carried out. Below is the description of some of the work which uncovers the principles of cell fate acquisition and its stability in terminally differentiated cells.

1.5. Mechanism of Terminal differentiation

Cellular reprogramming or differentiation process leads to a stable cell fate for example, Cardiomyocytes, Muscle cells, or Neurons. The success of reprogramming and differentiation in normal development depends on how a stable and functional yield of cell population has been achieved. Similarly, stable or terminally differentiated cells must take strong measures to ensure the maintenance of a differentiated state with a distinct gene expression, which is maintained for years or months to come. The following are the measures that are hallmarks of terminal differentiation.

- i) Global change in General transcription machinery**
- ii) Distinct composition of General transcription factor p-TEFB**
- iii) Change in composition and function of co-factor complexes**

1.6. Global change in General transcription machinery

Acquisition of a new cell fate requires activation of a distinct gene set which creates a network of activities leading a cell to perform a specialized function such as expression of myogenin (Pandurangan et al., 2014) to help muscle contraction and myotube maturation or expression of GFAP (Wislet-Gendebien, Leprince, Moonen, & Rogister, 2003) and Tuj 1 (Ying, Gonzalez-Martinez, Tilelli, Bingaman, & Najm, 2005) genes for the facilitation of electrical pulses in the neurons. Most of these genes are associated with transcription through a RNA-Pol-II transcriptional program. RNA-Pol II is a multi-subunit complex with each subunit made up of multiple proteins (Hieda, Winstanley, Maini, Iborra, & Cook, 2005). The composition of RNA-Pol-II (RNAPII) complexes varies within cell types and

during development. The basic machinery consists of five main subunits (TFIIA, TFIIB, TFIIE, TFIIF, TFIIH) (Deato & Tjian, 2007a) each with a specialised function. During terminal differentiation, a cell loses an essential component of the TFIID complex (TATA-binding protein) along with other TATA-binding protein-associated factors such as TAF1, TAF10, TAF9, and TAF4), which eventually cause the extinction of the whole TFIID complex (Deato & Tjian, 2007a). This extinction is rescued by the expression of other transcription factors such as TRF3 and TAF3 (Hart, Santra, Raha, & Green, 2009). This vital change in global transcription machinery could be a determinant step in the terminal differentiation, as it may result in the permanent activation or repression of selective genes associated with specialised functions.

1.7. Pol-II elongation complex interaction differs in the terminally differentiated cells

Generally, RNA-Pol II assembles a preinitiation transcription complex on the chromatin sites in the cells (Luse, 2013). This initiation complex is stable but stops the transcription by Negative Elongation Factor (NELF) few bases (Aida et al., 2006). PTEF-B (Positive Transcription Elongation Factor) is another complex that then phosphorylates the CTD domain of RNA -Pol II at the Ser2 residue, which results in the productive elongation of a gene (Price, 2000). PTEFB consists of several subunits. One of these units is a phosphorylase CDK9 (Zhou & Yik, 2006). CDK9 normally interacts with another general transcription factor, CycT1, to perform its function.

Analysis of PTEFB regulation in differentiating myoblast into myotubes shows the fundamental change in the interaction of PTEFB. Instead of interaction with CycT1, PTEF-B interacts with CycT2 (Simone et al., 2002), this change is caused by the selective degradation of CycT1 degradation in the myotubes by the expression of E3 ubiquitin ligase. Similarly, during terminal differentiation of granulocytes into macrophages, PTEF-B

interaction changes from CycT1 to CycT2, with later being unable to be degraded by the PEST domain (Liou, Haaland, Herrmann, & Rice, 2006).

The expression of CycT1 is mainly a characteristic of undifferentiated and rapidly dividing cells, while the regulation of terminally differentiated cells largely depends on the CycT2. This again shows the distinct dynamics of terminally differentiated cells. It is also interesting to know that this particular interaction of PTEFB is very peculiar to the transcription factor domains such as basic Helix domain of MyoD (De Luca, De Falco, Baldi, & Paggi, 2003) as opposed to more general transcriptional activators like Vp16.

1.8. Role of transcription factors in maintaining the differentiated state.

Transcription factors are broadly divided into many categories which are selected on the base of either their family such as Basic Helix loop Helix transcription factors Ascl-1 and MyoD (S. Jones, 2004), or they are named on the motifs where they bind such as Sox2 or SRY box and Oct family on the name of Octamer binding proteins (Tantin, 2013). There is also a second order of TF classification, which is purely based on the function of a transcription factor. The main example of such classification is pioneer transcription factors, Ascl-1, MyoD (Iwafuchi-Doi & Zaret, 2014), and Oct4. These factors have the ability to engage with previously closed chromatin states and have the potential to start a gene expression program. Similarly, there are also some transcription factors known as “Master regulators,” which may or may not be pioneer transcription factors but exhibiting an ability to change a cell’s fate to another (Hobert, 2008). The last category to mention is a terminal selector gene; these TFs are involved in the maintenance of cell identity such as CHE-1 in the *C. elegans* neuronal maturation and Ascl-1 in terminally differentiated cortical neurons (Chang, Johnston, & Hobert, 2003).

Acquisition and stability of cell fate or identity results after a combinatorial transcriptional code by different transcription factors. Master regulators start a cascade of transcriptional events, followed by the expression of effector transcription factors. Together, the effector and Master regulator transcription factors create a code which intricates a new cell fate. During this dramatic change in a cell's fate, Master regulators often Auto-regulate their expression (Crews & Pearson, 2009). Some examples in *C.elegans* show how the simple combinatorial TF codes can maintain and acquire cell fate, e.g., expression and heterodimers activity of MEC-3-UNC-86 and TTX3-CEH-10 control and maintain the mechanosensory neurons and AIY neurons in *C.elegans* (Y. Zhang et al., 2002), and Cholinergic motor neurons are controlled by the cis- regulatory signature and continuous expression of a transcription factor UNC-3 (Kratsios, Stolfi, Levine, & Hobert, 2012).

The above-mentioned regulatory dynamics have been studied in non-mammalian species such as *C.elegans*. But a question arise that is there any existence of such code exists in higher mammals? The answer to this question is neither yes or no. A similar terminal selector gene mediated differentiation phenomenon has been seen in the photoreceptors of the mouse retina, where a homeodomain transcription factor CRX (Corbo et al., 2010) is controlling the expression of a variety of genes. On the contrary, most of the mammalian combinatorial codes are different from the ones in *C.elegans* e.g. AST-1 expression is associated with expression of genetic program leading to the specialization into the dopaminergic neurons; however, AST-1 (Flames & Hobert, 2009) mammalian counterpart ETV1 is not associated with dopaminergic neurons. It is often observed that mammalian combinatorial codes depend on many terminal selector genes regulating different effector genes to maintain a specialised state (S. Wang & Turner, 2010).

Surprisingly, in some cells the cellular identity is guarded by a single or a combination of transcription factors. Ablation of these genes have caused the reversal of cells fate to

progenitor or sometime to another fate. For example, ablation of Pax5 changes the B cell identity into B cell Progenitor cells (McManus et al., 2011), which can be converted into T cells and macrophages. Similarly, the ablation of Fork-head 12 (Foxl2) transcription factor in adult mouse ovary induces a rapid cell fate change from granulocyte to the Sertoli cells (Schmidt et al., 2004). Weeks after ablation of Foxl2 a complete physical structure like testis has been seen with a testosterone production (Uda et al., 2004) in the females. Antagonistically, ablation of a transcription factor Dmrt1 in the Sertoli cells, convert them into Granulosa Ovary cells (Masuyama et al., 2012).

Most of these single gene ablations associated with cell fate change phenotypes have been seen in the cells with more genomic plasticity, such as B cells and T cells. However, this is not that simple in some complex cell types like liver cells. The ablation of terminal selector gene in liver cells, (HNF4 α) the early stages of differentiation, disrupts the hepatocytes transcriptional program (Hayhurst, Lee, Lambert, Ward, & Gonzalez, 2001). While, the late ablation of HNF4 α causes alteration in gene expression but does not change the cell's fate. In the similar context the ablation of Lmx1b (Song et al., 2011) in the terminally differentiated neurons is associated with altered gene expression without a profound effect on cell's fate and morphology.

Through the analysis of literature, it is evident that the transcription factors play a very essential role in guarding the cell's fate after playing a crucial role in its acquisition. However, it is still very unclear that how these transcription factors can maintain the expression of their target genes for several decades. One hypothesis is that these transcription factors bind very tightly to their chromatin sites while staying there for hours or days. The second hypothesis is that, the general transcription machinery in terminally differentiated cells or the co-factor pool plays an essential role in the stabilization of transcription factors. There is no evidence that the long dwell time of a transcription factor showing to have a profound effect in cell's identity.

But the long dwell time has been associated with the acquisition of a cell fate. Sox2 prolonged binding in 4 cell mouse embryos is associated with ICM specification (White et al., 2016). By using FRAP a long dwell time of Sox2 has been recorded as 200 milliseconds. Contrarily, RAP1 competition ChIP in the yeast cells shows an occupancy time of 90 minutes (Lickwar, Mueller, Hanlon, McNally, & Lieb, 2012a), and TBP competition ChIP associated with different promoters (RNAP-I II and III) shows a variable promoter specific dwell time. For instance, TBP stay on the promoter binding site for more than 90 minutes when associated with RNAP-III promoter as opposed to 30 minutes on RNAP-II promoters in HEK 293T cells (Hasegawa & Struhl, 2019a).

These finding suggest a new mode of transcription factor regulation in different cells types. Perhaps, the prolonged binding of these transcription factors is a requirement for a robust transcription that may lead to an alternative gene expression program resulting in cell fate change or its stabilization. One such example is the control of dual cell fate by Ascl-1. In the mouse Neural Stem (NS) cells an oscillatory expression of Ascl-1 leads to renewal of stem cell fate, while sustained expression pattern of Ascl-1 leads to terminal differentiation of NS to cortical Neurons (Imayoshi et al., 2013).

1.9. Formulation of the problem:

Regulation of transcription in the terminally differentiated cells with respect to the role of a transcription factor binding dynamics is elusive. Many of the combinatorial codes which guard mammalian cell identity are unknown. Moreover, very little is known about the dynamics such as dwell time of transcription factor on multiple binding sites, their co-factor interaction, and chromatin permissiveness. Understanding of these mechanisms could be of considerable biological interest. However, due to the division limitation, most of these investigations are difficult to perform in terminally differentiated cells. Furthermore, assays like ChIP-seq provide the information of fixed cells, where the proteins are cross-linked by

Formaldehyde and thus do not provide dynamics of protein complexes or of their co-factor interactions. Another problem in studying the behaviour of transcription factors is the limiting amount of co-factor molecules because of the limited division potential of these cells. Without considering the transcription factor complexes, it is harder to elucidate a mechanistic model of transcription factor action in terminally differentiated cells.

To address these problems, we aim to use *Xenopus* oocyte, because of its greater size and easy availability. *Xenopus* oocyte is a non-dividing cell which is arrested at the first meiotic prophase (Tokmakov et al., 2014), until it is activated by the progesterone (Huchon, Crozet, Cantenot, & Ozon, 1981). The *Xenopus* oocyte maintains its transcription for several days in culture and thus provides a great alternative to other non-dividing specialised cells for studying the transcriptional regulation.

Xenopus oocyte has been used for decades to study transcriptional regulation in the germ cells. An example of this is the study of regulation of Histone H2B gene (Hinkley & Perry, 1991), study of TATA binding protein TBP2 (Akhtar & Veenstra, 2009a) and its role in the early development of Frog. In the oocyte, the molecules can be injected at a precise location with a desired concentration without disturbing the host cell co-factor pool. This provides an immense advantage over other non-dividing cells like mature neurons and muscle cells. Where over-expression of transcription factors can disturb the nuclear dynamic environment, such as concentration and composition of other co-factor molecules, which in return may not present the actual transcriptional state of the cells. In contrast, over-expression of a transcription factor through mRNA in *Xenopus* oocyte has a negligible effect on the oocyte-specific transcription.

1.10. Studying Ascl-1 binding dynamics in the Xenopus oocytes.

Xenopus oocyte is a non-dividing specialised cell with a diameter >1.3 mm when fully grown. During the oogenesis of *Xenopus*, an oocyte grows a lot from the stage 1-VI, stage VI being the mature oocyte with a high degree of polarity. A typically mature *Xenopus* oocyte has

maternally stored mRNA and proteins (Winata & Korzh, 2018), which are essential for supporting the transcription in the earlier stages of embryogenesis (Sheets et al., 2017). Due to its large size, *Xenopus* oocyte can be manipulated in many ways, such as isolation of nuclei or GV (Germinal Vesicle) and injection of large volumes of molecules such as DNA and mRNA.

In the *Xenopus* oocyte microinjection of several molecules can be introduced in very precise quantity. An example of this is the injection of mRNA encoding for Rabbit globin protein into the oocyte (Brown and Gurdon 1964), and by the *in vivo* expression of an exogenous mRNA by the transcription of a cloned cDNA into *Xenopus* oocyte GV. In addition to microinjection and protein expression, the *Xenopus* oocyte has been used to isolate the transcription factors (Engelke et al., 1980), which underpinned the discovery of many regulatory processes in the cells such as regulation of SnRNA genes (Friend, Kolev, Shu, & Steitz, 2008), (Wilczynska, Git, Argasinska, Belloc, & Standart, 2016) as well as identification of nucleolus as a site for ribosomal RNA transcription (Brown and Gurdon 1964).

A *Xenopus* oocyte can bear about 100 nl of mRNA injected into the cytoplasm and about 23 nl of a solution containing purified DNA or dyes like DAPI can be done. Recently, purified nuclei have also been injected into the oocyte GV (Halley-Stott et al., 2010) to study the regulation of transcription in the process of cellular reprogramming and chromatin modifications (Pasque, Jullien, Miyamoto, Halley-Stott, & Gurdon, 2011a). Most importantly, *Xenopus* oocyte can be induced to go through germinal vesicle breakdown *in-vitro* and transformed into an egg that can be fertilized. By this method of *in-vitro* maturation of an oocyte (Miyamoto, Simpson, & Gurdon, 2015), the effect of several molecules can be studied in development. Recently, a new method called Trim 21 (Clift, So, McEwan, James, & Schuh, 2018) has been used to degrade the maternal transcription factors in the *Xenopus* oocytes (Data unpublished). Endogenous degradation of proteins in the *Xenopus* oocyte can help in studying their role in complex developmental processes.

Another most important quality of *Xenopus* oocyte is the presence of a homogenous pool of transcription and co-factors. Effect of any molecule injected into the oocyte can be studied either on the development after in-vitro maturation or on the directly available chromatin or plasmid DNA injected into the oocyte germinal vesicle. Above mentioned characteristics of *Xenopus* oocyte makes it a specialized cell which is best suited for studying the transcriptional dynamics with respect to transcription factor behaviour on its chromatin binding site. One such transcription factor is *Ascl-1*, which exhibits pioneer transcription factor properties and is well known for its role in neural differentiation and induced neurogenesis.

Ascl-1 is a pioneer transcription factor belongs to the bHLH family of transcription factors. bHLH transcription factors are distributed widely between species from yeast to human and play a very crucial role in the development and sex determination (Bhandari, Sadler-Riggelman, Clement, & Skinner, 2011). Most of the bHLH transcription factors contain a conserved but functionally distinct domain with 60 amino acids; at the N terminal of this domain, a basic amino acid sequence is mainly present, which allows these transcription factors to bind to a consensus Hexanucleotide sequence known as Ebox (Yutzey & Konieczny, 1992). bHLH transcription factors are broadly categorised into VI subclasses which include E proteins, ID proteins, and lineage determination factors such as *Ascl-1* and *MyoD*.

Ascl-1 is a transcription factor with many diverse functions, including the induction of neural cell fate during the development in vertebrates and in mammals (Vasconcelos & Castro, 2014). The deletion of *Ascl-1* gives rise to mice with a compromise in the nervous system, especially in olfactory neurons (Krolewski, Packard, Jang, Wildner, & Schwob, 2012). Further to its developmental activities, *Ascl-1* is also largely involved in the conversion of cell fates e.g. the conversion of MEFs and NHDF into the induced Neurons (iN)(Wapinski et al., 2013). *Ascl-1* alone shows the potential to convert fibroblasts into neurons, which makes its role very special in reprogramming (Chanda et al., 2014). However, the resultant neural cell type

acquisition is dependent on the combinatorial expression of Ascl-1 with other transcription factors such as Sox2, or Neurog. This combinatorial expression can give rise to variable neural lineages (Araújo et al., 2018). The function of Ascl-1 in converting cell fate is also visible in the conversion of mouse Müller glial cells into neurogenic retinal cells by over-expression (Hufnagel et al., 2013).

In addition to Ascl-1 role in development and reprogramming, Ascl-1 also has been a marker and driver of a variety of different carcinomas such as Neuroblastoma (L. Wang et al., 2019), Glioblastoma (Rheinbay et al., 2013) and the most aggressive Small Cell Lung Cancer (SCLC) (Augustyn et al., 2014). The Pioneer transcription factor activity of Ascl-1 is perhaps associated with the global opening of chromatin, which allows other transcription factors to start an aberrant gene expression program, which may lead to the heterogeneity in cancer. Recently, it has been shown that some aggressive cancers like neuroblastoma can be controlled by the ablation of the Ascl-1 activity (Augustyn et al., 2014).

The above mentioned literature illustrates the important roles of Ascl-1 in development and diseases. However, a little is known about its promoter specific regulation. Understanding of Ascl-1's promoter-specific dynamics may open new arenas in therapeutics or increase the knowledge of neural induction in developing embryo.

1.11. This Thesis:

In summary, the regulation of transcription by a master regulator or lineage determination transcription factors in the terminally differentiated cells is not well known. Some lineage determination transcription factors like Ascl-1 are more diverse in their function apart from their ability to change a cell fate. Understanding the mechanism of how they are regulated in different cell systems would be of considerable biological interest. Additionally, regulation of transcription in *Xenopus* oocyte is unique, as the injected DNA into the GV of an

oocyte forms stable transcriptional complexes that remain coherent for several rounds of transcription. Mechanistic insights in stable transcriptional complexes in oocyte can provide an invaluable tool for building a hypothesis on the role of stable transcriptional complexes in the maintenance of cellular identity.

In this thesis, *Xenopus* oocyte has been used as a model system to understand the binding dynamics of Ascl-1. I propose to analyse several parameters of the regulation of Ascl-1 mediated transcription in the *Xenopus* oocyte. This includes promoter specificity for productive transcription, in which I aim to analyse the transcription elongation, which is a result of Ascl-1 binding onto differential binding motifs. Further, I aim to study the dwell time or occupancy time of Ascl-1 on its binding site by several competition experiments. Third, I aim to study the role of *Xenopus* oocyte transcription factors in stabilising gene expression and their effect on the binding dynamics of Ascl-1. I aim to re-analyse the general model of transcriptional regulation in *Xenopus* oocyte.

Significance of this work:

The work proposed in this thesis will allow us to understand the fundamental mechanism of gene regulation in non-dividing cells. This information also contributes to the understanding of how cells maintain their differentiated state for prolonged periods. Another aim of this study is to characterise the transcriptional regulation of a germ cell. A part of this quest is to understand how the molecules in the oocyte support the transcription of the first few cell divisions in the embryo before zygotic genome activation.

Materials and Methods

In this chapter a detailed description of materials has been provided. Additionally, a detailed and revised version of the scientific protocols have been listed for reproducibility of the work.

Materials

2.1.1: Modified Barth solution (MBS) for *Xenopus* oocytes culture at 16-18°C.

Isolated *Xenopus* oocytes were kept in Modified Barth's Solution (MBS) at its 1X concentration. The MBS solution was supplemented with 10µg/ml penicillin 10µg/ml Streptomycin, collectively known as Penstrep before injections (Wlizla, McNamara, & Horb, 2018). The concentration of different components of MBS has been given in table 1.

Components	Concentration (mM)
NaCl	88
KCl	1
CaCl ₂	0.4
Ca (NO ₃) ₂	0.33
MgSO ₄	0.8
TRIS-HCl pH 8.0	5
NaHCO ₃	2.4

Table 2.1: Xenopus oocyte Modified Barth's solution

2.1.2: GV Extract Medium:

Germinal vesicles of the oocytes were isolated and kept in this medium to make an extract with retention of transcriptional activity, it means that the extract made by this procedure is able to transcribe a DNA template when provided. The composition of GV extract buffer is given below in the (Table 2.2)(Lehman & Carroll, 1991).

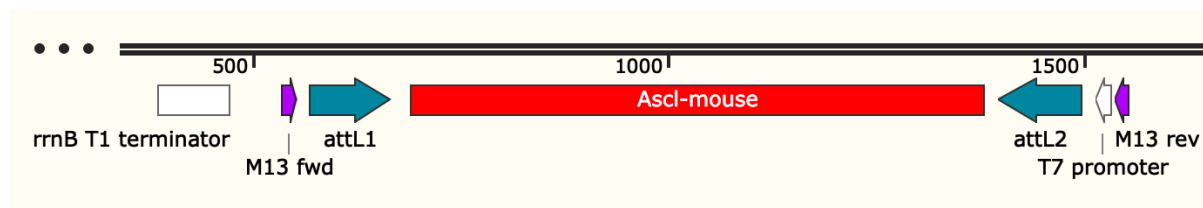
Components	Concentration (mM)
NH ₄ Cl	70
MgCl ₂	7
EDTA	0.1
DTT	2.5
Glycerol	10% (v/v)
HEPES pH 8.0	10

Table 2.2: Composition of *Xenopus* Germinal vesicle extract medium

2.1.3. Plasmids for Ascl-1 production:

Most of the experiments performed in this thesis have used Ascl-1 3HA mRNA to perform cytoplasmic microinjections in the *Xenopus* oocyte. To make the mRNA encoding for Ascl-1 protein, a cDNA sequence of mouse Ascl-1 NM_008553.5 (NCBI) was used to clone in PENTR™/D-TOPO™ plasmid from Invitrogen (Cat: K240020 which was later recombined to a destination vector containing 3X HA tag. Plasmid map of a typical pENTR and final destination vector is given in the Figure 2.1 A and 2.1 B respectively. An SP6 promoter is then used to synthesize mRNA from a linearised DNA template.

A)



B)

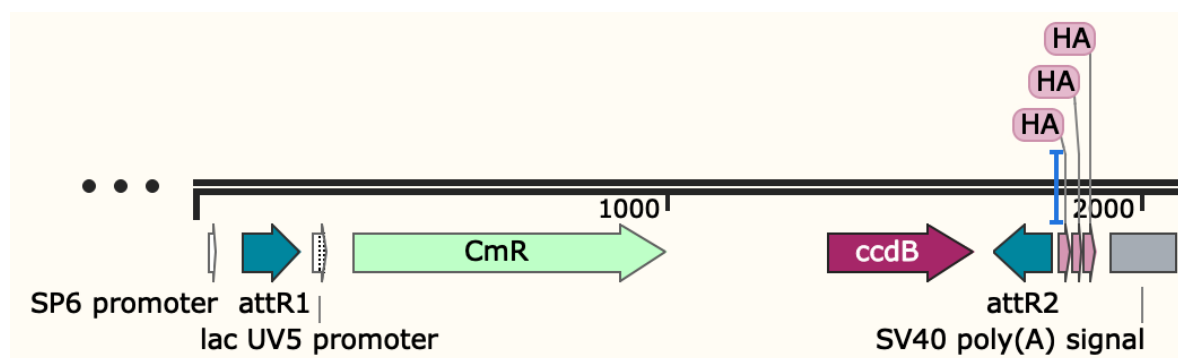
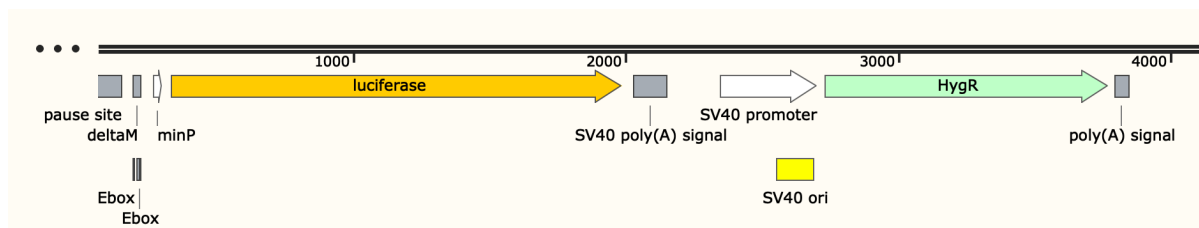


Figure 2.1: Map of plasmid DNAs A) showing the cloned mouse *Ascl-1* in *PENTRTM/D-TOPOTM*. While B) shows the Destination vector with C terminal 3X HA tags. The Maps have been generated by the Snapgene software, the digits written presents the relative position of the *Ascl-1* cDNA and attL1 sites used for the recombination for the generation of multiple tagged versions of *AScl-1* such as *Ascl-1 GFP* or *Ascl-1 myc*.

2.1.4. Reporter plasmid DNAs containing *Ascl-1* binding sequence

For direct analysis of productive transcription, PGL.4.26 plasmid DNA from Promega was used to clone the Ebox-PoU-Ebox sequence from mouse *DLL3* gene. Upon binding to this site, *Ascl-1* starts the transcription of Firefly reporter which is shown in Figure 2.2.

A)



B)

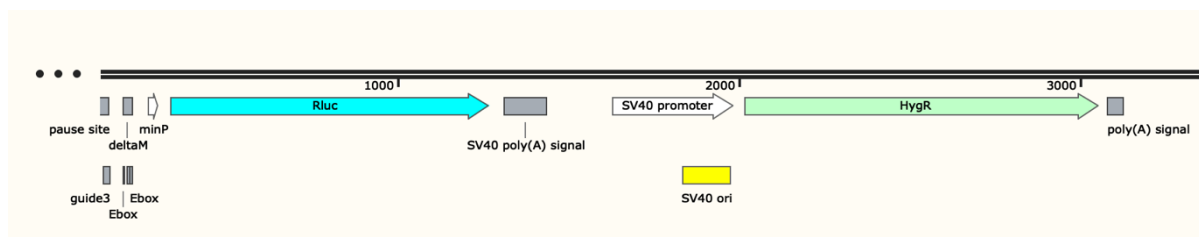


Figure 2.2: Map of reporter plasmid bearing the binding site for Ascl-1. A) Firefly reporter plasmid with Ascl-1 (Delta LL3 gene) binding site cloned upstream. B) Renilla reporter with a binding site for Ascl-1.

These plasmids were given to us by Prof. Anna Philpott, a mutant version of these plasmid has been used to study the effect of Ascl-1 binding. The mutant sequence of the binding site of Ascl-1 has been shown where the red nucleotides are shown to be mutated in Ebox-PoU-Ebox (Figure 2.3).



Figure 2.3: Shows the mutations in Ebox-PoU-Ebox sequence

2.1.5. Cells used for nuclear transfer:

Normal MEFs isolated from mouse embryo were purchased from Cambridge Stem Cell institute. While the cells used for chromatin permissiveness Normal Human Dermal Fibroblasts (NHDF) and Normal Human Epidermal Keratinocytes (NHEK) had been purchased from Lonza biosciences with the catalogue numbers NHDF (CC-2511) which were grown in a commercially available medium from Lonza (Cat: CC-3131). NHEK (Cat: 00192627) were grown in a commercially available medium from Lonza biosciences (Cat: 00192060). MEFs were grown in **DMEM** (Dulbecco's Modified Eagle Medium) Supplemented with 10% FBS and 1% Penstrep.

2.1.6. Cell lysis Buffer

Prior to western blotting, oocytes were lysed in a lysis buffer having the following different components and their final concentration. 50mM Tris HCl, pH 7.4, 150 mM NaCl 1mM PMSF (Enzyme inhibitor), 1mM EDTA, 5µg/ml Aprotinin, 5µg/ml Leupeptin, 1% Triton X-100, 1% Sodium Deoxycholate and 0.1 % SDS.

2.1.7: Streptolysin (SLO)

Streptolysin O (Sigma Aldrich) was prepared by dissolving 25,000 Units in 1.25 ml of PBS. After the complete dissolution of SLO 0.01% BSA, and 62.5 µl of 0.1M DTT was added. After addition of the components the solution was incubated at 37 °C for 2 hours. 25µl aliquots were prepared and immediately stored at -20°C or -70 ° C for further use.

2.1.8 Gels

Agarose and polyacrylamide gels were prepared as described in Sambrook and Russell (2001). Gradient gels were prepared from BioRad with a (Cat: 4561096).

2.1.9. Measuring the concentration of RNA/ DNA and Protein.

The DNA and RNA concentrations were measured by using the NanoDrop ND1000 according to the manufacturer's instructions. Protein competition was determined by using the Bradford assay according to the manufacturer's instructions.

2.2. Methods

2.2.1: Injection of the mRNA into the cytoplasm of the oocytes

A Needle puller was used to pull the needles with a very small opening by using the program number 49 on the needle puller machine. Oocytes were washed twice with 1X MBS before placing them into the dish for microinjections. Purified mRNA dissolved in RNase/DNase free water was filled into the needle and injected into the oocyte at the volume adjusted with the arrangement of the buttons as (UDUU). Concentration of mRNA to be injected is predetermined and varies according to the needs of experiments.

2.2.2: Injection of DNA into the Germinal Vesicle of the oocyte

DNA containing the binding site of Ascl-1 was diluted according to the concentration required for the experiment. First, Oil was injected into the oocyte, by doing this a needle was marked at which the injection is most likely to hit the middle of an oocyte's GV. The volume of the injector is adjusted to desired injection volume. The oocytes were washed twice after injections with MBS and stored at 18°C for overnight. The next day, oocytes can be injected further or can be used for a variety of different assays.

2.2.3: RNA isolation

The oocytes were injected with mRNA, followed by the DNA injection in the form of plasmid or as permeabilised chromatin from human or mouse cells. A pool of 8 oocytes per condition was picked up and frozen immediately at -70°C on dry ice for further use. In many circumstances, the oocytes were lysed and processed for the RNA isolation by using RNeasy Mini Kit (Qiagen Cat# 74104) according to the manufacturer's protocol. A small change has

been made in dealing with the oocytes, where after lysis, the oocytes were briefly centrifuged to settle down the debris containing pigment and excessive yolk by spinning down the lysate for 2000g for 5 minutes.

2.2.4: cDNA synthesis

To synthesise cDNA from isolated RNA from the *Xenopus* oocyte a lab formulated protocol was used. The detailed description of protocol is given as following.

1-2 μl of isolated mRNA was checked by the agarose gel for its integrity, followed by the brief quantitation using NanoDrop to analyse the purity of the sample. 12 μl of RNA is then mixed with 0.5 μl of 10 mM dNTPs followed by 0.5 μl of OligodT primers into a sterile RNase DNase free Eppendorf tube. The mixture was pipetted up and down for 5 seconds and then incubated at 65°C for 5 minutes in a thermostat. After incubation the mixture was briefly spun to settle down any evaporated residues from the tube cap. 1 μl of DTT, 1 μl of dd.H₂O, 0.5 μl of RT enzyme, 0.5 μl of RNase inhibitor, and 4 μl of FS RT buffer was added to the mixture, which was then incubated at 50°C for 1 hour. After the incubation 70 μl RNase free water was added and sample were again stored at 80°C for RT inactivation.

2.2.5. qPCR of cDNA

After the synthesis of cDNA, 5 μl of sample is used to make a master mixture of 25 μl reaction. SYBR -green Taq mixture (Sigma S9930-250 RXN) was used to amplify the cDNA by sequence specific primers for the desired loci.

Table 3 shows the concentration of each component used in the amplification of cDNA. All the qPCR shown in this study has been analysed by the similar protocol, unless indicated otherwise.

Component	Volume in (μl)
SYBR-green	12.5
RNase-DNase free H₂O	7.36
Primers (FWD-REV)	0.14
Template cDNA	5

Table 2.3: Composition of qPCR reaction with a total volume of 25 μ l.

2.2.6. Firefly and Renilla Luminometer assays

Firefly and Renilla assays were performed using an assay kit from Promega (Dual Luciferase Reporter Assay System Cat: E1980). The detailed protocol is given as follows:

8 individual oocytes from each experimental condition were picked up by a glass pipette and transferred into the PCR strip. All of the excess medium was removed, and oocytes were lysed in the 1X passive lysis buffer (Promega commercial, 5X diluted into 1X with ddH₂O). otherwise the oocytes were stored immediately on dry ice for delayed use. 20 μ l of 1X lysis buffer was added to each tube in the PCR strip and oocytes were lysed by pipetting up and down. Immediately after the lysis, the oocyte lysate was placed on ice. 20 μ l of 1X Firefly reagent (Promega) was added into the each tube and then mixed by pipetting thoroughly. The mixture was then transferred into a 96 well plate (opaque) and then transferred to illuminometer for analysis.

Following are the options which were selected during the analysis using GloMax.

Number of injections	0
Delay time	2 seconds
Integration time	5 seconds
Number of Runs	1

Table 2.4. GloMax Software chart showing the selection of parameters while performing the Firefly and Renilla Assays.

After, taking the firefly values, 20 μ l of Stop Glo reagent was added to each well containing the oocyte lysate and Firefly mixture. The Stop Glo reagent was made by mixture of two ingredients at 5:250. This reagent then provides the assay for Renilla in the same samples. For detailed mechanism see (Promega E 1980 protocol). The plate with Stop glo reagent is read under the same setting as Table 2.4.

2.2.7. Western Blotting

There are two types of main western blotting procedures have been done in this thesis i.e. blotting of isolated germinal vesicle of the oocyte, and whole oocyte western blots. The procedure first mentioned is about the whole oocyte western blot.

2.2.7.1. Whole oocyte Western blot:

A desired amount of mRNA encoding for Ascl-1 and other transcription factors were injected into the cytoplasm of the oocytes. The oocytes (10 oocytes) were then incubated for overnight production of protein from the injected mRNA. On the next day, the oocyte was taken out by glass pipette and washed thoroughly with the 1X MBS. Most of the liquid from the oocytes is then removed before lysis in 50 μ l of lysis buffer (10 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 1mM EDTA and 1X proteinase inhibitor cocktail from Roche. The oocytes lysate is then spun for 10 minutes at 4°C at 16000 g. Protein concentration was measured with

BCA assay (BioRad) according to the manufacturer's instructions. 50 ug of protein was mixed with 1X SDS loading buffer under the reducing conditions and sample were either boiled for 3 minutes at 90°C or 20 minutes at 70°C. The boiled samples loaded on the 12% polyacrylamide gel which is then immersed in SDS Running buffer (25mM Tris, 192mM glycine, 0.1%SDS and pH 8.3) and gel was run at voltage of 200mV for 2 hours.

2.2.7.2. For cytoplasm and GV only

For analysis of protein concentration and localisation into cytoplasm and GV of the oocytes a western blot of these separate compartments was performed. Oocytes were taken out from the MBS medium and transferred into another dish containing Sterile mineral oil from (Sigma). All the excess water was removed from the oil and medium interface. An oocyte was separated from the clump and pierced with the forceps. With the help of another forcep, the GV was pushed out and cytoplasm can be pushed away in the dish. By repeating this process, enough material can be collected to use for the western blot. After isolation of the GV, a pool of 10-15 GVs are boiled in 1X SDS buffer with reducing agents and then loaded on the polyacrylamide gel for further analysis.

2.2.7.8. Transferring the blots:

After the completion of gel running duration, gel containing the samples were washed with 1X SDS Running buffer and then placed on the activated PVDF membrane. The gel and PVDF membrane are sandwiched in between 5 layers of blotting paper (immersed in Transfer buffer Sigma PGCE-3011) each side and then placed on the semi dry transfer machine. Transfer was run at 100mV for 25-30 minutes.

After the transfer, the PVDF membrane was blocked by the addition of 4% milk in TBS tween for 1 hour. After the incubation, Primary antibody was used at the dilution of 1:2000 for overnight. After the overnight incubation with primary Antibody, the membrane was washed thrice for 5 minutes with TBS 0.1% tween at room temperature. After the washing step a

secondary antibody is applied at the ratio of 1:10000 and membrane was again incubated at the room temperature under dark conditions for about an hour before analysis.

2.2.8. Chromatin Immunoprecipitation

In this thesis, a variety of chromatin immunoprecipitation experiments have been performed. The general injection plan used for sample preparation is illustrated below figure 2.4. For a typical ChIP experiment, oocytes were injected with an mRNA encoding for Ascl-1 protein at the final concentration of 210 pg -1.4 ng/oocyte. The oocytes were then incubated overnight and followed by the DNA injection at typical concentration of 200-300 pg/oocyte. Pool of 15-20 oocytes were selected and washed repeatedly for 10 minutes before proceeding for ChIP by (Stewart, Tomita, Shi, & Wong, 2006), with minor modifications.

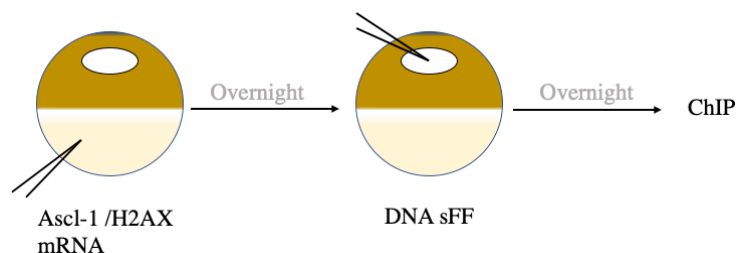


Figure 2.4: Schematic diagram of mRNA and DNA injection used for a typical Chromatin precipitation in the *Xenopus* oocyte. On day 0, a desired amount of mRNA is injected in the cytoplasm of an oocyte. On day 1, mRNA injected oocytes are then injected with a DNA plasmid bearing the binding site of a transcription factor or a general transcriptionally active promoter such a CMV. the next day, oocytes are the picked up and fixed with the formaldehyde before proceeding them for Chromatin Immunoprecipitation.

2.2.9. Protein Competition ChIP

Protein competition ChIP was performed to understand the dwell time of transcription factors for a single binding site. This was achieved by the injection of two differentially tagged Ascl-1 mRNAs into the *Xenopus* oocyte separated by 24 hours time. The illustration of the injection plan is given in the figure 2.5. To explain in detail, *Xenopus* oocytes were injected with a Ascl-1 mRNA encoding for the Ascl-1 GFP protein at a final concentration of 1.4 ng/oocyte. After a day of protein synthesis by overnight incubation, a DNA, either Firefly or Renilla, was injected into the GV of same oocytes. After the nuclear injection, the oocytes were incubated at 18°C for overnight. On the next day another mRNA encoding for Ascl-1 3HA NLS was injected at 10 times more concentration than Ascl-1 GFP (i.e. 14ng/oocyte). The oocytes were then incubated for 24 hours to ensure the proteins can have enough time to compete before the oocytes are processed for ChIP procedure by (Stewart et al., 2006).

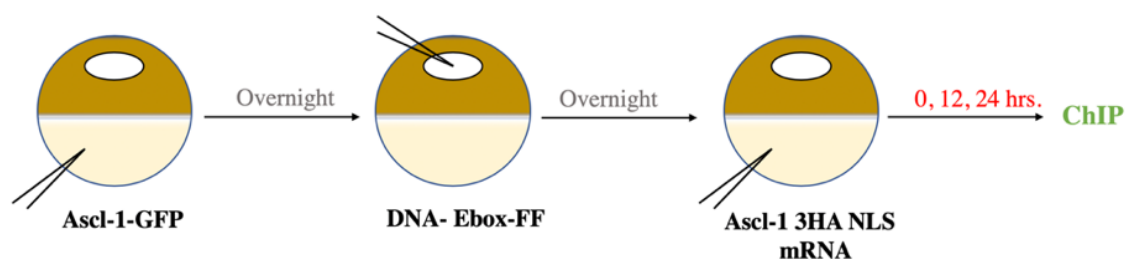


Figure 2.5: Schematic diagram of injection of a protein competition ChIP performed in *Xenopus* oocyte. The oocytes were injected with a limiting amount of Ascl-1 GFP in the cytoplasm, followed by a DNA-FF injection in the GV. On the next day, same injected oocytes were then given a cytoplasmic injection of a competitor Ascl-1-3HA NLS at 10X concentration. The oocytes were then fixed with 1% Formaldehyde and analysed through a competition ChIP.

In protein competition experiment, three different antibodies were used to perform an IP. The antibodies with their catalogue numbers are listed in table 2.5.

Antibody	Purpose	Catalogue number
Anti-Ascl -1	Detects whole Ascl-1	Ab-740295
Anti-GFP (ChIP grade)	Detects Ascl-1 GFP only	Ab-290
Anti-HA (poly)	Detects Ascl-1 3HA NLS	H-6908

Table 2.5: Antibodies used in the analysis of protein competition ChIP by Ascl-1 and Oestrogen receptor in *Xenopus oocyte*

2.2.10. DNA competition ChIP

A DNA competition experiment has been performed to analyse the dynamics of transcription factor Ascl-1 and to analyse the binding of transcriptional machinery such as TBP2 and RNA-Pol-II with Ser 2 and Ser5. To perform this experiment, Ascl-1 mRNA in a limiting amount is injected into the oocyte (210pg to 1.4 ng) per oocyte and oocytes are then incubated overnight for protein synthesis. On the next day, oocytes were injected with 210 pg of DNA-I (amount varied among experiments and indicated in particular experiments). After an overnight incubation, the same oocytes receive another nuclear injection with the DNA-II, with at least 5X more concentration than the first DNA. After another 24 hours, the oocytes containing all these injected components were collected as pool of 20 oocytes/condition. Collected oocytes were then washed twice with 1X MBS before processing for ChIP by (Stewart et al., 2006).

Antibody	Purpose	Catalogue number Abcam
Anti-Ascl -1	Detects whole Ascl-1	Ab-740295
Pol-II ser2	Detect CTD-Ser2	Ab-5095
Pol-II ser5	Detect CTD-Ser5	Ab-5131

Table 2.6: Shows the Antibodies used in DNA competition ChIP experiments.

2.2.11. Fluorescent labelling of the plasmids:

Fluorescent labelling of DNA-FF and DNA-Ren was performed by the covalent modification of G residues. The labelling was performed by commercially available kit by **MirusBio**. To retain transcriptional activity of labelled plasmids, slight modification of the protocol was made. The detailed method is given below.

Label IT Cy3 and Cy5 were reconstituted from the lyophilised pallets according to the manufacturer's (**MirusBio**) protocol (Cat MIR-3625 for Cy3), and (Cat MIR-3725 for Cy5). The following is a table 2.7. with components and their concentration for labelling of Firefly and Renilla Plasmids.

Name of reagent	Final volume (μ l) Cy3	Final volume (μ l) Cy5
Ultra-pure H ₂ O	37.5	37.5
10X labelling Buffer A	5	5
μ g/ μ l DNA sample	5	5
Label IT reagent	2.5 (original value 5)	2.5 (original value 5)
Total volume	50	50

Table 2.7: Showing the concentration of the components of Mirus Label IT kits. (Red) are the original values on the kit, which were changed to half to render DNA plasmid transcriptionally active.

2.2.12. Sample preparation for confocal Microscopy

In this section, method of preparation of oocyte GV for Confocal microscopy has been described in detail. *Xenopus* oocytes were injected with fluorescently labelled DNA followed by an overnight incubation. Depending upon the experimental condition, the oocytes are injected with another DNA into the GV and incubated for another day. If not, oocytes are then collected and GVs were isolated in oil, as mentioned in section (2.2.7.2) page 30. The isolated GVs were pipetted in and out to make sure that any yolk if attached will be released. Pool of

5-10 GVs were transferred into a glass bottom (**ibidi Bioscience**) ibidi dish 60 μ Dish (cat: 81158). The use of this Dish is critical as, GVs remain intact for longer duration and imaging such as time-lapse can be done for relatively longer durations. Along with GVs 10 μ l of sterile mineral oil is placed on the sides of the GVs to localise them into a specific area. It needs to be assured that GVs are completely submerged in the oil.

Imaging:

SP8 microscope inverted Confocal microscope was used to visualise the injected DNA in the intact GV. 20X and 60X oil immersion lenses have been used to image the GVs. Sp8 confocal microscope uses white lenses, which can be tuned into any wavelength depending upon the fluorophore used.

2.2.13. Transfer of nuclear material from the oocyte

A new approach was used which aids the transfer of nuclear content of one oocyte's GV into another without diluting it into any other buffer. Detailed method is given as follows. Pool of 10 healthy oocytes were selected and washed several times with 1X MBS. The oocytes were then transferred into a new plate containing sterile Mineral oil. GVs from all 10 oocytes were isolated with minimal damage and their nuclear membranes were ruptured. Then nuclear gel from all of the GVs is mixed and taken up by the microneedle connected with an injector. To ensure the quality of this procedure and of the transcription factors, the nuclear material was immediately injected into the recipient oocyte at variable concentrations, which depends on the experimental conditions. In a typical nuclear content transfer experiment, 13 nl of material was transferred into the GV of a recipient oocyte. After the nuclear content transfer, about 35% of the oocytes die, while others survive if the concentration of BSA in the MBS medium is high as 0.4 %.

2.2.14. Generation of mutant plasmids

P1008 DNA has been mutated into the Δ Ebox and Δ PoU domain by Prof. Anna Philpott and have been gifted to us. However, the main plasmids which were generated in this study are p1008 with Δ SV40 and Δ Transcription (p1008 with no transcriptionally active sites). Site directed mutagenesis was performed by the **NEB kit** for site directed mutagenesis. All the steps were followed according to manufacturer's (New England Biolabs) protocol.

To mention briefly, Primers for site directed mutagenesis for Δ SV40 and Δ Transcription was designed by the guidelines provided by the NEB Base changer software, (URL <https://nebasechanger.neb.com/>). The whole SV40 region was deleted by the PCR and the resultant plasmid was grown to make aliquots for microinjections.

2.2.15 Preparation of oocyte cytoplasmic extract

To prepare the *Xenopus* oocyte extract, a large quantity of the *Xenopus* oocytes has been used. A large number of protocols are available containing variety of different methods to prepare the extract. The one preferred here does not use any of the buffer or dilution medium. A detailed step protocol of *Xenopus* oocyte extract procedure is given as following.

2.2.16. Whole oocyte extract:

Approximately, 400-600 healthy oocytes which are freshly defolliculated from ovary or maximum 1 day older are selected. The oocytes are then picked up by a glass pipette and transferred into a 1.5 ml sterile tube. Followed by transfer to the tube, the oocytes were washed repeatedly with 1X MBS containing 1X proteinase inhibitor. The oocytes were then vortexed for about 10 minutes until a greyish mixture of thick liquid has been seen in the tube. The tube is then placed in a centrifuge in a cold room and spun at the maximum speed i.e. 16,000 g for 20-25 minutes. A thick needle syringe is then used to take out the fraction of clear extract by puncturing the 1.5 ml tube. Collected extract was then used immediately for the assays or could

be stored at -70°C for future use. The extract made by this method is active for at least 6 months with a minor decrease in quality.

2.2.17. GV Extract preparation

Large number of germinal vesicles, ~ 200 , were isolated in a GV extract medium (2.1.2) and then transferred to a new tube with a minimal volume. The total GV were centrifuged for 5 minutes at 500 g to collect the supernatant which is then stored for future use. To have most reproducible results, GV extract has been made on same day of experiment.

2.2.18. Primer and DNA oligos used in this Thesis (ChIP and Competition ChIP)

Primer name	FWD 5'-3'	REV 5'-3'
DNA-FF Ebox	CTAACTGGCCGGTACCTGAG	AACAGTACCGGATTGCCAAG
Ebox-FF II	CGCTCTCCATCAAAACAAA	CGACGCTCAGCTGTATGGTA
Control	GGCGCTTTCTCATAGCTCAC	AGTCGTGTCTTACCGGGTTG
DNA-FF gene body	AAAAGAAGCTACCGATCATA	AATGGCACCACGCTGAGGATA
DNA-Ren Ebox	CGCTAGCCTCGAGAGAGCAG	TCATAAACTTTCGAAGTCAT
DNA-Ren Gene body	GAAAAAATGGTTTTGGAGAATA	ATATATTTTCCATTTCATC

Primer used for the creation of mutant plasmids

FF primers	CGCTAGCCTCGAGAGAGCAG	CGCTAGCCTCGAGAGAGCAG
Δsv40	TACCCTCTAGTGTCTAAGC	TACCCTCTAGTGTCTAAGC
$\Delta\text{TATA box}$	GGAAGCTCGACTTCCAGC	TACCCTCTAGTGTCTAAGC
Sv40 primer	ACCAGCTGTGGAATGTGT	GAGCCTGGGGACTTTCCA
hygro p1008	CCGGCAGCCCTCGTCTGC	CTGCGGACGATGGCATCG
GFP Primers	CTGGTCGAGCTGGACGGCGACG	CATGGTCCTGCTGGAGTTCGTG

Primers used for qPCR of genes in Normal Human Dermal Fibroblasts

ATOH8	TGGGCAGAAGCTGTCCAAACT	GTGGTCGGCACTGTAGTCAAG
GAPDH	TGACTTCAACAGCGACACCCA	CACCCTGTTGCTGTAGCCAAA
DLL1	GATGTGATGAGCAGCATGGA	CCATGGAGACAGCCTGGATA
DLL3	AATCGCCCTGAAGATGTAGACC	GCACCACCGAGCAAATACAA
Sox2	GCGGAGTGGAAACTTTTGTCC	GGGAAGCGTGTACTTATCCTTCT
Sox11	AAGAACATCACCAAGCAGCA	TCCAGGTCCTTATCCCACCAG

List of the SgRNA used for the dCAS9 mediated Locus pull down

Guide Name	Guide RNA sequence	Region of interest
Ebox- FF	CCAGAACATTTCTCTGGCCT	Firefly specific Ebox
Ebox Renilla	AAACAAACTAGCAAATAGG	Renilla specific Ebox
Control region	ATCTCAATTAGTCAGCAACC	Control region (no Ebox)
Scramble	TTTAAACTCTAGAAAGCTGC	No known target

The *Xenopus* oocyte as an Assay system to study transcription factor binding dynamics

Introduction:

The *Xenopus* oocyte has been used for decades to study eukaryotic transcriptional regulation. The *Xenopus* oocyte is a large cell containing many maternal proteins and mRNAs that support the early phases of development (Sheets et al., 2017). The large nucleus and size of a *Xenopus* oocyte permit the injection of different molecules of interest without the restriction of time and concentration. Moreover, a *Xenopus* oocyte contains a surplus amount of transcription factors such as RNA-Pol I, II, and III to transcribe the genes multiple times. The factors present in the *Xenopus* oocyte can reprogram somatic cell nuclei injected into the Germinal Vesicle of the oocytes (Miyamoto, 2019).

Additionally, the oocyte has a pool of transcription factors that are sufficient to reprogram about 300-500 somatic cells into an oocyte-like gene expression program (Jullien et al., 2012). The mechanism of transcription of somatic cell nuclei in an oocyte suggests incorporating linker histones and the expansion of chromatin. Within 24 hours of nuclear transplantation, the somatic cell components are replaced by the oocyte transcription components (Jullien et al., 2012). Reprogramming of somatic cells by an oocyte is one of the fastest and successful methods.

The *Xenopus* oocyte has been well studied for the regulation of transcription for many human and murine origin genes. The regulatory elements are cloned into plasmids, which are then injected into the oocyte GV (W. L. Wang & Shechter, 2016). A variety of assays such as confocal microscopy (Robb & Wylie, 1999), electron microscopy (Allen et al., 2007), and other molecular methods such as chromatin Immunoprecipitation (Stewart et al., 2006) then allows those molecules to be studied in precise details.

In this chapter, the *Xenopus* oocyte has been studied with a prospect of a detailed analysis of the in-situ behaviour of transcription factor Ascl-1. To do this, *Xenopus* oocyte has been injected with an mRNA encoding for Ascl-1 protein. A precise dose of Ascl-1 mRNA can be injected by either changing the injection volume or the concentration of mRNA injected in the oocyte. After mRNA injections, oocytes can be stored overnight to synthesize Ascl-1 protein, which is then transported into the oocyte's GV. On the next day, the same Ascl-1 injected oocytes are then injected with a DNA containing an Ascl-1 binding site followed by a reporter gene such as Firefly or Renilla. The activity of Ascl-1 binding can be detected with the reporter expression by illuminometer values. This chapter optimises the necessary methods for A) Analysis of kinetics of Ascl-1 on its chromatin binding site in the oocyte. This includes optimization of its dose at which it saturates its binding site, the concentration of Ascl-1, which can be maximally transported to the nucleus of the oocyte, optimization of CHIP for analysis of Ascl-1 direct binding to Ebox in the oocyte. Further, it includes the regulation of the Ascl-1 promoter in the *Xenopus* oocyte and analysis of injected Ascl-1 on the injected chromatin isolated from Human primary cells. B) Dynamics of Ascl-1 interaction with *Xenopus* protein has been studied by a site-specific chromatin fragment isolation by different methods such as biotin labelling of the plasmid DNAs and by locus pull down by CRISPR system.

3.1: Injected mRNA for Ascl-1 encodes for a functional protein in a dose dependence manner

Expression of mRNA across many species can be halted at the transcriptional or translation level (J. R. Yang, Maclean, Park, Zhao, & Zhang, 2017). It is important that the Ascl-1 mRNA which we use to express the protein is well expressed in the oocytes and that the protein has the correct post-translational modifications. If not so, the function of protein will be altered, or the protein remains inactive in all ways. I asked a question whether the mRNA injected into the *Xenopus* oocyte encodes for a functional Ascl-1 protein? To study the

expression of Ascl-1 mRNA and its function in *Xenopus* oocytes, I used a reporter plasmid, which bears the binding site for Ascl-1 protein. I assumed that an effective translation of Ascl-1 mRNA will activate the reporter when it binds to the Ebox-PoU-Ebox DNA sequence in the plasmid. This assay provides the translational and functional optimisation of Ascl-1 in the oocytes.

3.1.1. Experimental procedure:

An Ascl-1 mRNA with a dose ranging from 1 ng to 14 ng was injected into the oocyte on day 0 (Figure 3.1). The oocytes were then incubated in 18 °C incubators for overnight. Overnight incubation of oocytes make sure that the mRNA has been translated and protein has been successfully localised to the targeted organelles, which is nucleus in the case of Ascl-1. The same injected oocytes were then injected with DNA-FF 300-350 pg in the nucleus (Figure 3.1 A). Time course analysis suggests that injected mRNA for Ascl-1 gets translated into the protein in 2 hr (visible signal at high magnification) and translocated into the GV of an injected oocyte. After the injection of DNA into nucleus, the oocytes were then incubated for another 16 hrs in 18 °C incubator for Ascl-1 DNA interaction in the presence of oocyte specific co-factor molecules. On day 2, the oocytes were taken out and selected on the basis of GFP signals on the membranes which has resulted in the expression of a CMV-mGFP plasmid which is co-injected with DNA-FF. The selected oocytes were then frozen individually in PCR tubes. Eight individual oocytes were then analysed by FF and Renilla expression depending upon the reporter used. Ten pooled oocytes were used for the western blot analysis for the detection of Ascl-1 protein abundance.

3.1.2. Results:

Protein expression analysis by western blot suggests that mouse Ascl-1 mRNA injected into oocyte is highly expressed in a dose dependent manner (Figure 3.1B). Membrane GFP (mGFP) signals in 95% of the oocytes confer a high nuclear target rate for DNA injections (Figure 3.1C).

Furthermore, the dose of mRNA injected into the oocyte was assayed for its functional output by activation of firefly reporter on DNA-FF. To understand the dose dependent functional output of Ascl-1 I used a fixed amount of DNA i.e. 350 pg/ oocyte. Figure 3.1D shows the increase in Firefly signals for a fixed amount of DNA-FF. I next asked if a similar trend is observed when an increasing dose of Ascl-1 is preceded by the DNA-Ren. Figure, 3.1E shows similar trend of Renilla reporter activation as in Figure 3.1D. This result shows that Ascl-1 function depends more on the binding sequence than the reporter downstream of its binding site. Another part which needed special attention was a variety of Ascl-1 mRNAs used for the experiments. An example of this is Ascl-1 GFP mRNA and Ascl-1 NLS HA. I wanted to know the effect of differential tags on Ascl-1 function in the oocyte, (Figure 3.1F) shows that the presence of an NLS significantly changes the firefly activation after 24 hours as compared to Ascl-1 GFP. This is because concentration of Ascl-1 NLS protein is greater than Ascl-1 GFP.

3.1.3. Conclusion

The experiments such as over-expression of Ascl-1 through mRNA injections in the oocyte confirm a dose dependent translation of Ascl-1 in the Xenopus oocyte. It also provides the evidence that the injection of even high doses of Ascl-1 mRNA is not toxic for the oocyte. The firefly activation by Ascl-1 confirms the correct folding and functional retention of mouse Ascl-1 in the oocytes. Overall, these experiments suggest that further optimisation of Ascl-1 binding dynamics in the oocyte can be proceeded.

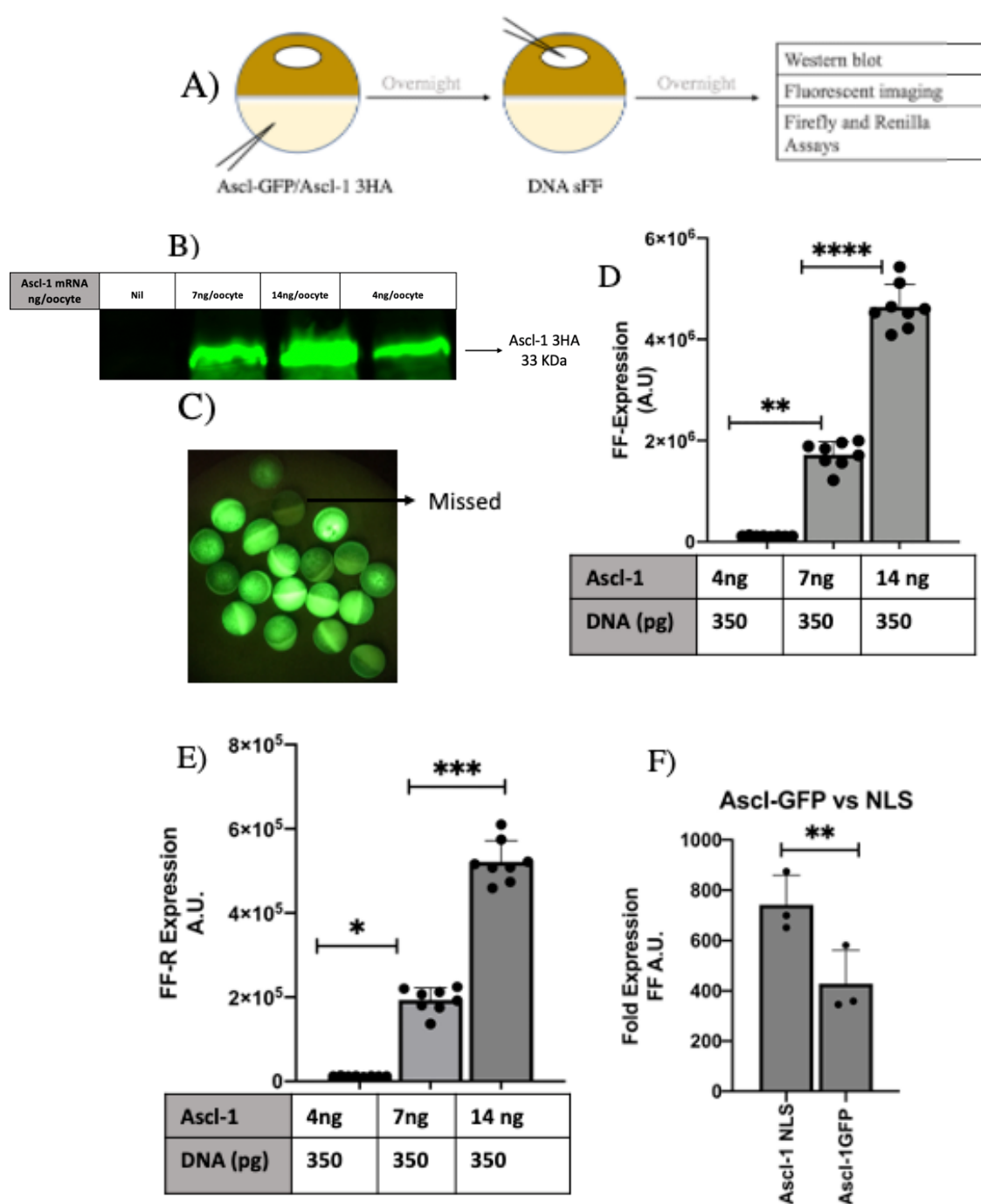


Figure 3.1: Injected mRNA in oocyte encodes functional Ascl-1 Protein

A) A schematic diagram for Ascl-1 mRNA injection into oocyte dose ranging from (4ng-14 ng of mRNA/oocyte). B) Anti-Ascl-1 western blot of 10 oocytes injected with 4-14 ng of Ascl-1 mRNA/oocyte. Oocytes were picked 24h post injection. C) GFP-marker for successful GV injection aids in selection of oocytes for further analysis. D) Dose dependent activation of Firefly Luciferase (FF-L), 8 individual oocytes were used for the assay. E) Dose dependent activation of Firefly Renilla (FF-R) injected into GV of oocyte. F) Firefly activation by Ascl-1 GFP and Ascl-1 NLS.

*($p < 0.07$) **($p < 0.001$) ***($P < 0.001$) ****($P < 0.0007$).

3.2. Ascl-1 needs Dll1 Ebox sequence for activation of transcription by Firefly DNA:

Ascl-1 binds to a canonical Ebox sequence, the chances of which is to be repeated every 4096 bp in a given plasmid DNA or in a chromosomal DNA. I performed a similar analysis to find out exact number of Ebox like sequences in the DNA-FF and DNA-Ren. The real question was to determine which Ebox sequence in the plasmid is used by Ascl-1 for initiation of functional transcription? To answer this question, I used the mutated version of the DNA-FF called (Δ Ebox-FF). In this plasmid the Dll1 derived Ascl-1 binding site was mutated as shown in (Figure 2.3 Materials and Methods section). While in another case, I used the backbone plasmid which contain no Ebox-PoU-Ebox sequence derived from the Dll1.

3.2.1. Experimental procedure:

An mRNA encoding for Ascl-1 was injected at day 0, followed by the injection of WT DNA-FF to the Ascl-1 injected oocytes. Some of Ascl-1 injected oocytes were injected with the mutant versions of DNA. One mutant version has a mutated Ebox Sequence. While, other version of plasmid is the base vector called PGL 4.26, which does not contain canonical Ebox Sequence derived from Dll1 gene Mouse. One day after nuclear injection of DNA, the oocytes were lysed and used for Firefly luminometer assay (Figure 3.2 A).

3.2.2. Results:

Alignment analysis showed approximately 12 and 13 Ebox like (CANNTG) sequences in DNA-FF and DNA-Ren. Firefly activation through Ascl-1's binding on DNA showed that 80% of Firefly expression declined when a mutant plasmid is used (Δ Ebox) as a template (Figure 3.2B). Similarly, it also has shown that when the Ascl-1 injected oocytes are injected with backbone plasmid 4.26, the transcription was about 2% as compared to the Wild type DNA-FF (Figure 3.2 C).

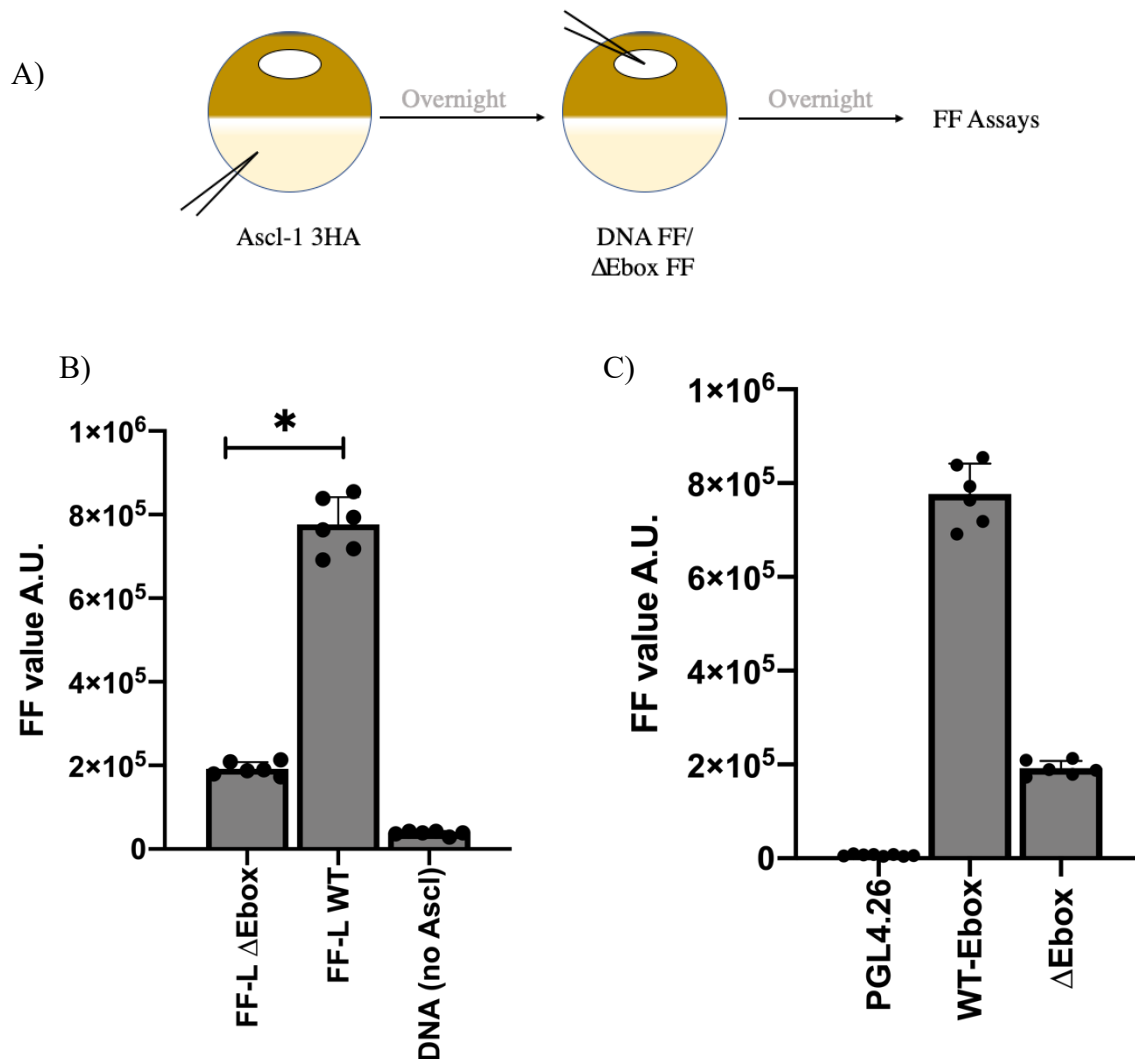


Figure 3.2: Ascl-1 mediated transcription depends on Dll1 derived Ebox sequence in oocyte

A) Injection plan showing the injection of Ascl-1 mRNA 1.4ng per oocyte followed by the injection of 300 pg of FF WT DNA or FF- Δ Ebox DNA into the GV of preinjected oocyte. B) Firefly luminometer values shows the Dll1 depend Ascl-1 mediated transcription, 8 individual oocytes used per experiments ($n=3$). C) Shows that activation of Ascl-1 dependent transcription does not start from the Ebox-like sequences present in FF-plasmid.

*($P < 0.001$) $n=3$ for figure B and C. Each experiment contained 8 individual oocytes. Statistical significance is shown by Student T test.

3.2.3 Conclusion:

Comparison of firefly activation by Dll1 containing WT DNA-FF and Δ Ebox plasmid has shown that, in the *Xenopus* oocyte, Ascl-1 activates the transcription by a single binding site, which is Ebox-PoU-Ebox. Only a little activation was observed with a mutant Δ Ebox plasmid. This further validates the use of the oocyte as a system to analyse the binding dynamics of a transcription factor.

3.3. Ascl-1 retains its pro-neural activity in the oocyte

To further characterize a system for the Ascl-1 dwell time analysis, I asked a question whether the expressed Ascl-1 in the *Xenopus* oocyte retains its activity of activating the neural specific genes? I answered this question by the replacing the nuclear injections of plasmid DNA with an intact chromatin (nuclei) prepared by SLO mediated permeabilization of NHDF cells. I aimed to see if the genes such as Dll1, Dll3 and Atoh8 are upregulated when these nuclei are transplanted into the oocyte's GV. The reason for selecting these genes is because of their early expression (T=48 hours) in trans-differentiation of NHDF into induced Neurons (iNs).

3.3.1. Experimental procedure:

Variable doses of Ascl-1 ranging from 2ng-14 ng per oocyte were injected into cytoplasm of the *Xenopus* oocytes (Figure 3.3A). The oocytes were then incubated overnight for Ascl-1 protein production and localisation into GV. On the next day, preinjected oocytes were injected with ~ 300 SLO (Liu, Chu, & Ng, 1993) permeabilised (Methods section 2.1.7) NHDF nuclei (Wapinski et al., 2013a). Some oocytes were frozen immediately to be used as a T=0 controls. Injected oocytes were further incubated for 48 hours before their collection for RNA isolation.

3.3.2. Results:

Oocytes containing homogenous NHDF nuclei in the GVs were selected through the mGFP signals (Harvey, Lukovic, & Ucker, 2001). RNA isolation from those oocytes was performed as mentioned in (Methods section 2.2.3). Amplification of cDNA through qPCR showed the upregulation of genes associated with neurogenesis. Comparison of activation of these genes in the oocytes injected with a different dose of *Ascl-1* mRNA showed a direct relation of gene activation, i.e. higher the *Ascl-1* dose, higher will be the gene activation (Figure 3.3B). Furthermore, variable trend in the activation of *Ascl-1* dependent genes were observed i.e. *Atoh8* (Masserdotti et al., 2015), expression is at least 2.5-fold higher than *Dll1* and *Dll3*. This means that some of the oocyte factors favour in the activation of *Atoh8*, through co-operative binding of *Ascl-1* to *Atoh8* regulatory sequences. Similarly, *Sox11*, which is activated in NHDF in response to *Ascl-1* is minimally activated in all three doses of *Ascl-1* mRNA. This may be because co-factors which promote *Sox11* expression in *Xenopus* oocytes are limited.

I analysed the effect of *Ascl-1* expression in the pluripotency genes in the oocytes, by amplifying the cDNA related to *Sox2* and *Phox2A*, genes. Results show that *Ascl-1* dose in fact, does increase the expression of pluripotency genes such as *Sox2* (Figure 3.3C), and it also activates a neural specific gene *Phox2A* in the oocyte. *Phox2A* is a direct target of *Ascl-1* in *Xenopus* embryos, however its expression in the oocyte transplanted with nuclei, as a response to *Ascl-1*, has been observed first time in this experiment.

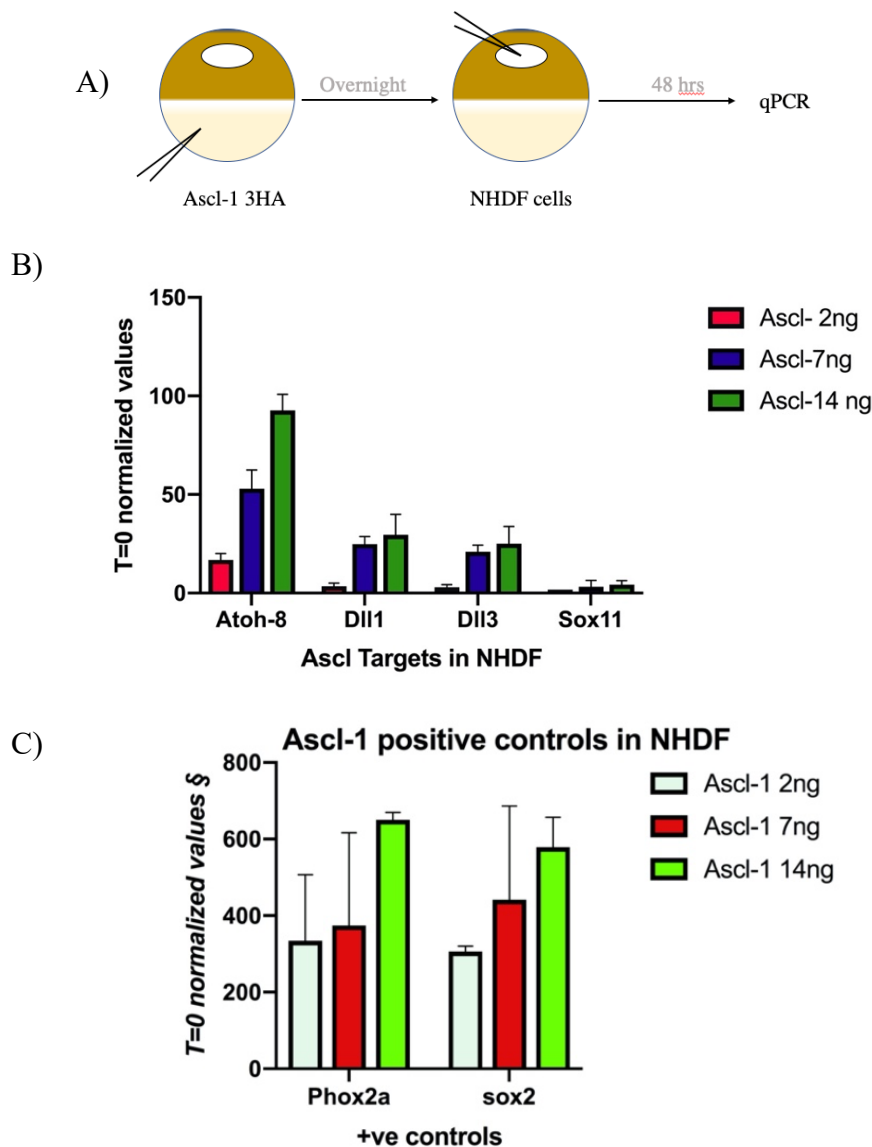


Figure 3.3: Ascl-1 activates the neural genes on injected nuclei in the GV of an Ascl-1 injected oocyte.

*A) Schematic diagram of injection of Ascl-1 mRNA doses (2,7, and 14 ng/oocyte) followed by the injection of nuclei by NHDF permeabilized with SLO. The sample were collected at time 0 (T=0) and T=48 hours post nuclei injection. B) Ascl-1 dependent activation of neural precursor gene targets shown by the qPCR analysis. Gene expression values have been normalised with the T=0 timepoint. C) qPCR results showing the activation of known Ascl-1 positive control gene in *Xenopus* early development and a pluripotency gene Sox2.*

Error bars shows the SEM of n=3 experiments, with each sample containing pool of 8 oocytes

3.3.3. Conclusion

Activation of neural specific genes in a dose dependent manner from the transplanted NHDF nuclei in the *Xenopus oocyte*'s GV show that *Ascl-1* retains its function to induce neural gene expression. However, some genes such as *Sox11*, and *Dll3*, expression has been seen with an altered level of expression as compared to NHDF alone. This alteration in expression pattern may be because of the oocyte co-factors or epigenetic status of chromatin that interact with *Ascl-1*.

3.4. *Ascl-1* specifically binds to the Ebox-PoU-Ebox sequence on injected plasmid

Ascl-1 belongs to the bHLH transcription factor which use a preferred binding motif called Ebox (CANNTG), which varies a little between the family members. However, over-expression of transcription factor often yields in non-specific binding (Chua et al., 2006), which can lead to the activation of unrelated genes. In this section, I asked whether the over-expression of *Ascl-1* in the *Xenopus oocyte* leads to the non-specific binding on Ebox like sequences on the reporter DNAs. For this, I performed a chromatin Immunoprecipitation experiment, in which, I compared the binding efficiency of *Ascl-1* on the Ebox -like sequences to the *Dll1* derived Ebox-PoU-Ebox sequence. It is important to know which Ebox sequence is the most actively used by *Ascl-1* for transcription initiation. This will allow to narrow down to a single binding site, where all other binding assays can be performed. For a positive control a Histone H3 variant was injected, which does not have any sequence specificity and would allow to estimate the success of ChIP procedure.

3.4.1. Experimental procedure

An mRNA encoding for *Ascl-1* protein at the concentration of 7-14 ng of /oocyte (Figure 3.4A) was injected into the cytoplasm of the *Xenopus oocytes*. In another group of oocytes, a same of amount Histone H2A was also injected as a positive control. On the next day, the preinjected oocytes were injected with DNA and incubated for another day in the

incubator. After 16 hours of incubation, the oocytes were taken out and cross-linked with the Formaldehyde and then processed for ChIP as stated in (Methods section 2.2.8).

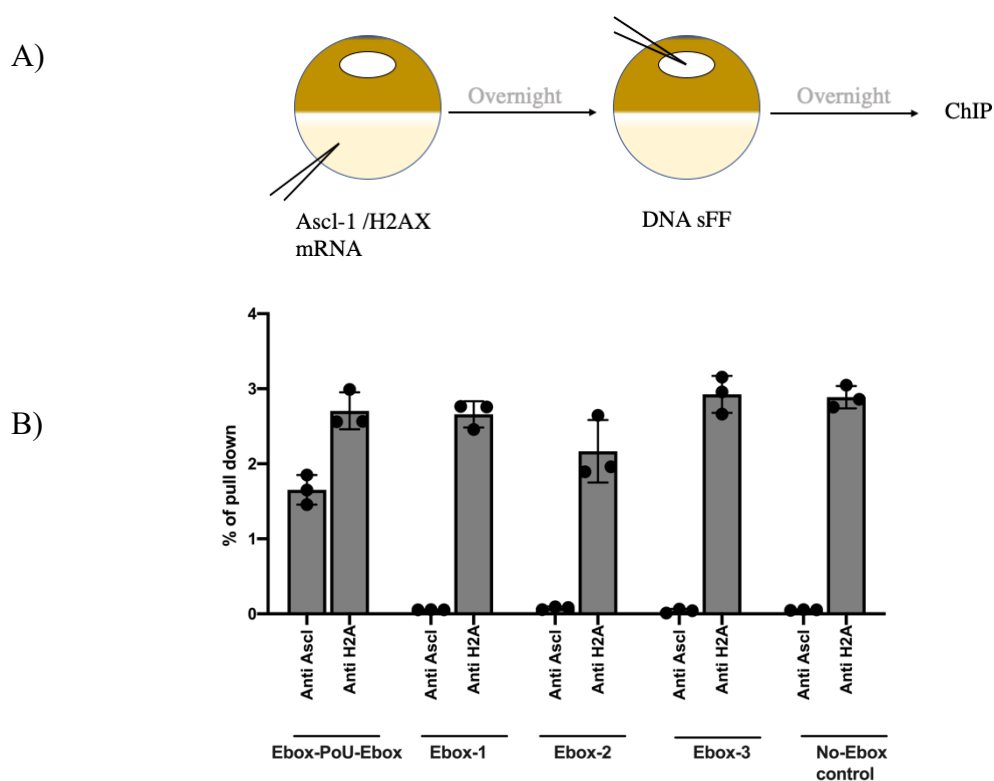
3.4.2. Results

To estimate the binding of Ascl-1 on Ebox like sequence, specific primers (section 2.2.16) were used which amplify three Ebox like sequences other than Ebox-PoU-Ebox, present on the DNA-FF. Amplification of pulled down DNA shows the binding of Ascl-1 (Figure 3.4B). Surprisingly, comparative analysis of Ascl-1 binding to Ebox like sequences and to the Ebox-PoU-Ebox sequence shows a remarkable difference of at least 90% Figure 3.4B, Anti Ascl bars. This means that despite the long incubation time and with over-expression, Ascl-1 remained more confined onto Ebox-poU-Ebox binding site. This observation can be explained by the presence of a poU factor, which may guide or stabilise Ascl-1 on the Ebox-poU-Ebox sequence. Clearly, there is no poU sequence has been seen adjacent to other Ebox like sequences.

Furthermore, as distribution of Histone H2A on Ebox-like sequences and on the Dll1 derived Ebox-PoU-Ebox sequence shows a homogeneous binding (Figure 3.4B), Anti H2A bars. This is because, the Histone H2A does not have a sequence specificity.

Conclusion:

The ChIP experiment shows that distribution of histone can be seen all over the plasmid. However, amplification of Ebox like sequence and Ebox-PoU-Ebox sequence show that the Ascl-1 binds specifically to the Dll1 derived sequence, not to any of the random Ebox sequence present. This finding allows to further analyse binding dynamics of Ascl-1 on Ebox.



(Figure 3.4: Ascl-1 binds preferably to the Ebox-PoU-Ebox sequence derived from Dll1.

A) Schematic diagram showing the injection plan of Ascl-1 and Histone H2AX in the cytoplasm of oocyte (7ng/oocyte). Oocytes were picked 16h after DNA injection.

B) qPCR amplification of DNA pulled down through Anti -Ascl-1 and Anti-HA antibody (which recognizes H2AX-3HA only). Primers for different Ebox-like sequence have been used to amplify the DNA pulled down. Primers specific only to the Ebox-PoU-Ebox sites were used to amplify the DNA. No-Ebox control region has been used as a negative control for Ascl-1 ChIP.

Error bars show SEM of n=3. Each experiment contained three replicates, with each replicate as pool of 15 oocytes.

3.5. Ascl-1 and histone bind to injected plasmid within same time frame

The previous experiment (section 3.4) confirmed the binding of Ascl-1 to Ebox- PoU- Ebox domain is crucial for Ascl-1 mediated Firefly expression. However, a concern arises about whether the physiological site of Ascl-1 action is chromatinised. In the *Xenopus* oocyte, the DNA bearing the binding site of Ascl-1 is injected in an Ascl-1 preloaded GV. This may mean that all the transcription we see results from a naked DNA. Previous finding suggests that a naked plasmid DNA injected into the oocyte's GV is bound by histone within 6 hours. The ultimate question arises, asks what is chromatin state of plasmid DNA when has been injected in the Ascl-1 preinjected oocyte's GV? To answer, I performed a competition ChIP of histone, and Ascl-1. In the competition ChIP I determined the time frame at which histone and Ascl-1 associate themselves with the injected reporter DNA.

3.5.1. Experimental procedure:

The oocytes were injected with the mRNA encoding for Ascl-1 and Histone H2AX protein in the cytoplasm. Those preinjected oocytes were then again injected with a reporter DNA on the next day. After the DNA injection, oocytes were X-linked with 1% formaldehyde at different time points (0, 2, 6, 12, 24 hours) and processed for Anti Ascl-1 and Anti-H2AX (3HA) ChIP.

3.5.2. Results:

Amplification of DNA pulled down through Anti-HA and Anti Ascl-1 ChIP show where in the plasmids these two proteins bind. Two unique sets of primers used to analyse the binding of Ascl-1 and histone H2A on DNA-FF. One pair analyses the binding of Ascl-1 and Histone on the Ebox-PoU-Ebox sequence. Amplification of this region shows the enrichment of either Ascl-1 (Red) or Histone (Black) over the period of 24 hours Figure 3.5B). Analysis shows that histone binds much quicker than the Ascl-1 on the injected DNA-FF. However,

binding of Ascl-1 can only be detected after 2 hours of DNA injection. The main reason for delayed binding could be a limitation of detection. Histone can bind more tightly to the DNA, while transcription factor binding to the DNA is mainly through co-factor interaction etc. However, a steep increase in Ascl-1 occupancy on the Ebox sequence was observed from 6-24 hours. This steep increase of Ascl-1 follows the same trend as the histone binding (Figure 3.5B).

Furthermore, the analysis of 2nd primer pair (Amplifies, non-Ebox region on DNA-FF) show similar binding pattern of Histone on the non-Ebox sequence. As, expected, Ascl-1 has not shown any binding to that region during 24 hrs time period (Figure 3.5C).

3.5.3. Conclusion:

Histone and Ascl-1 competition time-course ChIP shows that as a DNA-FF injected into the Ascl-1 preinjected GV, both transcription factor and Histone binds to it in a similar time frame. By the time, transcription has started, DNA-FF has become chromatinised. This experiment negates the hypothesis that the DNA injected in Ascl-1 preloaded GV may never get chromatinised.

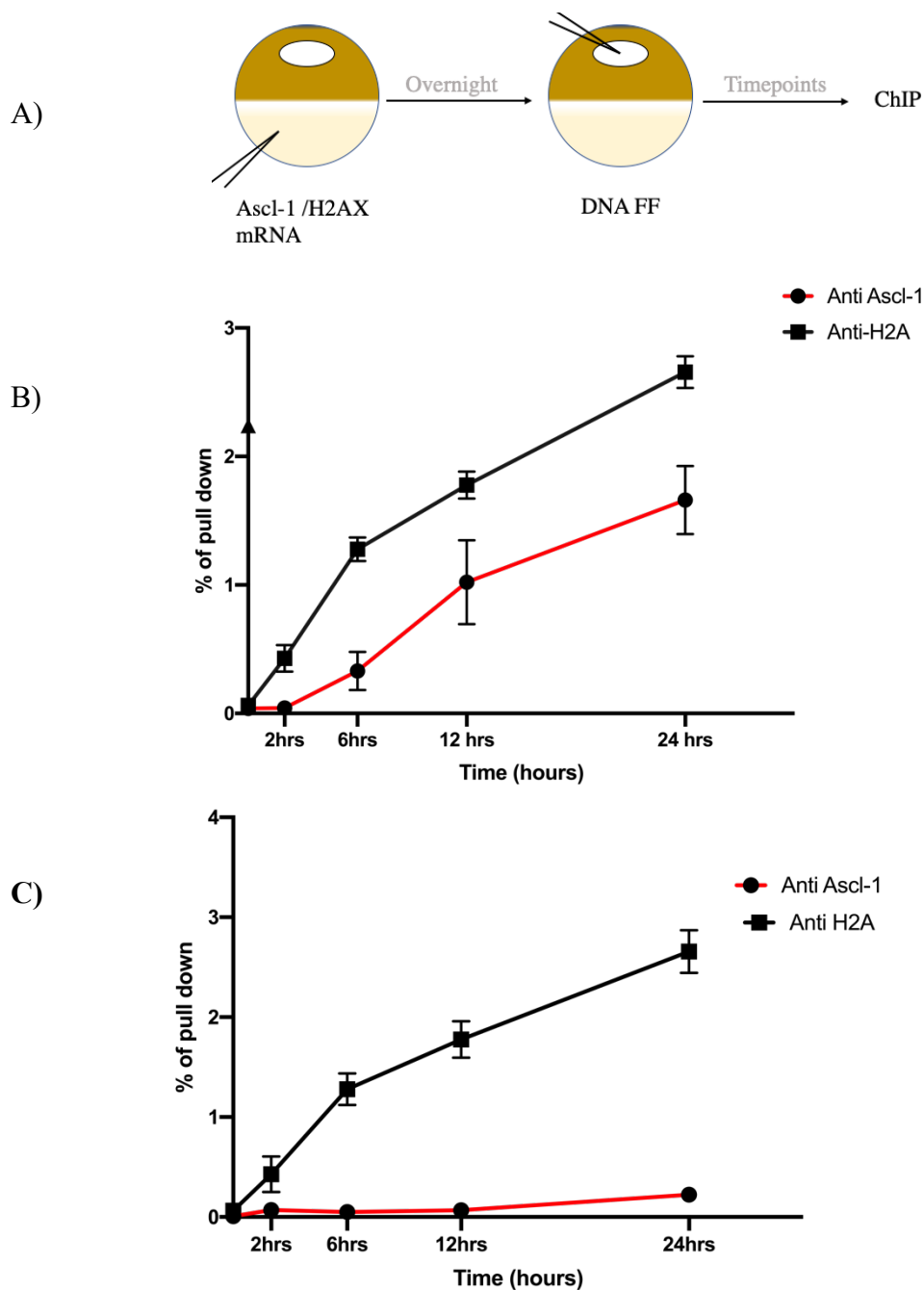


Figure 3.5. Ascl-1 and histone H2AX binds to chromatin in a relative kinetics

A) Schematic diagram of mRNA injection into the oocyte followed by the injection of DNA after 24 hrs. The oocytes were picked after 0, 2, 6, 12 and 24 hrs) and assayed for Anti-Ascl-1 and Anti- Histone H2AX-3HA ChIP. B) qPCR mediated amplification of pulled down DNA specific to *Dll1* derived Ebox-PoU-Ebox sequence on FF-L plasmid. Red line shows the amplification of regions pulled down by Ascl-1 (black) line and also shows the region pulled down by H2AX. C) qPCR mediated amplification of non-Ebox target sequence in FF-L plasmid.

Error Bars shows the SEM for n=3 with each experiment containing 15 oocytes

3.6.: General transcription machinery starts the transcription within 2 hrs of DNA injection:

Section 3.5 shows the relative binding of Ascl-1 and histone H2A on the injected DNA-FF in a time-course manner. In that section, it is evident that histone can be seen easily in 2 hrs after the DNA injections into the GV. However, the binding of Ascl-1 is clearly not seen just after 2 hours of DNA injection, it is detectable in 6 hrs post DNA injection. I asked a question, that at what time transcription from Ebox-PoU Ebox site starts when Ascl-1 and histone H2A are both present in GV? Understanding of this question will allow me to define a narrow window for studying the transcriptional dynamics on Ebox-PoU-Ebox sequence on the DNA-FF. To do so, I performed a similar time-course experiment as mentioned in (section 3.5.1). In this experiment I analysed the occupancy of Ascl-1, on the DNA-FF, Pol-II Ser5 and Pol-II ser2 at the Ebox and on a non-Ebox sequence. The Pol-II Ser5 and Ser2 antibodies measure the extent of productive transcription in two stages. First, Pol-II ser5 detects the formation of pre-initiation complex on the DNA, while second, Pol-II ser2 detects the elongation complex which shows the extent of productive transcription.

3.6.1. Experimental procedure:

In this experiment, oocytes were injected with the mRNA encoding for Ascl-1, followed by the DNA-FF on the next day. The oocytes were picked at different time points ranging from (0.5 hrs 2 hrs and 6 hrs) and fixed with 1% formaldehyde and proceeded directly for Anti-Pol II Ser5, Pol II Ser2 and Anti-Ascl-1 ChIP to investigate the binding kinetics of transcription initiation by Pol-II Ser5 and transcriptional elongation by pol-II-Ser2 (Figure 3.6A)

3.6.2. Results:

ChIP mediated pull down was performed by three antibodies. The DNA which was pulled down through each antibody was amplified by the qPCR. Anti Ascl-1 pull down (Figure 3.6B) shows the similar binding kinetics as to figure 3.5B) where its binding remained undetectable until 2 hours. Figure 3.6B (Black line) shows the negligible binding detection of Ascl-1 in first 0.5 hrs of DNA-FF injection. The binding of Ascl-1 surges to a higher-level post 6 hrs. Contrarily, on the non-Ebox sequence, Ascl-1 binding remained very low even post 6 hrs time (Figure 3.6C).

Amplification of DNA pulled down by RNA-Pol II Ser5 shows the formation of pre-initiation complex on the Ebox-binding site in as little as 0.5 hours (Figure 3.6 B Red line), this formation of pre-initiation complex grows until the 6 hours, where it reaches to a flattened like curve (Figure 3.6B). Surprisingly, formation of pre-initiation complex has also been observed on the non-Ebox sequence (Figure 3.6C Red line).

Lastly, amplification of DNA pulled down by pol-II Ser2 antibody shows that transcription on the Ebox site starts as little as 0.5 hours, which goes on to the near maximum state in about 2 hours (Figure 3.6 Green line). The rise in transcription coincides with the formation of pre-initiation complex. However, amplification of non-Ebox DNA shows little to no elongation of transcription (Figure 3.6C).

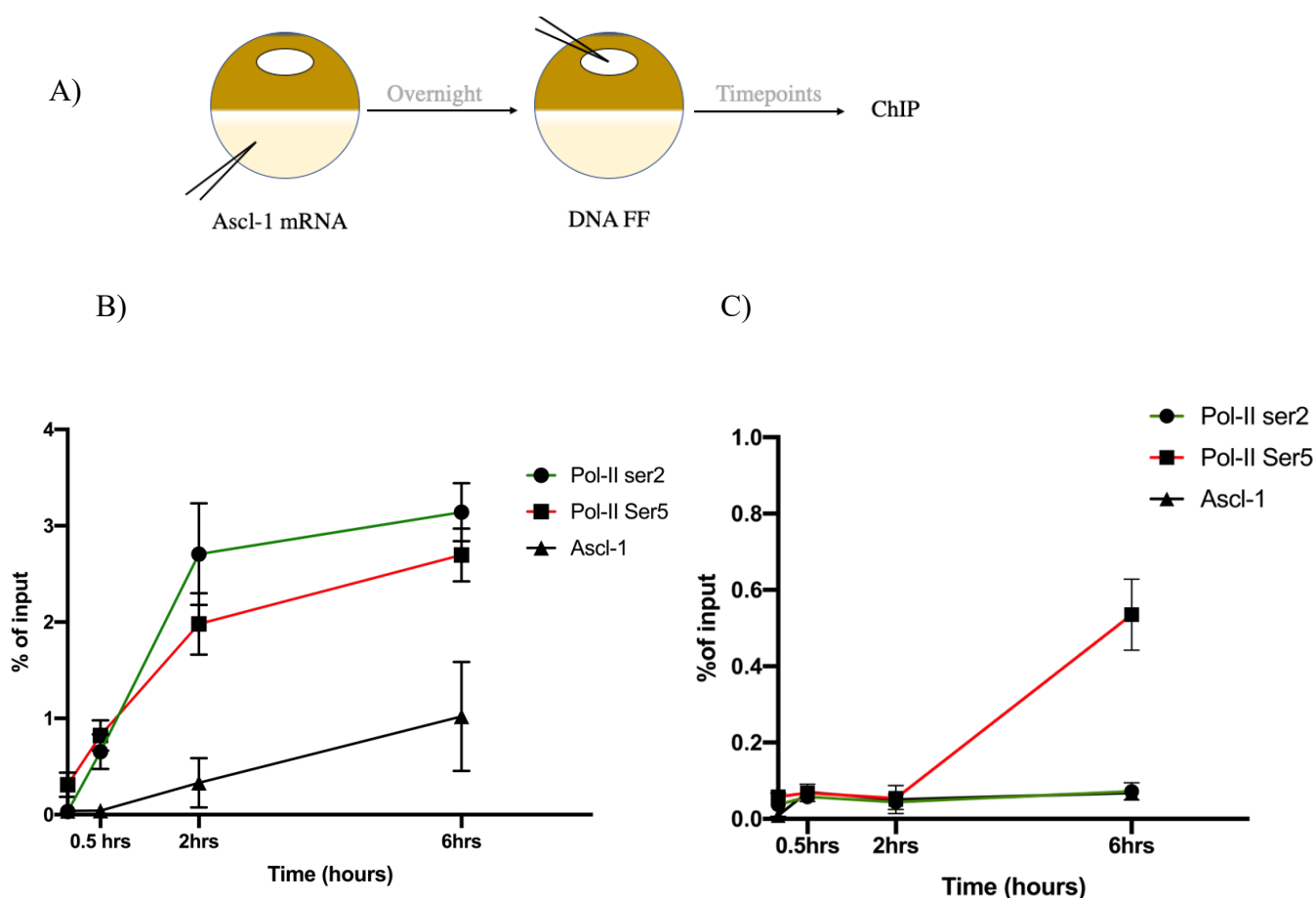


Figure 3.6: Transcription starts within 2 hrs of injection of FF-L DNA into *Xenopus* oocyte

A) Schematic diagram of injections. Oocytes were injected with mRNA encoding for Ascl-1 followed by DNA injection after a day. 15 oocytes per condition (in triplicates) were analysed through ChIP. B) qPCR mediated amplification of DNA fragment pulled down through Anti pol-II Ser2 (green) Anti -pol-II Ser5 (Red) and Anti Ascl-1 (black). A time course abundance of general transcription factor has been shown by the primer specific to Ebox-PoU-Ebox sequence capture. C) qPCR mediated DNA amplification of non-Ebox region shows the pull-down amount of DNA with Anti pol-II Ser2 (green) Anti -pol-II Ser5 (Red) and Anti Ascl-1 (black).

Error bars show the SEM ($n=3$) Each experiment was performed with triplicates each containing 15 oocytes.

3.6.3. Conclusion:

This experiment provides information about the actual transcription assembly and transcription starts on the Ascl-1 specific promoter. Through ChIP experiment I conclude that transcription of the injected template starts within 0.5 hrs to 2 hrs of DNA injection in the GV.

This experiment provides invaluable information about the window, where Ascl-1's binding kinetics can be studied.

3.7: Reporter DNA can be saturated by Ascl-1 mRNA

As the main aim of this study is to find the precise dynamics of Ascl-1 binding on a single binding site, it is necessary to determine the stoichiometry of the molecules injected into the oocytes. One of these parameters is to measure the amount of mRNA, which, upon translation, can saturate a fixed amount of DNA-FF. To do this experiment, I injected the variable doses of Ascl-1 mRNA ranging from 10 pg to 23 ng per oocytes followed by a fixed amount of DNA to 50 pg/ oocyte. A functional response of translated Ascl-1 was recorded by the Firefly reporter assay.

3.7.1. Experimental procedure:

A series of injections were performed on a variable group of oocytes each receiving a variable dose for Ascl-1 mRNA. Injected oocytes were incubated overnight and injected with DNA-FF (50 pg) on the next day. After a day, eight individual oocytes were picked and used for the reporter expression analysis through Firefly activity (Figure 3.7A)

3.7.2. Results:

FF expression mediated activity suggests that there is a sharp increase of reporter expression when Ascl-1 was increased from 0.1 ng to 1ng. Dose curve also shows that the activation of transcription by Ascl-1 reaches plateau at 4 ng for 50 pg DNA (Figure 3.7 B).

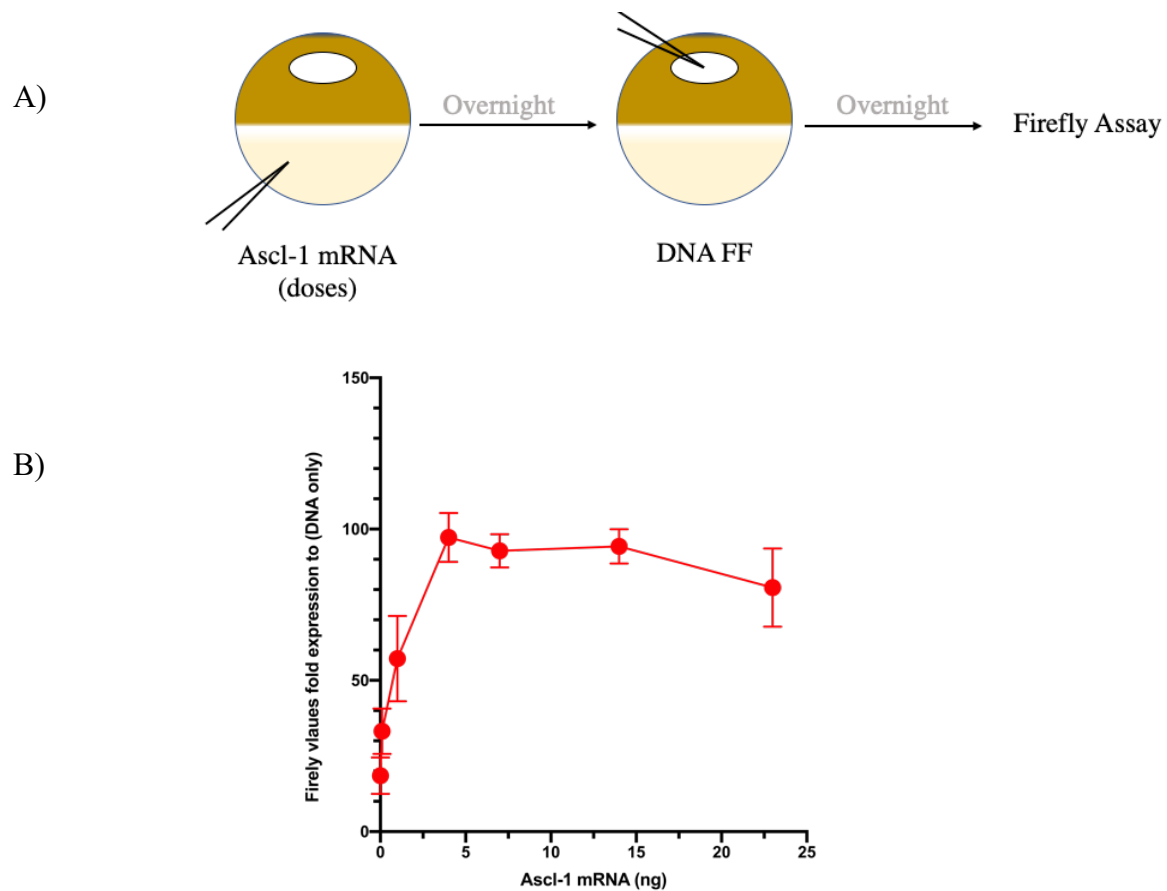


Figure 3.7: 4,6 ng of Ascl-1 mRNA is needed to saturate 50-60 pg of FF-L DNA

*A) Schematic diagram of *Xenopus* oocyte injected with variable doses of mRNA encoding for Ascl-1 protein (10pg to 23ng). The oocytes were then injected with DNA and assayed for FF-L. B) FF-L assay shows the saturation of Ebox-PoU-Ebox site with Ascl-1 mRNA.*

Error bars show SEM of (n=3), while each sample containing 8 individual oocytes.

3.7.3. Conclusion

This experiment shows that the transcriptional activity of DNA-FF can be modulated by the amount of mRNA injected. This information provides an important tool for making the mRNA limiting for the DNA and vice versa.

3.8 Chromatin loci can be isolated by RNA guide dCAS9 for proteomics study:

Transcription factor binding dynamics depends on the chromatin structure, its cognate binding site and on the host cell co-factor pools. Change in co-factor pool can lead to a differential gene expression programs in the cells (Donaghey et al., 2018). There are several approaches used to isolate the genomic loci and associated transcription factors to study the temporal dynamics of transcription. In this experiment I asked, whether it would be possible to optimise such a procedure in *Xenopus oocytes*? To answer this question, I pulled down the fragments of injected DNA-FF through sequence specific guide RNAs and associated dCAS9 protein.

3.8.1. Experimental procedure:

On day 0, 7ng/oocyte mRNA encoding for dCAS9 and Ascl-1 was injected into the oocyte cytoplasm. On the next day, the oocytes were injected with reporter DNA and guide RNAs which directs dCAS9 to Ebox and other proximal sequences on DNA-FF. The day after DNA and guide injections, oocytes were fixed with 1% formaldehyde and then processed for ChIP (Figure 3.8A).

3.8.2. Results:

Amplification of Pulled-down DNA through qPCR suggests that guide RNA 1 (directed to Ebox-sequence) has a highest ratio of pull down when compared to dCas9-SgRNA. Similar sequence specificity was observed by the amplification of sequence related to non-Ebox part of DNA-FF which is also called Control region (Figure 3.8B bar 3). However, scrambled guide-RNA injection shows some binding on the non-Ebox sequence Figure 3.8 Bar 4). This may be because of high concentration of dCAS9 mRNA.

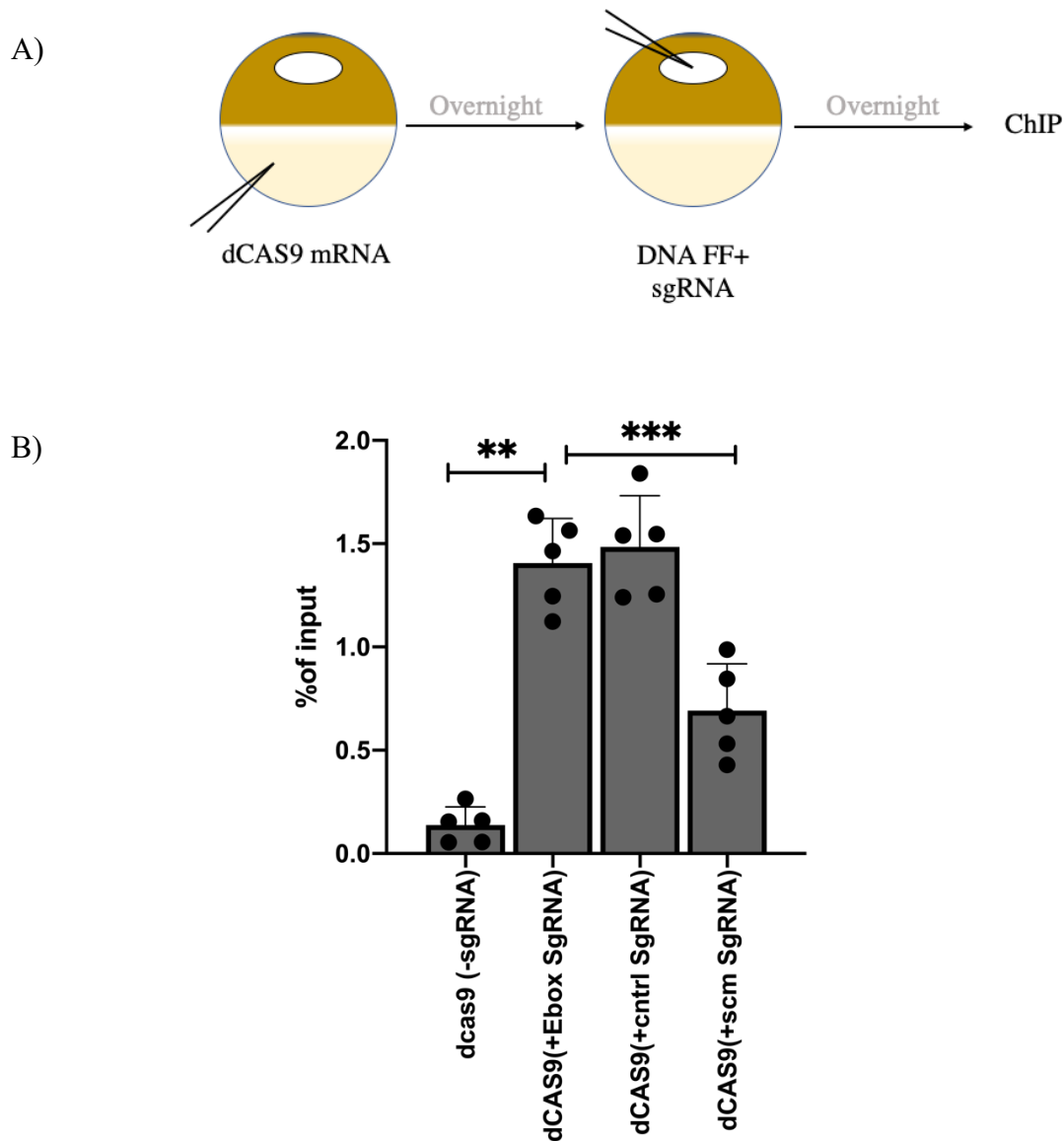


Figure 3.8: dCAS9 efficiently pulls down DNA region with sequence specific sgRNA

A) Schematic diagram of *Xenopus* oocyte used for the injection of dCAS9 mRNA followed by the GV injection of FF-L DNA. The oocytes were then incubated further overnight before processing for ChIP. B) qPCR mediated amplification of DNA pulled down through dCAS9 ChIP. Amplification of Ebox and non-Ebox control region was performed with sequence specific primers.

Error Bars show the SEM ($n=5$) each experiment contained triplicates with each sample having 15 oocytes pooled together.

** ($p < 0.007$) *** ($P < 0.001$) Student *t* Test was used for the statistical analysis.

3.9. Conclusion:

In this chapter, it has been concluded that a *Xenopus* oocyte can be used to study the binding dynamics of a lineage determination factor Ascl-1. This conclusion has been reached through a comprehensive analysis of the experiments performed in this chapter. Expression of Ascl-1 in a dose dependent manner in the oocyte indicates the likelihood that injected mRNA is encoding a functional protein which binds to the target site followed by activation of reporter DNA. ChIP analysis further confirms that the activation of reporter DNA is dependent on the cognitive binding site derived from Dll1 (mouse) and no other Ebox-like sequence in the plasmid is resulting the productive elongation. However, Anti-Pol-II Ser 2 and Ser5 ChIP shows the binding of Ascl-1 at the Ebox like sequences in the plasmid followed by the recruitment of Pol-II. However, it has been shown that due to the absence of many factor(s) Pol-II could not initiate the transcription.

Competition between histone and Ascl-1 for the reporter DNA suggest the accurate and precise capture of Ascl-1 DNA interaction. This precise capture of DNA interaction serves as a vital tool for the study of transcription factor binding dynamics with respect to its dwell time. Furthermore, dynamics of general transcription factors has also been investigated. The precise location and composition of RNA-pol II and co-factors are crucial for the understanding of transcriptional regulation.

Lastly, a DNA pull down method followed by qPCR is a promising foundation of pulling down chromatin fragments. This method can be further used to investigate the composition of proteins that bind to regulatory regions of Reporter DNA injected into *Xenopus* oocytes.

In summary, all the experiments have suggested that *Xenopus* oocytes can be used to study the dynamics of transcription factor Ascl-1 on its binding site

The stable transcriptionally active and inactive state of the same DNA template can co-exist in the same nucleus?

Introduction:

Chapter number 3 discussed that a *Xenopus* oocyte is a valid system to study the mode-of-action of a lineage determination transcription factor Ascl-1. Injected mRNA of the Ascl-1 transcription factor was successfully expressed in the oocyte in a dose-dependent manner and enabled firefly and Renilla reporter expression depending upon the DNA used (Figure 3.1). Furthermore, it has been found that a 4ng of Ascl-1 mRNA can saturate the binding sites available on the 50 pg of Firefly plasmid (Chapter 3 Figure 3.7). It has been demonstrated that the Ascl-1 mediated transcription in a *Xenopus* oocyte is very robust; typically, an oocyte preloaded with Ascl-1 starts the transcription of the DNA template within 2 hours by the recruitment of RNA-Pol-II and general transcription factors (Figure 3.6). Moreover, Ascl-1 can be detected bound to Plasmid binding site 72 hours after the DNA injection suggesting an association of Ascl-1 protein to its DNA is long-lasting.

In this chapter, the aim has been set to develop some assays which enable us to investigate Ascl-1's binding dynamics in precise detail. The major question asked in this chapter is; whether the transcription factor Ascl-1's binding to its DNA site in the *Xenopus* oocyte is long-lasting? Or the Ascl-1 molecules dissociate quickly from their binding site? To address this question, a novel strategy has been used to study the relative occupancy time of Ascl-1. The hypothesis and the strategy of a DNA competition experiment, and how it helps to decipher the Ascl-1's dwell time in oocytes is explained as follows.

Hypothesis:

Dwell time of a transcription factor is defined as the amount of time one molecule of transcription factor spends on its DNA site in a functional transcription complex (Suter, 2020).

To investigate the dwell time or occupancy time of Ascl-1 on DNA-FF in the *Xenopus* oocyte GV, I performed a DNA competition experiment, which is explained in detail below.

In DNA competition assay, two DNA (DNA-FF and DNA-Ren) containing an identical binding site are used. The only difference between these two plasmid DNAs is the presence of the reporters. In DNA-FF, binding of Ascl-1 expresses the Firefly reporter, while in DNA-Ren, a similar binding event causes an expression of the Renilla reporter. Both Firefly and Renilla can be measured through an illuminometer, and the results can be quantified (Branchini et al., 2018). In the experimental procedure, Ascl-1 mRNA is injected into the cytoplasm of the *Xenopus* oocytes on Day 0, and the injected oocytes are incubated overnight. On Day 1, the Ascl-1 injected oocytes are given DNA-FF through nuclear (GV) injections. On Day 3, the injected oocytes are again given a higher concentration of the DNA-Ren in the same GV. After the 2nd DNA injection, the triple injected oocytes are incubated overnight for competition and are assayed on the following day.

There are two possible outcomes of the DNA competition experiment, both of which are explained below.

- 1) **Activation of 2nd injected Renilla DNA**
- 2) **No-activation of 2nd Injected Renilla DNA**

In the first hypothesis, if the transcription factor is turning over from its binding site, it will activate the 2nd DNA, which essentially contains the same binding site. Turning over Ascl-1 molecules will not be able to distinguish between the two templates. The similarity between two binding sites will enable activation of the 2nd injected Renilla DNA. The ratio of Renilla and Firefly expression will allow us to estimate the general occupancy time of Ascl-1 molecules in the *Xenopus* oocyte.

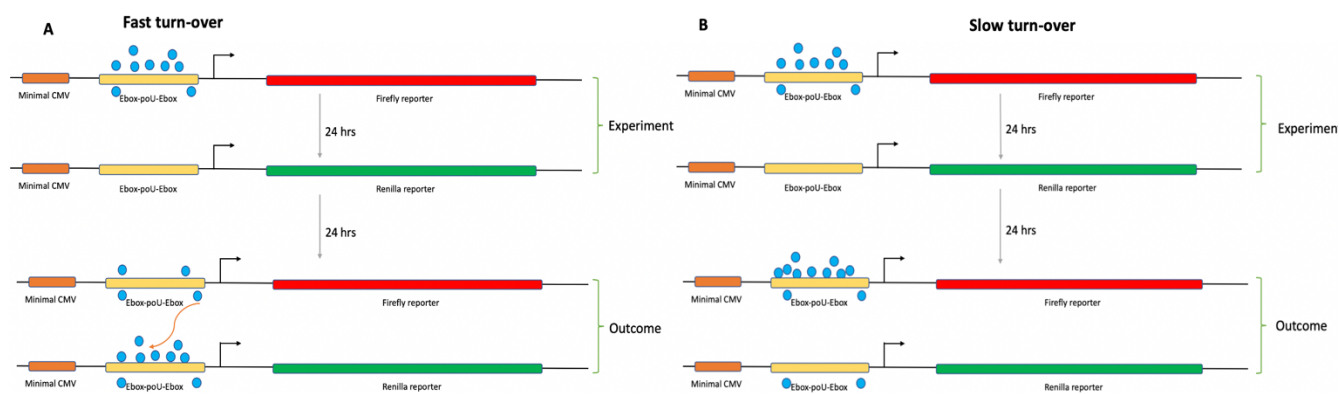


Figure 4.1: DNA competition as an assay to study the transcription factor turn-over rate in the *Xenopus oocyte*.

A) Shows the condition where *Ascl-1* turns-over rapidly and is available to 2nd Injected *Renilla* DNA for transcription initiation. B) shows tight binding of *Ascl-1* to 1st Plasmid and hence is not available to 2nd Plasmid for transcription even after 24 hours of competition.

4.1: A DNA competition assay reveals differential transcription of sequentially available DNA template within the same nucleus?

According to the hypothesis, if *Ascl-1* is turning over from its binding site, it will then activate *Renilla* reporter, which is supplied as a competitor plasmid with a non-distinguishable Ebbox site. The reason for this experiment is to study how effectively a reporter Plasmid, which is injected as competitor DNA, is expressed in the presence of a limiting amount of *Ascl-1*.

4.1.1. Experimental procedures:

To analyse the turn-over rate of *Ascl-1* from its binding site in the oocyte, a limiting amount of (210 pg) of *Ascl-1* mRNA was injected into the oocyte. The next day, oocytes were injected with the FF plasmid bearing the *Ascl-1* binding motif at the concentration of 210 pg per oocyte. During this time, *Ascl-1* establishes a transcription, and the signals can be seen by the Firefly (FF) assay. After 24 hours of the first DNA injected, the 2nd DNA with *Renilla* reporter (DNA-Ren) with *Ascl-1* binding site was injected into the oocytes and then incubated for another day (Figure 4.2 A).

4.1.2. Results

After the final incubation, the healthy oocytes were selected and then subjected to Firefly and Renilla assays to determine the expression of the reporters activated in response to the binding of Ascl-1. It was surprising to find out that the 2nd injected plasmid DNA (Renilla) was not expressed when the values were compared to Renilla alone Figure 4.2 B (Ascl-1>FF-L>FF-R). To confirm that this effect of Renilla under-expression is not because of its DNA sequence, I reversed the injection order of DNA-FF and DNA-Ren such as; DNA-Ren was injected on Day 1 and DNA-FF was injected on day 2, while Ascl-1 injections remained unaltered. Analysis of reporter expression further showed that DNA-FF is not expressed while DNA-Ren was activated by Ascl-1 (Figure 4.2B Ascl-1>FF-R>FF-L). This result showed that the DNA which is added sequentially in the *Xenopus* oocyte is under-expressed or not expressed at all.

Interestingly, when Firefly and Renilla plasmids were injected together, both of them expressed equally well with respect to their controls. Furthermore, a significant decrease was observed in Firefly and Renilla values when they were injected together as compared to injected alone in the presence of Ascl-1 when Ascl-1 mRNA is in limiting amount. This decrease in value is perhaps the reason for an equal distribution of transcription factors among these plasmids when they are co-injected.

Since the activation of DNA reporters depends on the binding of Ascl-1 to the Ebox sequence, it can be concluded that the Ascl-1 is tightly bound to the first DNA.

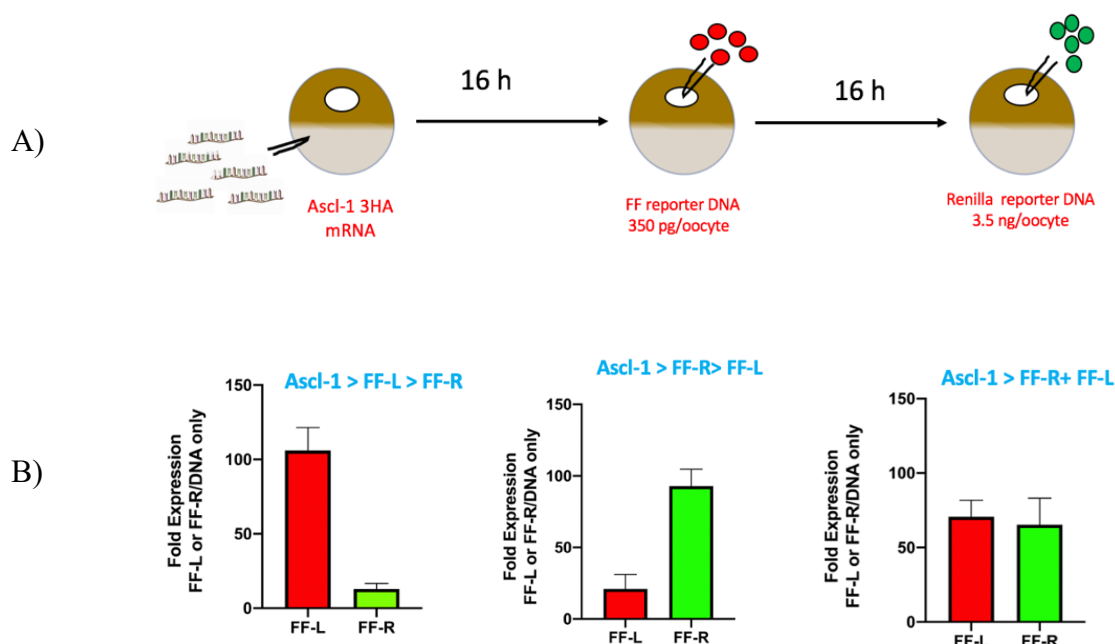


Figure 4.2: Non-competition state of 2nd sequentially injected Plasmid DNA into the *Xenopus* oocyte.

A) Schematic diagram of sequential injections into the *Xenopus* oocyte GV. Each injection was followed by 16-18 hours incubation at 18 °C. B) *Xenopus* oocytes were injected with 350 pg of *Ascl-1* 3HA mRNA, 350 pg of Firefly DNA, and 3.5 ng of Renilla DNA into the GV of an oocyte in three sequential injections. The oocytes were incubated overnight and then assayed for the *Ascl-1* mediated Firefly and Renilla reporter expression. Each sample contained eight individual oocytes.

** Data shows the average values of three independent experiments.

*** Statistic was performed by Student's T-test and Two-way ANOVA.

4.1.3. Conclusion:

DNA competition experiment reveals that the 2nd injected DNA is not well expressed in the *Xenopus* oocyte despite its high concentration. This finding tells us that, maybe, the injected limiting amount of *Ascl-1* is associated tightly to the 1st injected DNA and not available to the 2nd DNA.

4.2: Timing of injected DNA plasmid and chromatinization does not interfere with its expression

The previous experiment has shown that 2nd injected plasmid DNA in the *Xenopus* oocyte is not expressed despite of its high concentration to its predecessor. I ruled out that one possibility is that the injected limiting amount of Ascl-1 is tightly associated with the 1st injected plasmid DNA. However, another possibility may also explain this phenomenon, i.e., DNA, which is injected 1st may be converted into a transcriptionally active, mature state, while the DNA injected as 2nd is not mature enough to support the transcription. To test the 2nd possibility of transcriptional maturation of 1st Plasmid, an experiment was designed in which Ascl-1 was allowed to bind to the DNA, which already has stayed more than 24 hours in a GV of an oocyte.

4.2.1 Experimental procedure:

To test the hypothesis, a condition was established where Ascl-1 mRNA was injected on day 0, which is followed by the injection of reporter DNA-FF on day 1 (Figure 4.3 A). Contrarily, in another experimental condition, DNA-FF was injected on Day 0, while Ascl-1 mRNA was injected on Day 1 (Figure A1). After the sequential injections of the DNA and mRNA, the oocytes were incubated overnight. On the next day, oocytes were lysed and analysed by CHIP and Firefly and Renilla reporter assays. The term "Chromatinised" was used to refer to the DNA-FF, which was injected on Day 0. While the term "Unchromatinsed" DNA is referred to DNA-FF when it is injected on day 0.

First, I asked whether there is a difference in the extent to which Ascl-1 activates the Firefly reporter from DNA-FF when it's in either of the chromatinised or unchromatinsed forms. Firefly assay shows that irrespective of the chromatin state of the plasmid DNA, the Ascl-1 activates Firefly expression in a similar fashion (Figure 4.3B). Next, I asked whether the chromatin state of the Plasmid changes the binding strength of Ascl-1 on its binding site?

To answer this question, I performed a ChIP that detects the enrichment of Ascl-1 on the injected DNA. Figure 4.3C shows that the DNA injected on Day-0 supports more enrichment of Ascl-1 (Figure 4.3C red dots) on the DNA when compared to the DNA injected on Day1 (Figure 4.3C blue dots). But the difference seen in the binding is merely 10-15%.

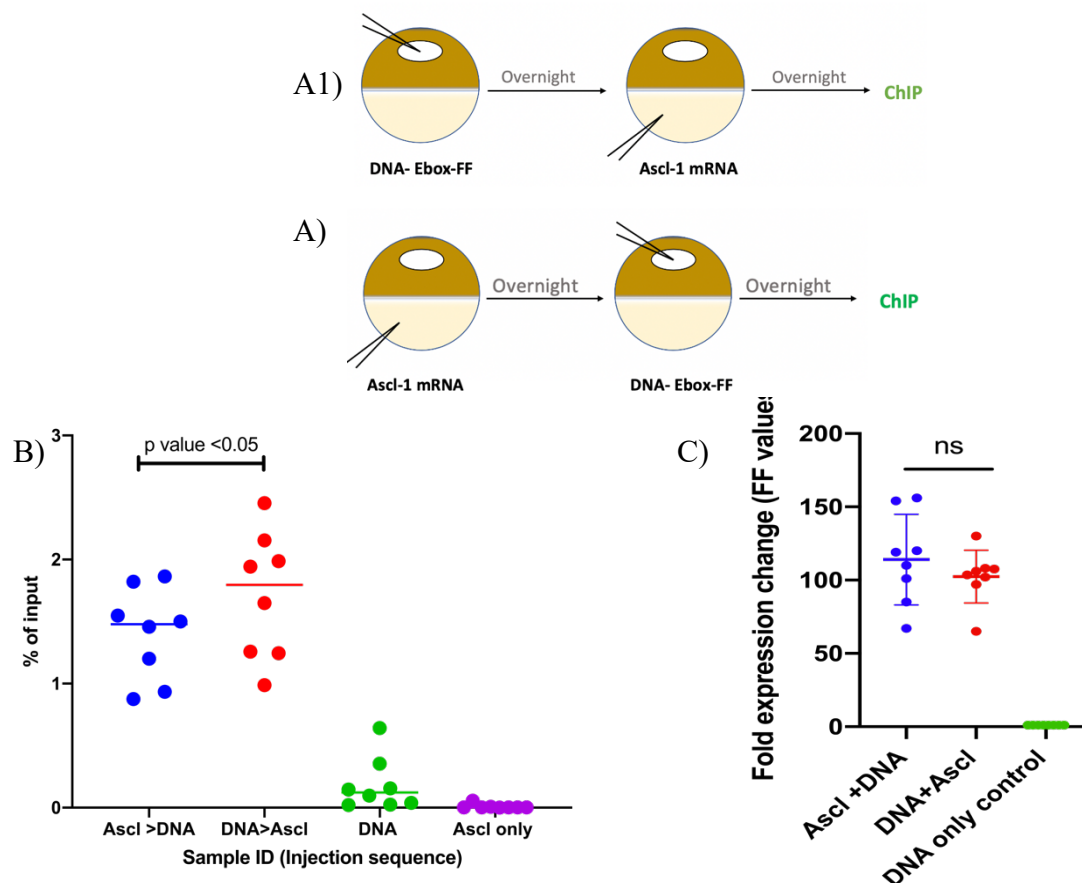


Figure 4.3: Chromatin state of injected DNA template influences Ascl-1 binding but not the resultant expression of the reporter.

A) Schematic diagram of injection into *Xenopus* oocyte. B) Differential binding strength of Ascl-1 on chromatinised and unchromatinised DNA. Oocytes were injected with DNA at Day 0 (chromatinised Red) and DNA at Day 1 (unchromatinised Blue) in the presence of Ascl-1. ChIP was performed by Anti-Ascl-1 Antibody 24h post the last injection. C) Non-significant change in the Ascl-1 mediated expression strength of chromatinised and unchromatinised DNA. The graph shows the fold expression of Ascl+DNA/DNA only.

** Student T-test was performed to know the statistical significance among the samples. Each sample contained 20 oocytes. n=3

*** Student T-test was performed to know the statistical significance among the samples. Each sample contained eight individual oocytes n=3

4.2.2. Conclusion:

The ChIP and Firefly assays performed on the chromatinised and unchromatinised DNA show the time a DNA spends in the GV of the oocyte does not change its ability to support the transcription by Ascl-1. Therefore, the hypothesis of 2nd Plasmid in a less mature state has not been supported by the experiment. It has been found that the rate of transcription of a reporter's DNA is the same and independent of its order of addition to the GV of an oocyte.

4.3. Dominance of first DNA template transcription arises at a threshold concentration.

Transcriptional efficacy depends upon the availability of transcriptional machinery. If the template exceeds the available general transcription machinery, the host cell would be able to express another template, and if the co-factors are tightly bound to the first template, the host cell would not be able to activate the template injected as 2nd. To further investigate the above-mentioned postulates, I asked whether the DNA injected as 1st has a threshold value which it would render 2nd DNA under-expressed in the *Xenopus* oocyte?

4.3.1. Experimental procedure:

A limiting amount of Ascl-1 mRNA (<1ng/oocyte) was injected into the cytoplasm of *Xenopus* oocytes. The preinjected oocytes were injected with different doses of DNA-FF, ranging from 10-500pg/ oocyte. On Day 2, the same preinjected oocytes were then injected with a fixed amount of DNA-Ren. Oocytes were then incubated overnight and then processed for the Firefly and Renilla assays (Figure 4.4A).

4.3.2. Results

Firefly assay shows the dose-dependent activation by the limiting amount of Ascl-1 (Figure 4.4B **Red line**). More the DNA-FF injected in the GV, more Firefly activation is observed. However, Renilla assay shows that its under expression starts when the DNA-FF dose exceeds the 200 pg/oocyte concentration (Figure 4.4B **Green line**). If the 1st injected

DNA-FF's concentration is lower than 200pg/oocyte, the activation of the second Renilla plasmid was observed. Analysis of DNA-FF and DNA-Ren plasmid sequences revealed the presence of a transcriptionally active SV40 promoter, which can be actively transcribed in the oocyte without any inducer. To check the contribution of the SV40 promoter, I deleted the Sv40 promoter sequence from DNA-FF, and the resultant Plasmid is called, Δ Sv40-FF. Injection of Δ SV40-FF DNA as a first DNA increased the threshold value to ~380 pg/oocyte (Figure 4.4B Black line). It means that 200 pg of WT DNA-FF is enough to create the competition state, while this amount goes up to about 40 % when the SV40 promoter is deleted from the DNA-FF. Firefly activation of Δ SV40-FF shows that the deletion of the SV40 promoter from the DNA-FF does not have a profound effect on the overall function of the Plasmid (Figure 4.4B Orange line).

4.3.3. Conclusion

Dose-dependent injection of DNA-FF and its mutant Δ Sv40-FF showed that there is a threshold concentration of 1st plasmid, which may saturate either Ascl-1 or General transcription factor. This saturation then results in the under-expression of 2nd injected Plasmid. The threshold dose ranges from 200pg/oocyte with dual promoters, while 380pg/oocyte with the Δ Sv40-FF.

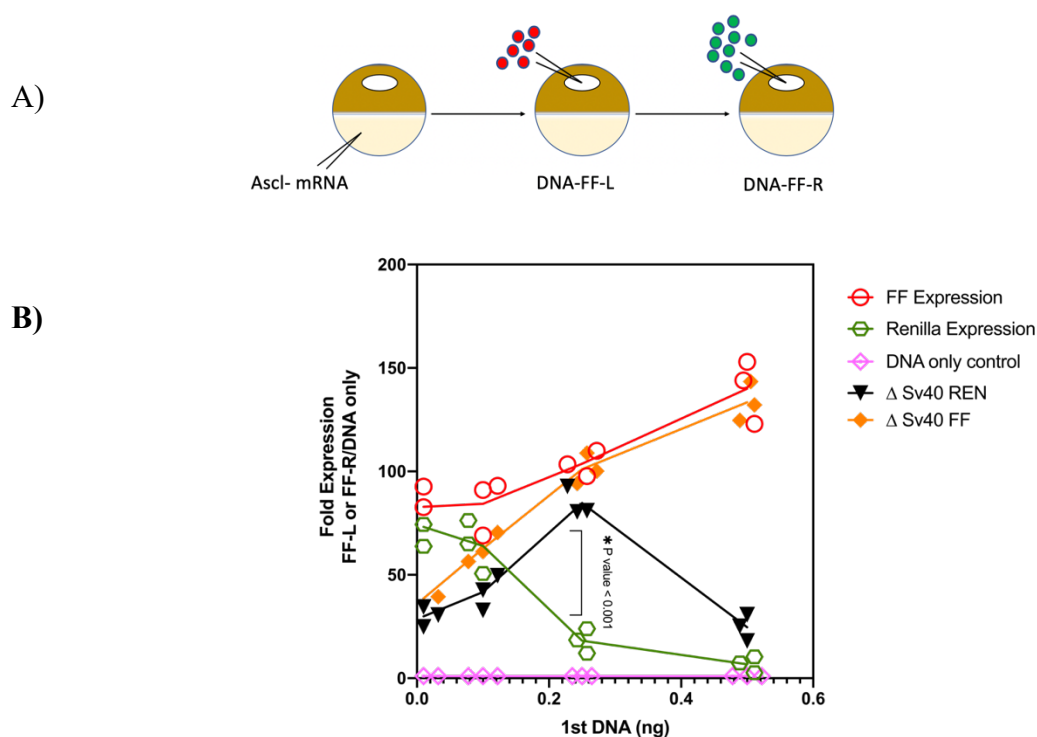


Figure 4.4: Resistance to 2nd DNA expression depends upon the concentration of 1st Plasmid and numbers of promoters.

A) Schematic diagram of the sequential DNA injection into oocyte B) Correlation of 1st plasmid concentration to the expression of 2nd Plasmid. Oocytes were injected with 10, 100, 200, 500 pg of FF DNA as 1st DNA followed by Renilla as ten times more than Firefly counterpart. Oocytes were assayed post 24 h of last DNA injection. N=3. B) Under-expression of 2nd injected DNA depends upon the number of transcriptionally active promoters. Oocytes were injected with 10, 200, 300, 400, and 500 pg of ΔSv40 promoter FF DNA as 1st DNA. Graphs show the FF and Renilla values taken 24 post 2nd DNA injection.

** Distribution pattern shows the average of n=3 values. In each experiment, eight individual oocytes were taken.

*** Statistical analysis was performed by the student t-test.

4.4: Dominance of first DNA template transcription is progressively established

In the standard DNA competition experiment, the injection of the two plasmids DNA is separated by 16 hours, and in this condition, only the first Plasmid is expressed (Figure 4.1 B). By contrast, if the DNAs are injected together, both are expressed in (Figure 4.1 D). I asked how much time 1st injected DNA-FF has to spend in the oocyte GV to become resistant to competition by another DNA? To answer this question, I performed a Time-course experiment.

In this experiment, 2nd DNA-Ren was injected in a time-course manner relative to 1st DNA injection.

4.4.1. Experimental procedure

A limiting amount of Ascl-1 mRNA was injected into the oocytes on Day-0. On Day 1, DNA-FF was injected into the Ascl-1 preinjected oocytes at the concentration of 300p/oocyte. A time-course injection of DNA-Ren was performed at T=0, T=30 min, T=60 min, T=120 min, T=600 min, and T=1400 minutes. The oocytes were collected after 24 hours of each DNA-Ren injection and assayed for Firefly and Renilla reporter expression (Figure 4.5A).

4.4.2. Results

Firefly and Renilla assay were performed to show the trend of DNA competition during the time-course. Renilla expression values show that if DNA-Ren is injected together with the DNA-FF, they both co-express (Figure 4.1B). However, if the time between the sequential injection of DNA-FF and DNA Renilla exceeds 6 hrs, the 1st injected DNA-FF becomes resistant to the competition (Figure 4.5 B)

4.4.3. Conclusion

The time-course injection of DNA-FF and DNA-Ren in the *Xenopus* oocyte revealed that 1st DNA needs approximately 6 hours to be able to become competition resistant.

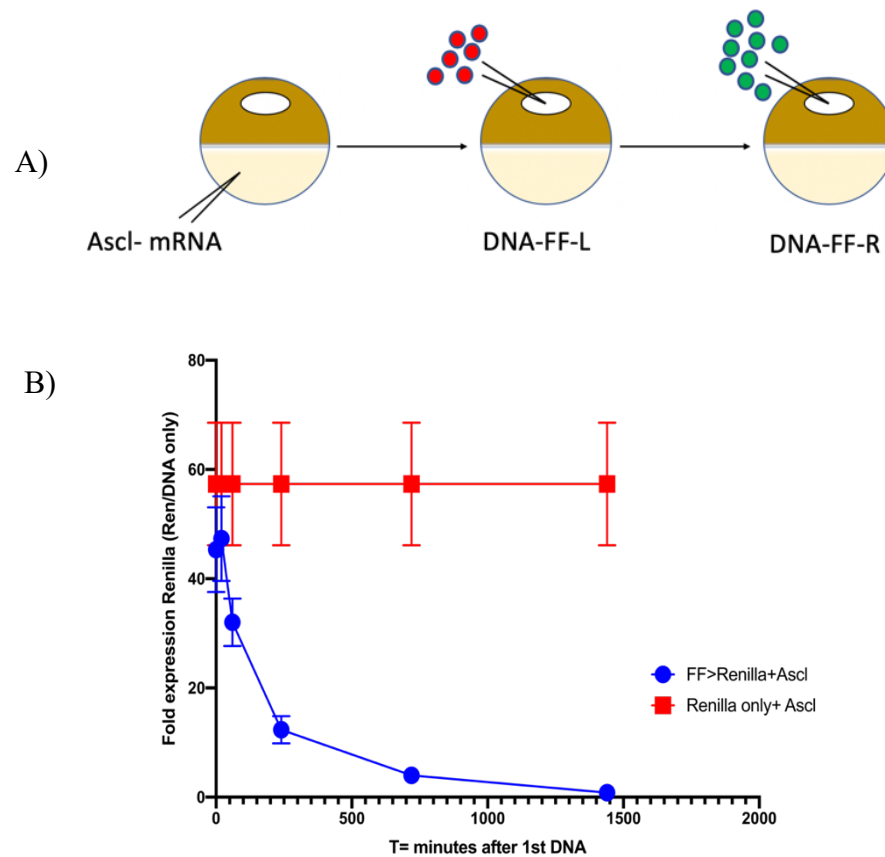


Figure 4.5: Resistance of 2nd DNA expression crucially depends on its time of injection after 1st DNA.

A) Schematic diagram showing the dose-dependent and sequential DNA injection into the oocyte. B) Expression of Renilla DNA injected at different times (minute: 0, 30, 60, 120, 600, 1400) after the first DNA. Renilla Assay was carried after 24 hours of 2nd DNA injection. One condition of 24 hours injections was taken the next day. The graph shows the Renilla values Experiment (Blue) vs. a line drawn by replicating the same value of Renilla only+ control (Red) to show the comparison.

** Error bar represent St Deviation of 3 independent experiments; each experiment contains eight individual oocytes.

4.5: Dominance of first DNA template transcription is established irrespective of the promoter driving expression

Fig4.4 suggests that the extent of transcription rather than the nature of the promoter driving transcription is important to establish dominant expression. The reason for this could be limitation of Ascl-1 or the factors needed by Ascl-1 to start transcription. This raises the

possibility that the differential expression of sequentially expressed plasmids stems from competition for components of the general transcription machinery rather than for gene-specific transcription factors. To formally test this hypothesis, a competition assay was set up using plasmids harbouring different types of promoters. The purpose of this experiment was to ask whether all types of promoters have a similar effect of resistance to DNA competition in the *Xenopus* oocyte?

4.5.1. Experimental procedure:

A limiting amount of Ascl-1 (<1ng) was injected into the cytoplasm of the *Xenopus* oocytes on Day 0. On Day 1, the same preinjected oocytes were injected with 210 pg of DNA-FF into the GV. In addition to DNA-FF injections, some Ascl-1 injected oocytes were injected with a variety of Ascl-1 non-specific DNAs such as CMV-GFP as the first DNA. After the 1st DNA injection, the oocytes were injected with the DNA-Ren, as a 2nd DNA at a high concentration of 3ng/oocyte. The triple injected oocytes were incubated overnight and assayed with Firefly, Renilla (Figure 4.6A)

4.5.2. Results

Expression of Renilla reporter preceded by a variety of Ascl-1 dependent and Ascl-1 independent DNA shows a variable trend in the activation. Renilla values show that when DNA-Ren is preceded by DNA-FF, it is not activated as we have seen in (Figure 4.1) and also in (Figure 4.6B WT-FF-L). Surprisingly, when DNA-Ren is preceded by CMV-GFP DNA, Renilla reporter expression shows the same trend, as it shows in the Ascl-1 dependent DNA-FF (Figure 4.6B CMV-mGFP). However, FF DNA with the deletion of all promoter Δ Transcription is injected as 1st DNA; the Ascl-1 present in the oocyte did activate the DNA-Ren (Figure 4.6 Δ Transcription FF-L). This finding suggests that a variety of transcriptionally

active DNAs that precedes the 2nd injected DNA-Ren in the oocytes become resistant to competition by another DNA.

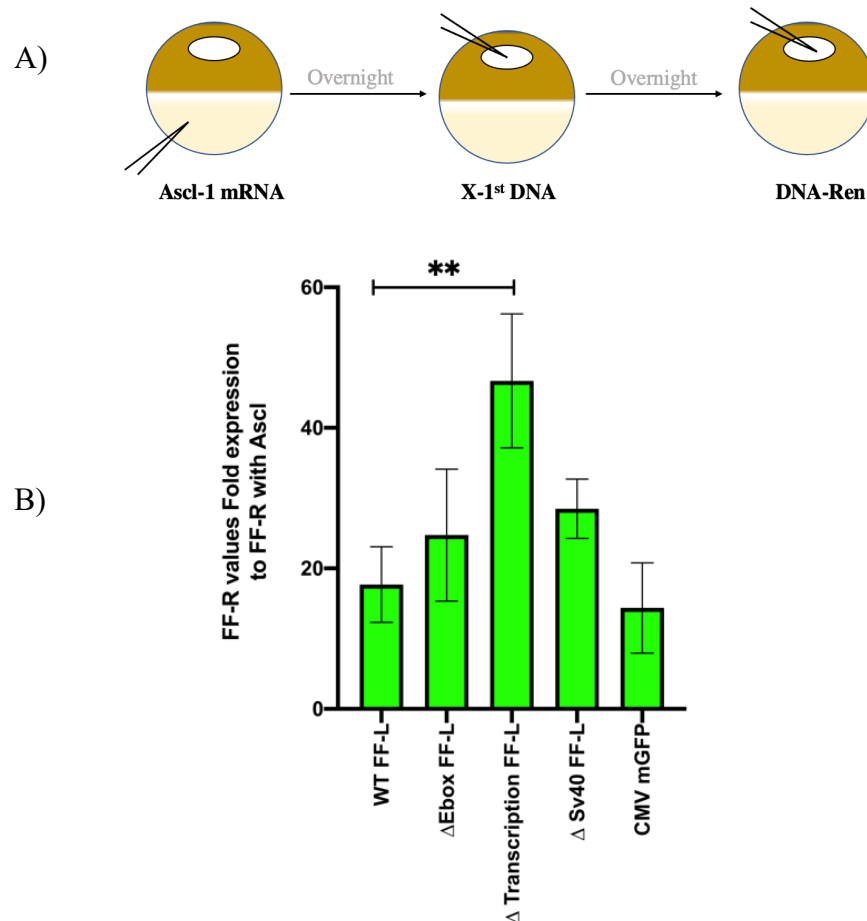


Figure 4.6: Resistance to the competition is not conserved to Ascl-1 mediated motif only.

A) Schematic diagram of the sequential DNA injections into *Xenopus* oocyte. B) Luminometer values for 2nd DNA expression preceded by the 1st DNA either transcriptionally active or inactive.

** Error bars show the SEM $n=3$. Each experiment contained eight individual oocytes per condition.

4.5.3. Conclusion

This experiment suggests a variety of DNAs can become resistant to competition by another DNA when injected into the oocytes. However, a part of this resistance comes from the limiting amount of Ascl-1. This experiment also suggests that the resistance results from the limitation of multiple transcription factors.

4.6 Conclusions

In this chapter, it has been shown that if a transcriptionally active DNA is injected into the *Xenopus* oocyte nucleus, which is preloaded with the transcription factor Ascl-1, resists the competition by the same type of DNA with another reporter even at the higher concentration. This type of behaviour is expected to arise from two possibilities. 1) Very tight binding of Ascl-1 to the 1st injected DNA. 2) Saturation of the 1st DNA with the general transcriptional machinery of the oocyte.

Lineage determination factors can stay longer than expected on their binding site for regulating the gene expression. However, little is known about their function in terminally differentiated cells. In the Ascl-1 limiting conditions, underexpression of the Ascl-1 driven FF-R in an oocyte preceded by another DNA suggests that the Ascl-1 transcription factor is not available for the 2nd DNA to start the transcription. Under-expression of 2nd DNA depends on the timing and concentration of 1st DNA. 200 pg of FF DNA with the dual promoter and 300 pg of DNA with single promoters are substantial for under-expressing the 2nd DNA injected into the oocyte. Furthermore, co-injection of both DNA successfully express Firefly and Renilla. Furthermore, By injecting constitutively expressing DNA into the oocyte as 1st, it has been shown that any transcriptionally active DNA has a tendency to compete for the factors necessary for the transcription of 2nd injected DNA.

Thus, the competition of factor (s), which are crucial for the 2nd DNA, could fall into two hypotheses. 1) Since the Ascl-1 injected in the oocyte is limiting and not available to the 2nd injected DNA to carry on the transcription of 2nd sequentially injected DNA. 2) General transcription factors are limiting to the 1st injected DNA and not available to the 2nd DNA when it is injected after a certain period of time. To further know about the possibility of the Ascl-1 low turn-over rate, it is necessary to establish an assay where the binding of Ascl-1 can be quantitatively measured.

Binding kinetics of Ascl-1 on injected plasmid DNA in *Xenopus* oocyte

Introduction:

In chapter 4, a series of DNA competition experiments illustrated that the DNA-FF injected into the *Xenopus* oocyte is converted into a competition resistant form and does not allow any other DNA to compete with it. Further experiments showed that this resistance to the competition is mainly because of the limitation of one or more transcription factor(s) (Figure 4.6). However, it is not known if the limitation is because of Ascl-1 being a limiting factor or another oocyte-specific transcription factor or if multiple factors are contributing to the phenotype of resistance to the competition. To investigate, if Ascl-1 is a limiting factor, it is suitable to perform an assay that measures the direct binding events of Ascl-1 on both of the plasmids.

In this chapter, *In situ* dynamics of the transcription factor, Ascl-1 is aimed to be thoroughly studied in a non-dividing *Xenopus* oocyte. Several molecular biology approaches such as ChIP and protein ablation techniques such as Auxin mediated degradation of degraon tagged proteins (AID) will be used to study the binding dynamics and kinetics of Ascl-1 on a DNA binding site on the injected plasmid. Three key questions of Ascl-1 binding dynamics in *Xenopus* oocytes are discussed here.

The first question asks, is the continuous presence of Ascl-1 is necessary for Ascl-1 mediated transcription by injected plasmid in the GV? In general, the transcription factors regulate transcriptions in different ways, e.g., some transcription factors bind to their binding sites to activate the transcription in bursts (Stavreva et al., 2019) an excellent example of this mode of action is Glucocorticoids receptors (GR), which activate their transcriptions in a bursting manner. While other transcription factors like FoxA2 co-operatively bind with other co-factors to differentially activate their target gene expression (Donaghey et al., 2018).

Additionally, there are some transcription factors that activate their transcription and are then no longer required for its maintenance (Čabart, Újvári, Pal, & Luse, 2011). Regulation of transcription can also vary depending on the cell types. For example, human ES cells transcriptional wiring is dense and very different when compared to differentiated cells types (S. Zhang, Tian, Tran, Choi, & Zhang, 2014) Transcriptional strategies of terminally differentiated cells also can vary from the actively dividing cells (K. A. Jones, 2007).

However, the dynamics of Ascl-1 mediated transcriptional regulation on an injected plasmid in the *Xenopus* oocyte is yet unclear. *Xenopus* oocyte has an ability to form stable transcriptional complexes, which are stable for several rounds of transcription (Darby, Andrews, & Brown, 1988). It is possible that the co-factor(s) or general transcription machinery of the oocyte is holding Ascl-1 to not be available to a 2nd DNA. The inability of 2nd sequentially injected DNA to activate the reporter gene in a limited amount of Ascl-1 can be addressed by degrading Ascl-1 in the GV at the time of 2nd DNA injection. The result can come in two possible ways. First, degradation of protein does not affect the Ebox mediated transcription. Second, degradation of Ascl-1 negatively affects the FF values.

The second question asks, how long a lineage determination factor can occupy its site in a terminally differentiated cell? Whether it binds to its chromatin site more tightly or turns over quickly. Understanding Ascl-1 behaviour on its binding site will help in the study of under-expression of 2nd DNA, which depends on Ascl-1 for transcription. This would be a way of stabilizing the transcription and expression of an initially introduced DNA.

The third question deals with the actual occupancy of Ascl-1 on its binding site. How much time one molecule or similar kind of molecule spends on its DNA binding site during the active state of transcription. To investigate further about the turn-over rate, a modified version of ChIP is used to understand the dynamics of a transcription factor on its DNA binding sites. By performing competition ChIP (Lickwar, Mueller, & Lieb, 2013) of Ascl-1 for a single

site on the injected plasmid, it would be possible to study the occupancy time of Ascl-1 in *Xenopus* oocyte. This method will also be used to understand the behaviour of non-lineage determination factors such as oestrogen receptor.

5.1: Establishing the protein competition Assay:

Competition of transcription factors can decipher meaningful information about transcriptional dynamics in a cell. An example of this is the competition of Rap 1 in yeast (Lickwar et al., 2013), and TATA-binding protein in HEK293 (Hasegawa & Struhl, 2019a) cells. The competition ChIP reveals the promoter-specific turnover rate of these transcription factors of 90 minutes and ~120 minutes respectively. This sort of information is very important to understand how a transcription factor behaves on its binding site. A similar procedure of competition ChIP was designed for the *Xenopus* oocytes to investigate about dwell time of Ascl-1 on its DNA binding site. In this assay, an mRNA encoding of Ascl-1 GFP is injected into the oocyte at Day 0. On Day 1, the same Ascl-1 GFP injected oocytes are injected with DNA-FF with the Ascl-1 binding site (WT and mutant). On Day 2, the pre-injected oocytes were further injected by an mRNA encoding for Ascl-1 3HA NLS (Ascl-1NLS) (Figure 5.1). The purpose of 2nd injected Ascl-NLS to create a state of competition of Ascl-1 for the injected binding site. After the injection of mRNA encoding for the competitor protein, the oocytes were incubated overnight, and the samples were processed for the ChIP.

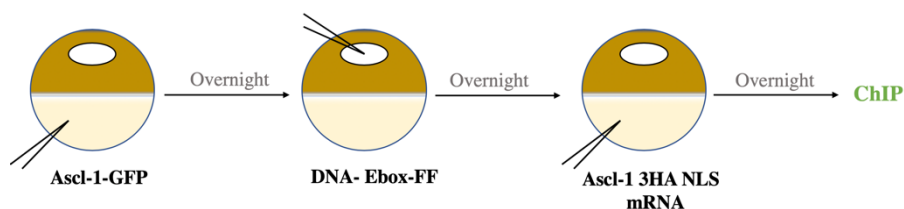


Figure 5.1: injection plan of Ascl-1 protein competition ChIP.

Oocytes were injected Ascl-1 GFP mRNA at 1.4ng/oocyte. On a day later, the same injected oocytes were injected with 300 pg of DNA-FF. On the next day, the preinjected oocytes were injected with a competitor, Ascl-1 mRNA, at 14ng/oocyte. Twenty-four hours post competitor injections, the oocytes were processed for Chromatin Immunoprecipitation.

5.2 Necessity of Ascl-1 on DNA at the time of 2nd DNA under-expression

Next, it was crucial to ask whether the continuous presence of Ascl-1 on the Ebox is necessary for E-box mediated transcription of 1st DNA at the time when the 2nd DNA is injected or, if it is just needed to initiate the process and is then no longer required? To address this question, Auxin mediated degradation system that enables inducible degradation of an AID tagged protein (Lambrus et al., 2018) was used to ablate the Ascl-1 in the oocyte. The rapid ablation of Ascl-1-AID will allow us to determine the continuous presence of Ascl-1 in the GVs of the oocytes.

5.2.1 Experimental procedure:

On Day 0, *Xenopus* oocytes were injected with 7ng of mRNA encoding for Ascl-1-AID and 7 ng of mRNA encoding for the TIR1. Some oocytes were injected with Ascl-1 3HA as a positive control. On the next day, the preinjected oocytes were injected with short-half-life DNA-FF and incubated overnight. On Day 2, the oocyte culture medium was supplemented with Auxin at the final concentration of 1mM (L. Zhang, Ward, Cheng, & Dernburg, 2015). After eight hours of Auxin supplementation, the medium was removed oocytes were washed and processed for Firefly reporter assay and for the Anti-Ascl-1 western blot (Figure 5.2 A).

5.2.2 Results

First, the extent of degradation of the Ascl-1 protein by Auxin was determined through western blots. The Anti-Ascl-1 western blot shows that Ascl-1 bearing the AID tag was rapidly degraded within 8 hours of the Auxin treatment (Figure 5.2B lane 4). It was observed that the Ascl-1 AID is stable in the oocyte if not supplemented with the Auxin (Figure 5.2 B lane 3), it means that there is no possibility through which Ascl-1 AID can be degraded without the presence of Auxin. Furthermore, it has also been confirmed that the addition of Auxin does not

degrade an Ascl-1 that does not contain an AID or degron tag (Figure 5.2 lane 2). Further, I asked if the degradation of the Ascl-1 by Auxin has an effect on the Ascl-1 mediated transcription from DNA-FF.

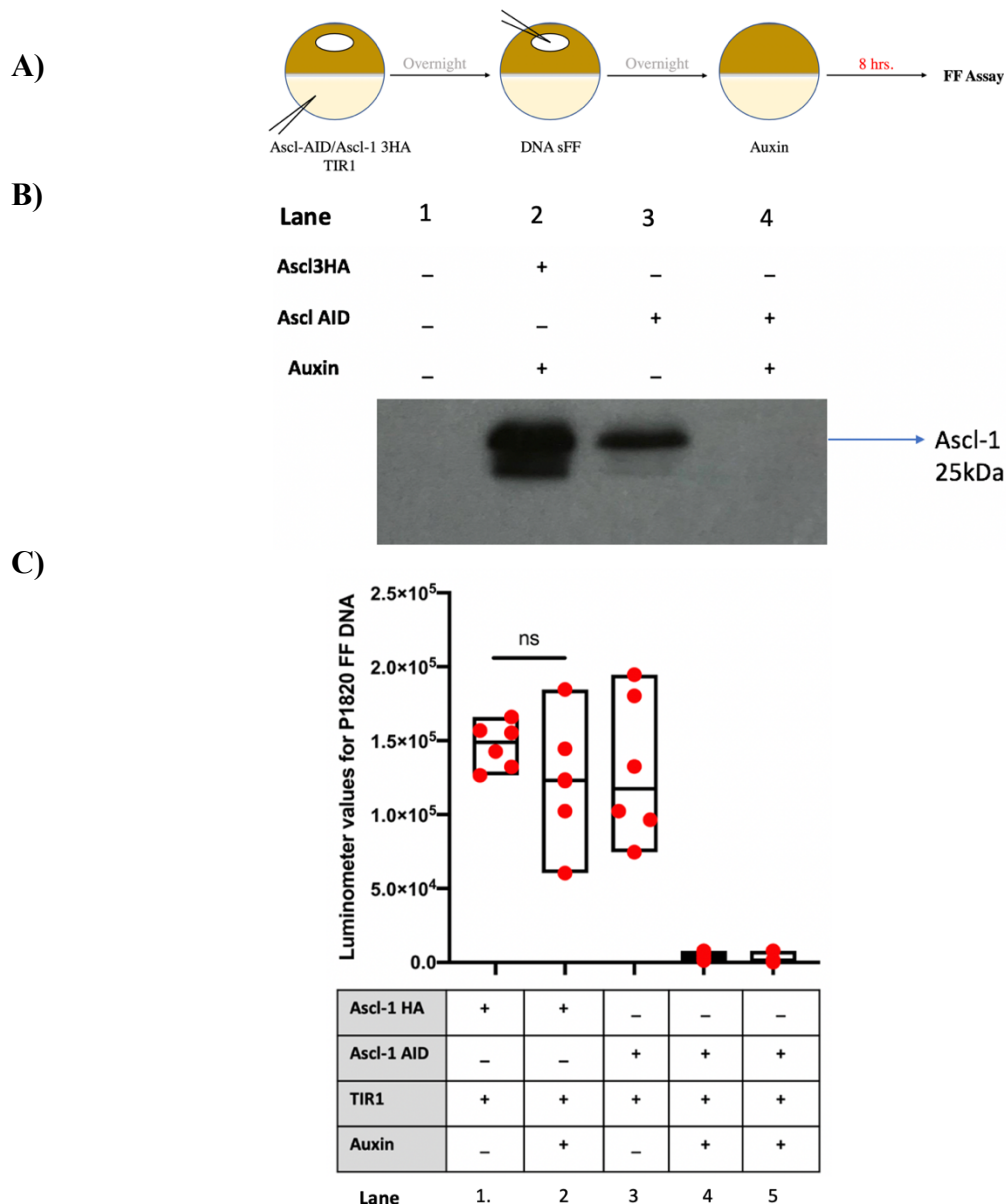


Figure 5.2: Continuous presence of Ascl-1 in the GV is necessary for Ascl-1 mediated transcription at the time of 2nd DNA injection.

A) Schematic diagram of injection of Ascl-1 AID into the oocyte. 7ng of Ascl-1 and TIR1 was injected, followed by DNA on the next day. Auxin was added to the system after 24 hrs. DNA injection. B) Western blot of oocyte treated with 8 hrs. Auxin in the medium and analysed by the Anti-Ascl-1 antibody. C) Firefly expression values were taken after treatment of Ascl-1 injected oocyte for 8 hrs. ns (Non-Significant).

In this experiment, a short half-life (sFF) Firefly has been used. The sFF has shown to be degraded in 0.84 (Leclerc, Boockfor, Faught, & Frawley, 2000) hrs as compared to the wild type enzyme, which can stay up to 4 hours in a living cell. The use of sFF ensures that we are observing the transcription happening in the last 40 minutes. Firefly assay after Auxin degradation shows that the degradation of Ascl-1-AID ablates the transcription (Figure 5.2C columns 5 and 6 from left). The Firefly value is at least 100 times less than a condition where Ascl-1 AID injected oocytes were not supplemented with Auxin (Figure 5.2C column 4). Moreover, it has been confirmed that injection of TIR1 and Auxin addition to the oocytes injected with Ascl-1 3HA injected oocytes do not degrade the protein; hence the reporter expression is stable even with overnight incubation in the Auxin (Figure 5.2C column 2).

5.2.3. Conclusion

Firefly assay after the degradation of Ascl-1AID through Auxin mediated degradation shows that Ascl-1 is necessary for the maintenance of transcription in the oocyte's GV at the time of 2nd DNA injection. This experiment points toward the possible limitation of Ascl-1 for 2nd DNA.

5.3: Continuous occupancy of Ascl-1 on plasmid DNA for 96 hours

Auxin mediated degradation of Ascl-1 AID has suggested that Ascl-1 is needed for the continuous transcription of the injected plasmid. However, the Auxin mediated degradation demonstrated the need for Ascl-1 in the GV for continuous Ascl-1 mediated transcription, but Ascl-1 presence on the actual binding site is yet to be determined. In this experiment, I asked whether, at the time of 2nd DNA (DNA-Ren) injection, the Ascl-1 is still bound to the DNA-FF? This experiment would indicate if Ascl-1 is a limiting factor for the under-expression of 2nd DNA expression.

To find out if Ascl-1's presence at its actual binding site, a ChIP was performed at different time points. This time-course ChIP is different because the mRNA injected in the oocytes contains a degradable AID tag. The purpose of the injection of AID tagged Ascl-1 for ChIP is to demonstrate that Ascl-1 can be deliberately ablated from the GV in a time-controlled manner.

5.3.1. Experimental procedure

An mRNA encoding for either Ascl-1 3HA or Ascl-1AID was injected into the oocytes at the final concentration of 7ng/oocyte on Day 0. On Day 1, the same preinjected oocytes were again injected with 300 pg of the plasmid in the GV. For performing the time-course experiment, oocytes were taken at different time points ranging from T=0-T=96 hours post DNA injections (Figure 5.3A). In a group of oocytes, Ascl-1 AID was degraded by the addition of Auxin, and two time-points were collected, namely; T=24 and T=48 hrs. The oocytes were fixed with 1% formaldehyde, and ChIP was performed by the Anti-Ascl-1 antibody, as mentioned in Chapter 2 in section 2.2.8.).

5.3.2. Results:

Time-course analysis of the general occupancy time of 7ng of Ascl-1 mRNA on the oocytes revealed that Ascl-1 could occupy the DNA binding site for a prolonged period of time (Figure 5.3 B). This prolonged binding can be as much as 96 hrs post DNA injection (Figure 5.3B). However, through ChIP, it has been observed that the binding of Ascl-1 on its DNA binding site is maximum at T=48 hours, which gradually declines toward the endpoint of T=96 hours. This experiment indicates that injected Ascl-1 can occupy the binding site for prolonged times. ChIP analysis of the oocytes where Ascl-1 AID was degraded by the action of Auxin shows that the occupancy has been declined (Figure 5.3B). This means that the addition of

Auxin also degrades the Ascl-1 bound to its binding site. This also suggests that the on-site binding of Ascl-1 is necessary for Firefly reporter expression from DNA-FF.

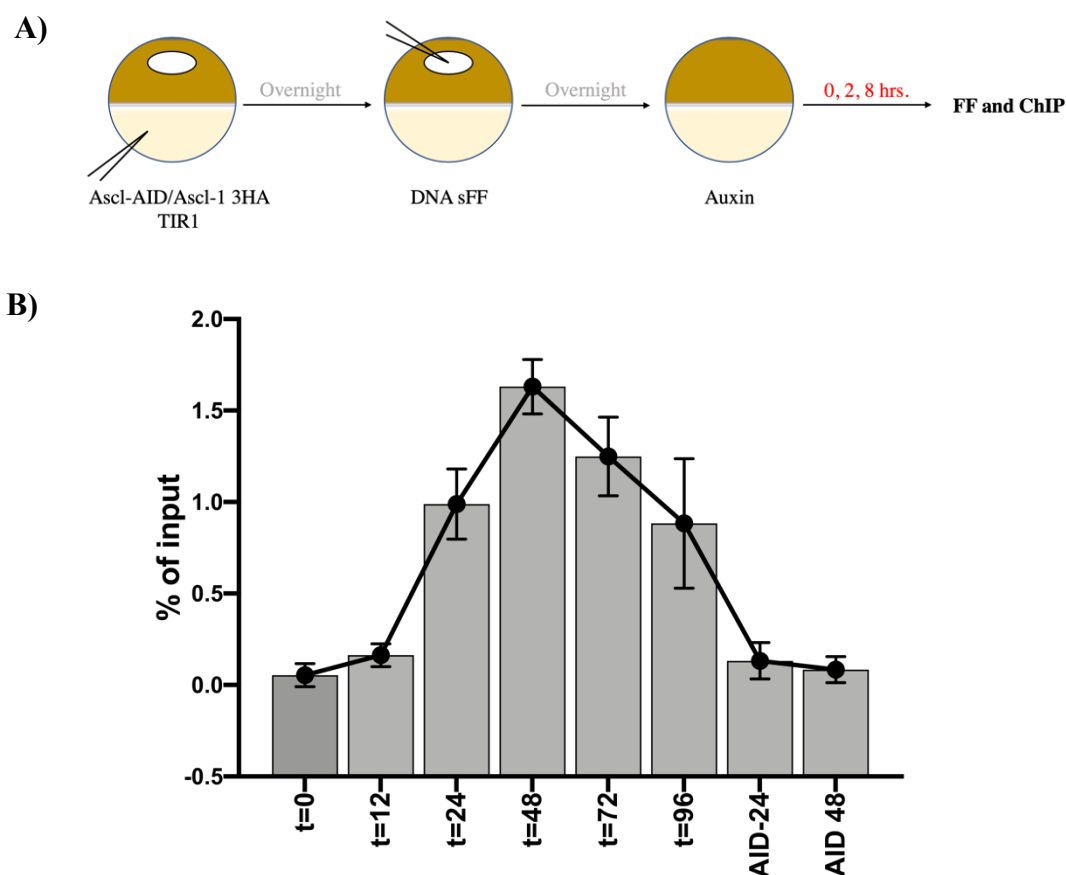


Figure 5.3: Ascl-1 needs to be at its binding site to govern the Ebox mediated transcription at the time of 2nd DNA injection.

A) Schematic diagram showing the injection plan for time Course AID ChIP in oocytes. 7ng of Ascl-1 AID and TIR1 was injected, followed by DNA injection. The next day oocyte was treated with Auxin in the medium for 8 hrs. B) Anti-Ascl-1 chromatin immunoprecipitation of Ascl-1 taken at different time points (0-96 hrs. With Ascl-1HA mRNA and (0-48) for Ascl-1 AID mRNA. Error bars represent st dv. in 3 independent experiments (n=3).

5.3.3. Conclusion

Auxin mediated degradation of Ascl-1 has shown not that just the presence of Ascl-1 in GV is important, but its presence on the binding site is necessary for reporter expression, when a 2nd DNA-Ren is injected. In summary, at the time of 2nd DNA injection, most of the Ascl-1 is engaged with the DNA-FF.

5.4: Ascl-1 competition ChIP in *Xenopus* oocyte

Time course Ascl-1 ChIP and decline of FF signals by Ascl-1 depletion through Auxin shows that Ascl-1 occupancy at the DNA binding site is high (Figure 5.3B). However, it is not known if the high occupancy of Ascl-1 on the first DNA is actually a tight binding or a long dwell time? Which does not allow the engaged Ascl-1 protein to be dissociated from DNA-FF and start the transcription on the DNA-Ren.

To determine if the reason for the prolonged occupancy of Ascl-1 is because of its long dwell time, a transcription factor competition assay was developed, as mentioned in (Section 5.1).

5.4.1. Experimental procedure

A limiting amount of 1.4 ng of mRNA encoding for Ascl-1-GFP protein was injected into the oocytes on Day 0. On Day 1, pre injected oocytes were injected with the 300 pg DNA-FF in the GV and incubated for another 16 hrs (Figure 5.4a). During this incubation time, pre injected Ascl-GFP establishes the active transcription with DNA-FF. On Day 2, 10X, more concentration of mRNA encoding for Ascl-1 3HA was injected into the same preinjected oocytes to establish the condition of transcription factor competition. The injected Ascl-1 GFP and Ascl-1 3HA were allowed to compete for a single binding site present on the injected DNA-FF. After overnight incubation, the oocytes in the Ascl-1 competition condition were fixed with 1% formaldehyde and then processed for the ChIP.

5.4.2. Results

First, to determine whether the competition condition has been successfully established in the oocyte, I performed a western blot of isolated GVs of the oocytes that were injected with Ascl-1 GFP and Ascl-1 HA NLS. Anti-Ascl-1 western blot shows the relative abundance of both Ascl-1 GFP and Ascl-1NLS (Figure 5.4B). Quantification of the western blot shows that competitor Ascl-1 NLS is at least 7 times more abundant than Ascl-1GFP. This result

confirmed that a state of competition had been established in the GV. The abundance of Ascl-1 NLS in the GV means that if the turnover rate of Ascl-1 is high, Ascl-1 NLS will have more chance to bind to the plasmid as compared to Ascl-1 GFP. To test this hypothesis, I performed ChIP with three different antibodies. The function of all these antibodies is explained in table 5.1.

Antibody	Purpose
Anti-Ascl -1	Detects whole Ascl-1 Irrespective of the tag
Anti-GFP	Detects Ascl-1 GFP only
Anti-HA	Detects Ascl-1 3HA NLS

Table 5.1: Antibodies used for competition ChIP and their functions

ChIP by Anti-Ascl-1 antibody answers a question of whether, at the time of competition, all the binding sites of DNA-FF were saturated or not? Anti Ascl-1 ChIP shows that the Ascl-1 GFP, which was injected in a limiting amount, was not able to saturate all the binding sites on DNA-FF (Figure 5.4 C see Anti-Ascl WT). The difference in Ascl-1 GFP and Ascl GFP> Ascl HA conditions show that the binding sites on the DNA-FF were not saturated with Ascl-1 GFP, which means that most of the Ascl-1GFP were associated with the DNA-FF.

ChIP by Anti-GFP measures the abundance of Ascl-1 GFP in the presence and in the absence of the competitor Ascl-1 NLS. Comparison of Ascl-1 GFP and Ascl GFP> Ascl HA conditions (Figure 5.3C see Anti-GFP) show that GFP occupancy from its binding site on DNA-FF has declined to about 40% in 24 hours. Next, I asked whether this occupancy is a general effect of competitor injection, or the reduction of Ascl-1 GFP from DNA-FF has been compensated by the binding of the competitor Ascl-1 NLS. ChIP by Anti-HA antibody showed that the reduction of Ascl-1 GFP had been compensated by the binding of Ascl-1 NLS (Figure 5.4C, see Anti HA).

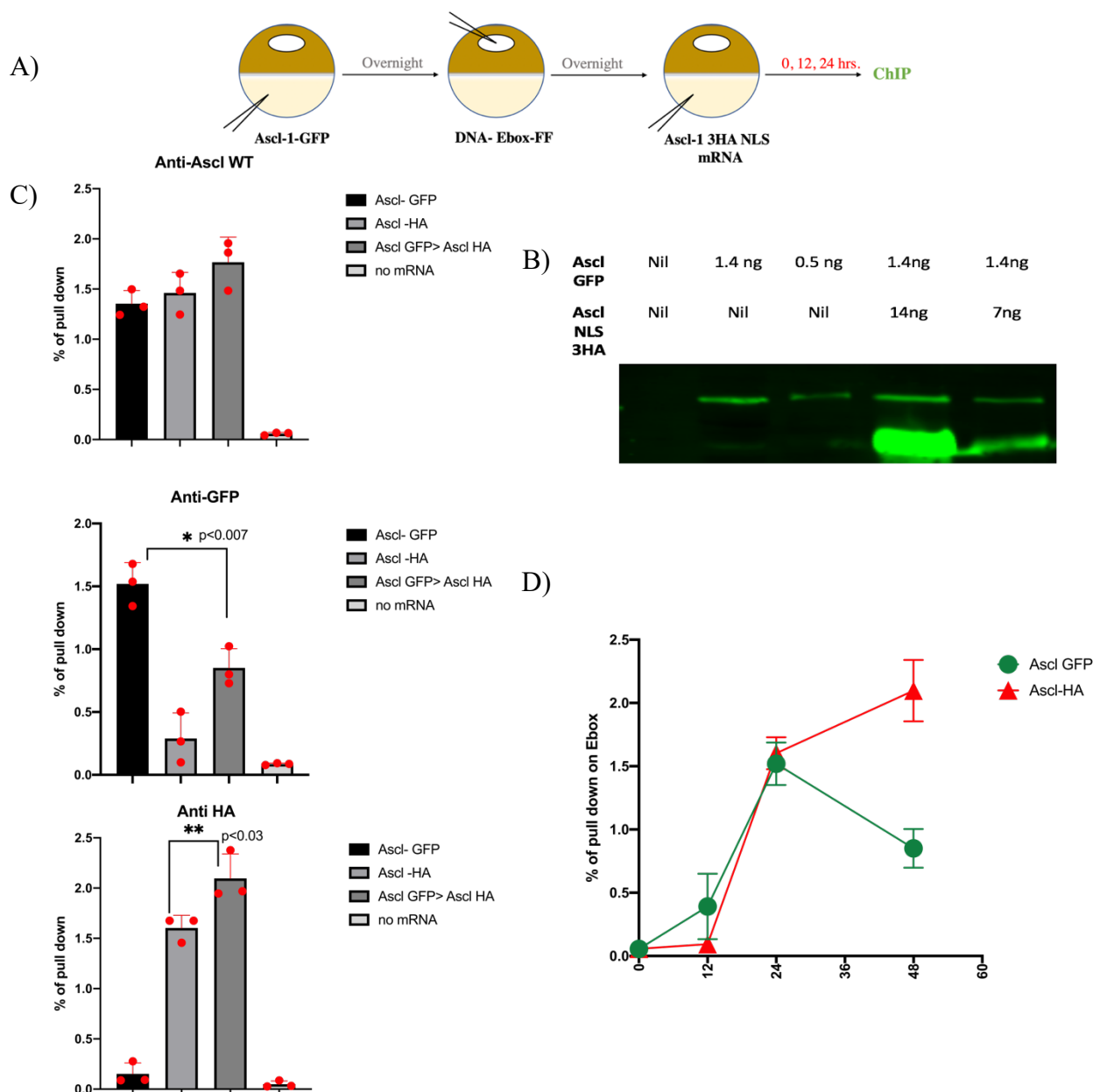


Figure 5.4: Ascl-1 dissociates from its binding site and can be replaced by the same competitor molecule provided later.

A) Schematic diagram of oocyte cytoplasmic and nuclear injection for competition ChIP. Oocytes were injected sequentially with Ascl-1 GFP mRNA followed by DNA and then Ascl-1 3HA NLS mRNA. ChIP was performed at (24hrs.) B) Anti-Ascl-1 western blot of isolated GV nuclei. The relative concentration of Ascl-GFP and Ascl-1 HA were determined in 10 separate GV nuclei. C) Site occupancy of competing Ascl-1 on the injected DNA. Anti Ascl-1 pull down to show the abundance of total Ascl-1 protein. Anti-GFP measures the abundance of Ascl-1 GFP in different conditions, and Anti HA measures the amount of Ascl-1HA at binding sites on the injected plasmid. D) Ascl-1 competition (occupancy trend) at different time points.

Error bars represent the mean value of 3 ($n=3$) independent experiments, each containing 45 individual oocytes. Statistics were performed using unpaired student's *t*-Test.

In another experiment, I determined the competition of Ascl-1 GFP and Ascl-1 NLS beyond the 24 hours' time-point. The question I asked is whether this competition persists even after 24 hours' time? (Figure 5.4D) shows the competition trend of the Ascl-1 GFP **Green line** and Ascl-1 NLS as **Red line**. The graph shows the trend in DNA-FF occupancy at the Ascl-1 binding site. Surprisingly, a sharp decline at T=48 has been observed, which shows that the turnover rate of Ascl-1 may accelerate after 24 hours of competition.

5.4.3. Conclusion

The competition ChIP of Ascl-1 has shown that the Asc-1 has a long residence time around its binding site. Only 40% of Ascl-1 GFP was replaced by the competitor protein in 24 hours. This result further confirms that Ascl-1 could be a possible limiting factor for 2nd DNA under-expression in the oocytes.

5.5: Ascl-1 turns over rapidly on a mutated binding site

The Ascl-1 competition ChIP in section 5.4 has shown a slow turnover rate from the binding site situated on DNA-FF. However, there is also a possibility that this prolonged dwell time of Ascl-1 has arisen from the supporting co-factors of *Xenopus* oocytes, such as poU 60 (Snir, Ofir, Elias, & Frank, 2006) (Cao, Siegel, & Knöchel, 2006). In this experiment, I asked whether a mutated binding site of Ascl-1 will show a similar long dwell time in the oocyte? To answer this question, I performed an Ascl-1 competition ChIP exactly, as mentioned in section 5.4, but the DNA injected was mutated for Ebox-PoU -Ebox domains. (See method section, Figure 2.3).

5.5.1. Experimental procedure

A limiting amount of 1.4 ng of mRNA encoding for Ascl-1-GFP protein was injected into the oocytes on Day 0. On Day 1, pre injected oocytes were injected with the 300 pg of a mutated DNA-FF in the GV and incubated for another 16 hrs (Figure 5.4a). During this incubation time, pre injected Ascl-GFP establishes the active transcription with mutated DNA-

FF. On Day 2, 10X, more concentration of mRNA encoding for Ascl-1 3HA was injected into the same preinjected oocytes to establish the condition of transcription factor competition. The injected Ascl-1 GFP and Ascl-1 3HA were allowed to compete for a single binding site present on the injected DNA-FF. After overnight incubation, the oocytes in the Ascl-1 competition condition were fixed with 1% formaldehyde and then processed for the ChIP.

5.5.2. Results

Surprisingly, Ascl-1 GFP and Ascl-1-NLS without the competitors showed a similar trend of site occupancy as it has been shown in (Figure 5.4C) also see (Figure 5.5 B). However, the main difference is observed in the competition condition when Ascl-1 GFP and Ascl-1 NLS were competing for the same binding site on the Mutated DNA-FF plasmid. (Figure 5.5B see Anti-GFP) shows the occupancy of Ascl-1 GFP on the DNA-FF with and without competitor (see condition “Ascl-GFP” and its comparison with a condition “Ascl-GFP>Ascl-HA” in the section (Figure 5.5 Anti-GFP).

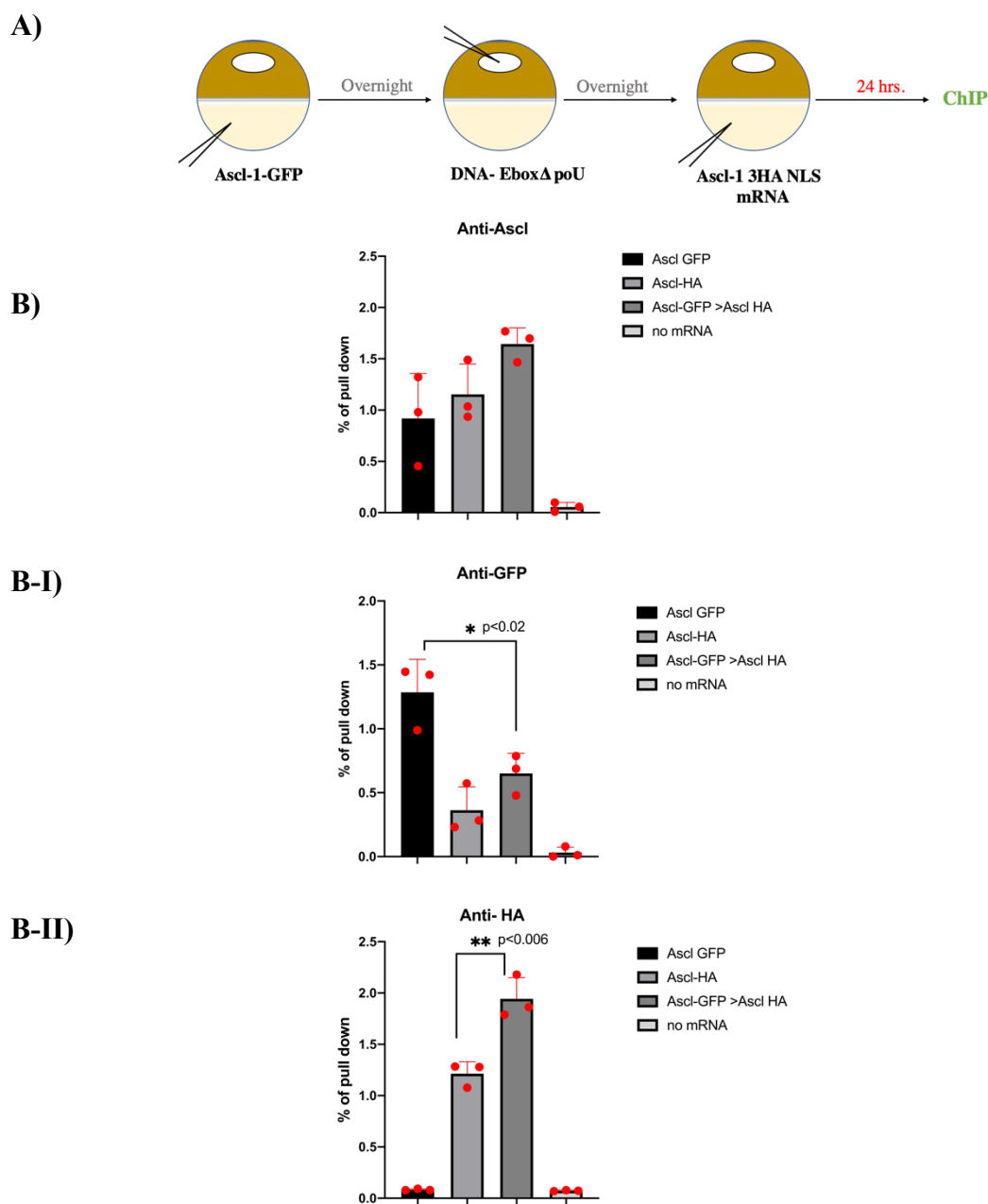


Figure 5.5: relatively rapid turnover rate of Ascl-1 on Δ PoU Ebox DNA binding site within 24 hrs.

A) Schematic diagram of Ascl-1 competition ChIP on Δ POU Ebox FF plasmid. The oocytes were injected sequentially with Ascl-1GFP followed by DNA. 10 times more concentration of Ascl-1 HA was injected a day after DNA injections. B) Shows the abundance and change of occupancy on a mutated binding site on Ebox-FF DNA. Anti Ascl-1 measures the total occupancy by Ascl-1 GFP and Ascl-1 HA. Anti-GFP measures the abundance of Ascl-1 GFP in different conditions, and Anti HA measures the amount of Ascl-1HA at binding sites on the injected plasmid

Error bars represent the mean value of 3 ($n=3$) independent experiments, each containing 45 individual oocytes. Statistics were performed using unpaired student's *t*-Test.

5.5.3 Conclusion

Ascl-1 competition experiment on the mutated binding shown that the Ascl-1 turns over from its binding site more quickly when compared to its turnover rate from DNA-FF. This indicates that the oocyte factors that bind with Ascl-1 may play an important role in stabilising the transcription.

5.6: Non-lineage determination factor behaves similarly to Ascl-1 in Competition ChIP

Lineage determination factor Ascl-1 shows a slow turnover rate from its binding site, as shown in (Figure 5.4). While the rapid turnover rate of Ascl-1 from a mutated binding site indicated support from the oocyte-specific factors. A question arises that if this stabilised transcription is a peculiar property of lineage determination factors or it's a general property of the oocyte-specific transcription? For answering this question, a competition ChIP was performed by using the Oestrogen receptor as a protein of interest. This experiment would allow us to decipher if oocyte, in general, forms the stable transcriptional complexes regardless of the transcription factor nature, or the stability of the transcriptional complex is directly associated with the nature of the transcription factor itself.

5.6.1. Experimental procedure:

An mRNA encoding for the limiting amount of Oestrogen Receptor (ER) was injected into the oocytes on Day 0, 1.4ng/oocyte. On Day1, the same injected oocytes were injected with a DNA-ERE at the concentration of 300 pg/oocyte. On Day 2, the pre injected oocytes were again injected with a competitor oestrogen receptor mRNA (ER-HA) at 10X more concentration 14ng/oocyte. The triple injected oocytes were then incubated overnight before processing for ChIP. In this experiment, the competition ChIP was performed with two antibodies, i.e., Anti-GFP and Anti-HA.

5.6.2 Results:

Amplification of DNA pulled down by Anti-GFP Antibody in the presence of the ER-HA competitor protein shows that occupancy of ER-GFP has reduced to about 50% (Figure 5.6 B see Anti-GFP), which is slightly more rapid than the reduction of Ascl-1 (40%). Anti-HA pull-down and the amplification has shown that the reduction of ER-GFP protein from the ERE binding site indeed has been replaced by the competitor ER-HA (Figure 5.6 B see Anti HA).

However, in this experiment, an antibody recognising the whole ER- protein, regardless of its tags, has not been used because of the unavailability of a reliable antibody for ChIP.

5.6.3. Conclusion

Competition ChIP experiment of Oestrogen receptor shows a similar extent of the turnover rate as of Ascl-1. This means that most of the transcription factor which functions with the oocyte-specific co-factors are likely to make stabilised transcriptional complexes.

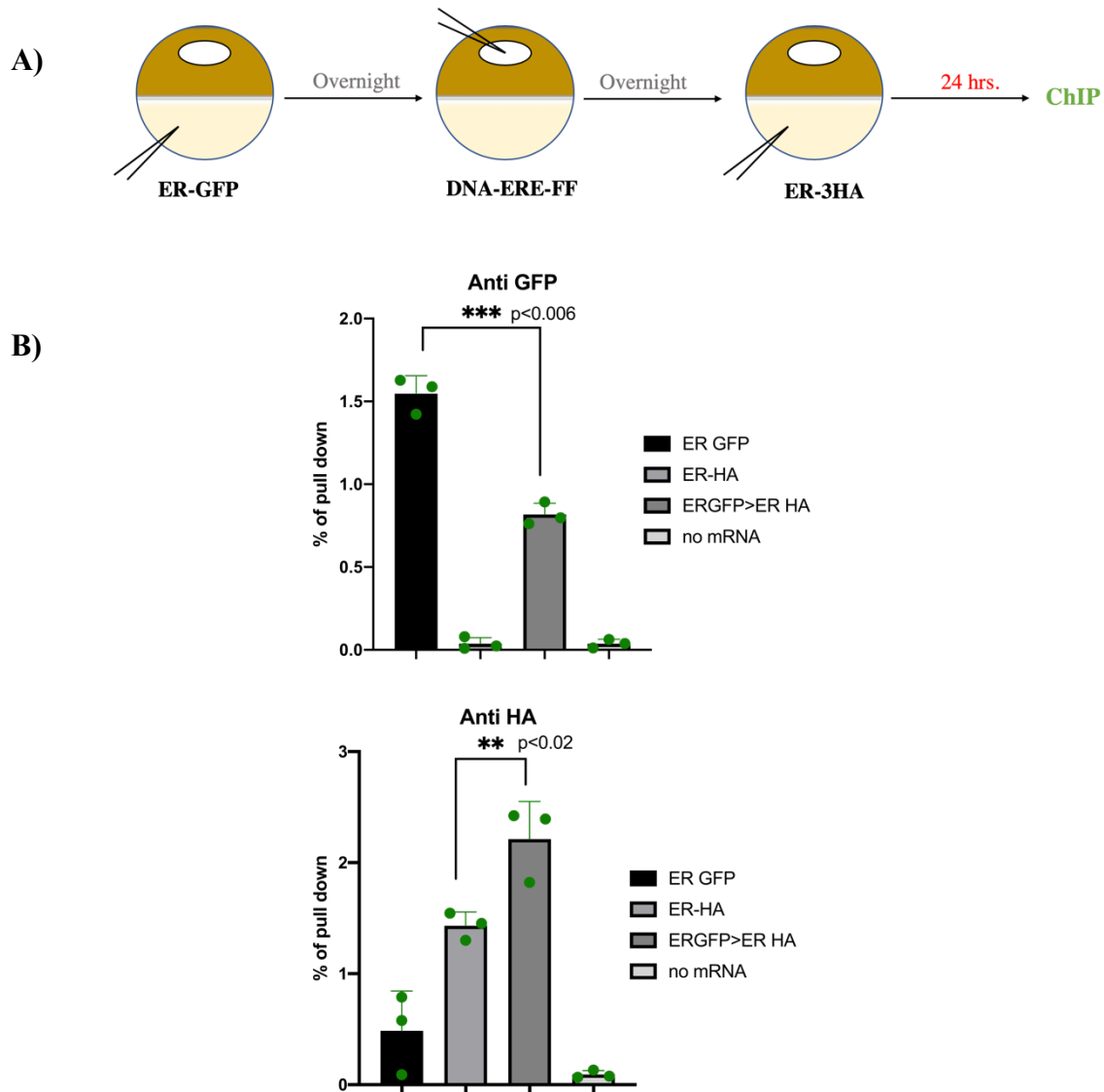


Figure 5.6: Slow turnover rate of a non-lineage determination factor ER in *Xenopus* oocyte.

A) Schematic diagram of *Xenopus* oocyte being sequentially injected by mRNA and DNA on consecutive days, followed by injection of a competitor mRNA on day 2. B) Occupancy time of oestrogen receptor in competition condition. 1.4 ng of ER-GFP was a competition against 14ng ER-HA for the same binding site. Anti-GFP measures the sites occupied by ER-GFP +/- competitor protein. Anti-HA measure the binding sites occupied by the ER-HA protein +/- competitor. Error bars show the mean of 3 independent experiments ($n=3$), with each experiment containing 45 individual oocytes.

5.7. Conclusion

In this chapter, a variety of competition ChIP assays have been performed to investigate the binding kinetics of transcription factor Ascl-1. It has been found that injected mRNA for Ascl-1 translates a functional protein that occupies its binding site for as long as 96 hours (Figure 5.3 B). Some experiments like Auxin mediated degradation of Ascl-1AID has shown that, when the 2nd DNA is in an under-expression state, most of the Ascl-1 at that time is associated with DNA-FF, which is injected 1st. Competition ChIP performed with two differentially tagged Ascl-1 shows that Ascl-1 turns-over very slowly from its binding site (Figure 5.3C). However, (Figure 5.4) shows the rapid turnover rate of Ascl-1 from a mutated binding site. Competition ChIP experiments performed with a non-lineage determination factor such as ER have shown that any transcription factor injected in the oocyte co-factor environments may preferably form stable transcriptional complexes, which are resistant to competition for at least 24 hours. This leads to a hypothesis that the stabilisation of transcription comes from oocyte-specific co-factors.

It led to another speculation that if Ascl-1 is turning over slowly from its binding site, is there still a possibility that it may be available to the 2nd DNA-Ren for transcription? To answer this question, I aim to perform a DNA competition experiment in which I will investigate the presence of Ascl-1 on the binding site of 2nd Injected, under-expressed DNA. I also aim to study the binding dynamics of some general transcription factors, such as TATA-Binding protein-like protein 2 (TBP2).

General transcription factor specific to Pol-II elongation is missing on 2nd DNA during the time of non-competition state

Introduction:

In chapter 5, the dwell time of Ascl-1 has been studied by using the Ascl-1 competition ChIP. Competition ChIP results on Wild type DNA-FF (Figure 5.3) and mutant DNA-FF (Figure 5.4) show that the stabilisation of the Ascl-1 mediated transcription complex may be caused by oocyte-specific co-factors. Moreover, Oestrogen Receptor (ER) competition ChIP showed a similar trend as Ascl-1 for prolonged dwell time. Therefore, a question arises that if Ascl-1 is turning over slowly, is it available to the 2nd injected DNA while the 2nd DNA is in the state of under-expression?

In this chapter, the causes of 2nd DNA's under-expression have been studied in detail. First, I aim to investigate the availability of Ascl-1 to the 2nd DNA when it is in an under-expressed state in the GV. For this purpose, I aimed to use DNA competition ChIP, which determines the binding of Ascl-1 on both 1st (DNA-FF) and 2nd (DNA-Ren) injected DNAs. Second, a DNA competition ChIP is also aimed to study the binding dynamics of oocytes specific TATA-Binding protein-like protein (TBPL2) on the injected plasmids. TATA-Binding protein has been shown to form stable transcriptional complexes, both in-vivo and invitro extracts. In HEK293 cells, TBP been has shown to exhibit a long dwell time of ~60-90 minutes. Investigation of TBP2 as a limiting factor may contribute to understanding the reason for 2nd DNA under-expression.

Third, I aim to perform a detailed analysis of the transcriptional state of both sequentially injected plasmids in the oocytes. For this, I aim to perform a competition ChIP, which will simultaneously monitor the status of active and paused transcription by C Terminal Domain analysis of RNA-polymerase II (CTD).

6.1. Ascl-1 is bound on the injected 2nd DNA in the oocyte under its under-expressing form

Protein competition experiments have suggested that Ascl-1 turns-over slowly from its binding site within matters of minutes to hours. However, it is yet unclear that if this dissociating Ascl-1 is available for the second DNA or it has been degraded after being used by the first DNA? To answer this question, I performed a DNA competition, ChIP, in which both DNA-FF and DNA-Ren have been investigated for the bound Ascl-1.

6.1.1. Experimental procedure.

A limiting amount of 1.4 ng of Ascl-1 was injected in the oocytes on Day 0, and the injected oocytes were incubated overnight. On Day 1, the preinjected oocytes were injected with DNA-FF at the concentration of 300pg/oocyte. On Day 2, the same injected oocytes were again injected with DNA-Ren at the final concentration of 3.0 ng/oocytes. The triple injected oocytes were fixed at two different time points after the 2nd DNA injection. The time points are 12 hrs and 24 hrs. The ChIP was performed as described in Methods section 2.2.8.

6.1.2. Results:

DNA-FF and DNA-Ren are remarkably similar to each other. The success of DNA competition ChIP relies on how the very similar Ebox-sequences on DNA-FF and DNA-Ren can be amplified distinctively. Careful analysis of plasmid sequences requires that a pair of primers that were chosen, to differentially amplify the Ebox specific to DNA-FF and another pair, which can amplify the Ebox sequence from DNA-Renilla.

Anti-Ascl-1 ChIP showed that when DNA-FF and DNA-Ren were injected together, they both receive Ascl-1 (Figure 6.1 B see condition FF+Ren), and it can be seen that DNA-Ren receives ~40 % more Ascl-1 because of its high amount, which is about 10 times than DNA-Ren. However, in 12 hrs, DNA-Renilla receives only 13% of Ascl-1 as compared to

DNA-FF when these plasmids are sequentially injected (Figure 6.1 see condition FF>Ren 12 hrs).

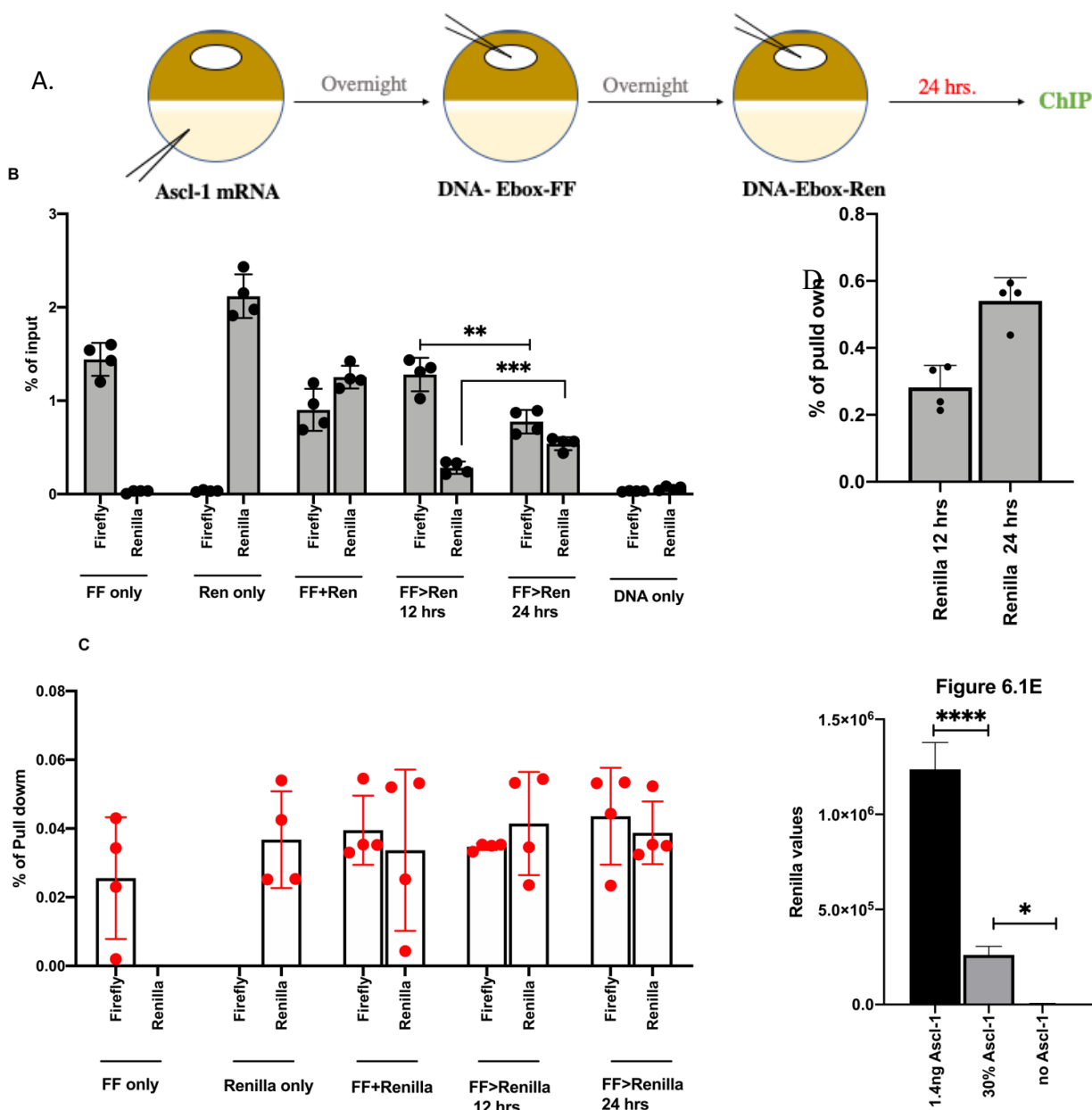


Figure 6.1: Abundance of *Ascl-1* on the Ebox site of 2nd sequentially injected DNA.

A) Schematic diagram of injection into the oocyte for DNA competition ChIP. The oocytes were sequentially injected with mRNA and both DNAs by 24 hours interval. B) qPCR amplification of Ebox Loci on the Renilla and Firefly reporter plasmids. The ChIP procedure was performed by the Anti-*Ascl-1* antibody. C) qPCR amplification of promiscuous Ebox sequence upstream of the Ebox-PoU-Ebox sequence region showing a very low enrichment of non-specific chromatin by *Ascl-1* during ChIP. D) Amplification of DNA pulled down during ChIP comparing the enrichment of *Ascl-1* on the Renilla reporter Ebox sequence in 12-24h time-points. E) Renilla expression assay shows the 30% *Ascl-1* (grey) can cause a significant expression of a reporter DNA if not preceded by 1st DNA.

Error bars show the St deviation of n=4 with each sample containing 15 oocytes.

* (p<0.05), ** (p<0.01), *** (P<0.005) **** (P<0.001).

Interestingly, the amount of Ascl-1 bound to the DNA-Ren increased from 13% to 30 % in 12 hrs, (from 12 hrs- 24 hrs) see (Figure 6.1B condition FF>Ren 24 hrs). A decrease in Ascl-1 from DNA-FF confirmed that bound Ascl-1 to DNA-Ren in 24 hrs might have dissociated from DNA-FF.

I next asked, is it possible that Ascl-1 dissociated from DNA-FF is binding only on the Dll1 derived Ebox, or it binds to any other Ebox like sequence in the plasmid? Amplification of a promiscuous Ebox like sequence upstream to transcription start site suggested that there is almost 1-2% off-target binding of Ascl-1 to the Ebox-like sequence (Figure 6.1C). This finding suggests that the dissociated Ascl-1 binds to the Dll1 Ebox on the DNA-Ren plasmid.

The DNA competition experiment showed that Ascl-1 is slowly turning over from its binding site on DNA-FF. The dissociated Ascl-1 is able to bind on the DNA-Ren in its under-expressed form. It means that the DNA-Ren, despite having Ascl-1 bound to its Ebox site, does not express. Two hypotheses can explain this: First, either the 30% dissociated Ascl-1 is not sufficient to support the transcription on DNA-Ren. Second, some oocyte-specific co-factor molecule is limiting, which, despite Ascl-1 presence, could not allow the transcription to be started.

To answer the first question of the inability of 30% of injected Ascl-1 to support the transcription, I injected 466 pg of Ascl-1 per oocyte (466 pg is 30% of initially injected 1.4 ng of Ascl-1), followed by the injection of DNA-Ren on the next day. The Renilla reporter value suggests a significant expression of Renilla reporter when compared to the no-mRNA control (Figure 6.1, E). However, this value of Renilla activation was much less than 1.4 ng mediated activation.

6.1.3. Conclusion:

DNA competition experiment revealed that within 24 hours of competition, only 30% of Ascl-1 is dissociated from DNA-FF and had been available to the 2nd DNA-Ren. Despite

receiving Ascl-1, the DNA-Ren could not express the reporter gene. However, it was confirmed that 30% of dissociated Ascl-1 is able to start transcription when injected in a separate experiment. This means some other co-factor molecule, which is of oocyte origin, is missing or limiting. Without that factor(s), Ascl-1 is unable to start the transcription.

6.2: Injected TBP2 is more abundant on 2nd injected plasmid than the 1st.

DNA competition experiments suggested that the reason for the under-expression of the DNA-Ren could be because of an oocyte-specific co-factor or a general transcription factor(s). I aimed to test the TBP2, which has shown to be able to bind very strongly to their promoter for prolonged periods of time (Hasegawa & Struhl, 2019b) and for its ability to initiate transcription by interaction with many other transcription factors (Ravarani et al., 2020). This experiment will help in understanding if a general transcription factor is limiting for 2nd injected DNA -Ren plasmid. The experiment with TBP2 is designed as DNA competition mentioned in section 6.1.

6.2.1. Experimental procedure:

An mRNA encoding for limiting the amount of Ascl-1 was injected into the cytoplasm of the oocytes on Day 0. On Day 1, the preinjected oocytes were injected in the GV with 300 pg of DNA-FF and incubated overnight. On Day 2, the injected oocytes were again injected with 3ng of DNA-Ren along with 7ng of TBP2-mCherry mRNA in GV (Figure 6.2A) and cytoplasm, respectively. After overnight incubation, the oocytes were used for reporter assays and for the ChIP, as mentioned in Section 2.2.8 in Methods.

Results:

First, I asked if the injection of TBP2 mRNA with 2nd DNA-Ren has some effect on the expression of Renilla reporter? Renilla and Firefly reporter show that the addition of TBP2 mRNA in the oocytes in competition condition has some rescue effect (Figure 6.2B see Renilla

expression black vs. Red). This ~5-7% increase in Renilla expression is significant. However, Firefly reporter shows a non-significant decrease in the values (Figure 6.2 see Firefly Black vs. Red).

Next, I asked what is the turn-over rate of TBP2 on injected plasmids? Before answering this question, it is important to mention that there is a significant amount of TBP2 present inside the *Xenopus* oocyte (Akhtar & Veenstra, 2009b). The only way to determine the turn-over rate of TBP2 is to analyse how much of the injected mCherry version of TBP2 has replaced the endogenous TBP2 on DNA-FF and DNA-Renilla. Figure 6.2C shows the binding of TBP2 on DNA-FF and DNA-Ren as detected by CHIP. Comparison of the control condition (no TBP2 injection, denoted as (-) TBP2 with the experiment where oocytes were supplemented with TBP2 mRNA denoted as (+) TBP2 shows the relative enrichment of TBP2mCherry on both of the injected DNAs. It can be seen that the TBP2 has been able to bind to both of the DNAs (Figure 6.2C). However, it is evident that the binding of TBP2 on 2nd injected DNA-Ren is much higher than DNA-FF (Figure 6.2C see REN (+) TBP2, and FF (+) TBP2).

Since, as mentioned above, the detection of TBP2 mCherry by CHIP points toward the relative turn-over rate of TBP2 on the plasmids, it seems that the TBP2 is turning over at a similar rate as it was Ascl-1. However, due to the lack of a reliable *Xenopus* TBP2 antibody, the total protein cannot be estimated.

6.2.3. Conclusion.

However, despite a large amount of TBP2mCherry binding, the DNA-Ren could not succeed in rescuing the reporter expression more than 10%. This experiment, therefore, suggests that there must be another transcription factor(s) that are cooperatively contributing to the phenotype of 2nd DNA under-expression.

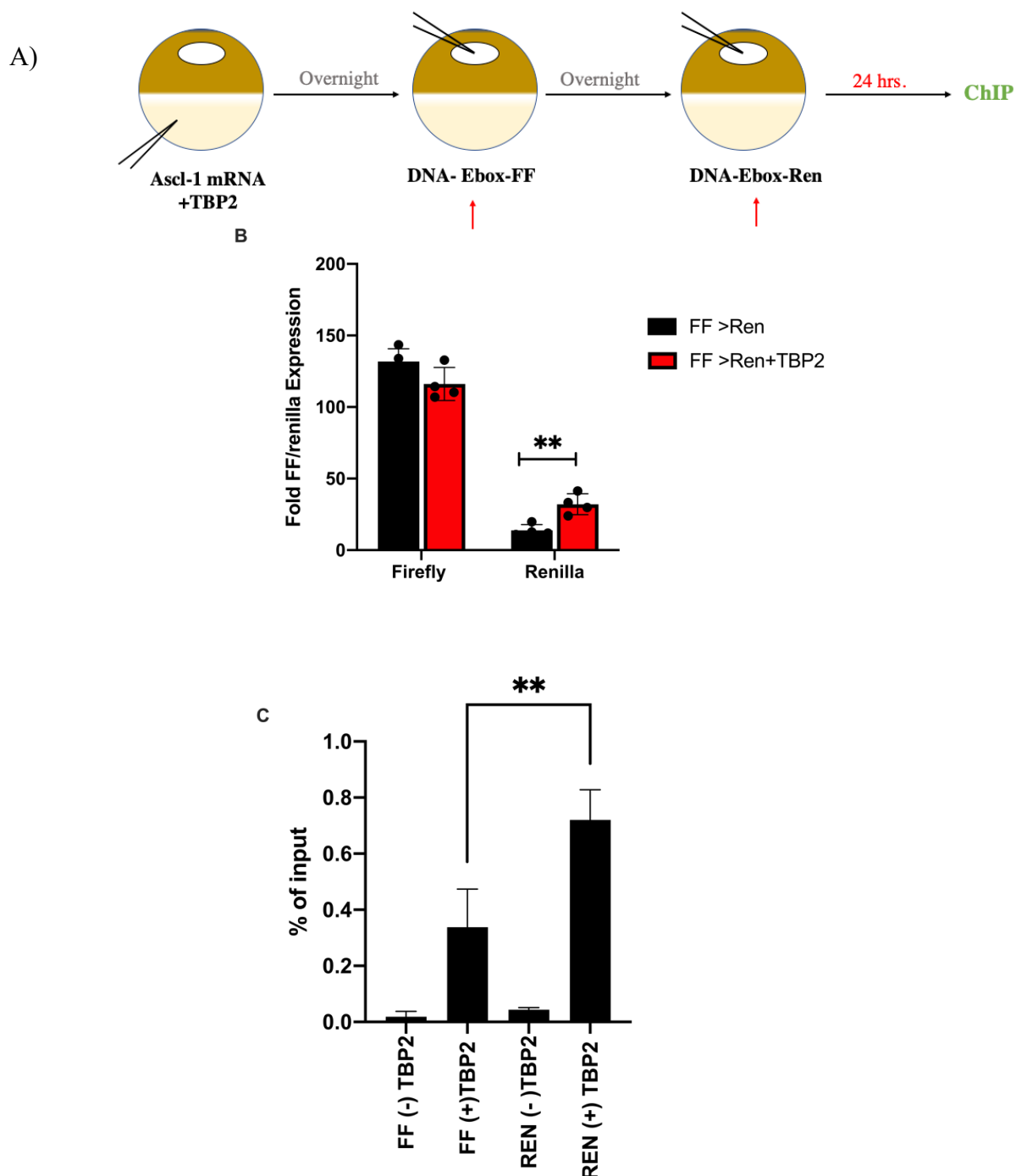


Figure 6.2: Injected TBP2 encoded protein is associated with both expressed and non-expressed plasmids.

A) Schematic diagram of injection of TBP2-mCherry mRNA injected into the oocytes at different time-points. B) Firefly and Renilla expression assay show a small rise in otherwise repressed DNA into the oocyte (Red-Renilla). Error bars represent SEM of $n=4$ experiments. C) Anti-mCherry ChIP of plasmids in the oocyte, which are injected with mRNA encoding for TBP2-mCherry. qPCR mediated amplification of Ebox sequences in Renilla and Firefly suggest turn-over of TBP2 at both loci. Error bars represent St.Dv of $n=4$; each experiment contained 3 replicates with each containing 20 oocytes. ** ($P<0.05$) (Student test in figure B) ** ($P<0.02$) (Two-way ANOVA)

6.3: 2nd injected DNA lacks RNA-Pol-II serine 2 phosphorylation:

TBP2 competition CHIP has shown that transcription initiation factors such as TBP2 and Ascl-1 may not alone contribute fully to the phenotype of 2nd DNA under expression. There must be an alternative mechanism(s) or some other factors which may be limiting for the 2nd injected DNA-Ren. In general, RNA-Polymerase II transcription is regulated at many points; however, two major points of Pol-II transcriptional regulation are i) Transcription initiation and ii) Transcriptional elongation. In transcription initiation, TBP2 and general transcription factors such as TFIIF and TFIID interact with RNA-pol-II and make an initiation complex on the promoter-proximal regions. This initiation complex starts the transcription of few bases after phosphorylation of its C Terminal Domain (CTD) at Ser5. Just after traveling a short distance, the transcription is blocked by a Negative Elongation Factor (NELF) (Aida et al., 2006), which stops the Pol-II and prevents Pol-II from shooting on to the gene body. In the 2nd step of transcriptional elongation, another complex called Positive Transcription Elongation Factor (P-TEFb), which is associated with a CDK9 Kinase (Price, 2000), binds to the paused complex and release it to the gene body by modification of Ser2 residue on CTD of Pol-II.

I asked a question that; is it possible that the 2nd injected DNA-Ren is deprived of the factors which are needed for transcription elongation or transcription initiation? The answer to this question provides a clue, with the help of which it can be deduced that at what stage the transcription is halted on the 2nd injected DNA?

6.3.1. Experimental procedure

A limiting amount of mRNA (1.4ng)/oocyte was injected into the cytoplasm of the oocytes at Day 0. On Day1, the preinjected oocytes were injected with 300 pg of DNA-FF. On Day2, the same preinjected oocytes were again injected with 3ng/oocyte DNA-Ren. Triple injected oocytes were incubated for 24 hours and then assayed for ChIP through Anti-pol-II ser2 and Pol-II Ser5 antibodies.

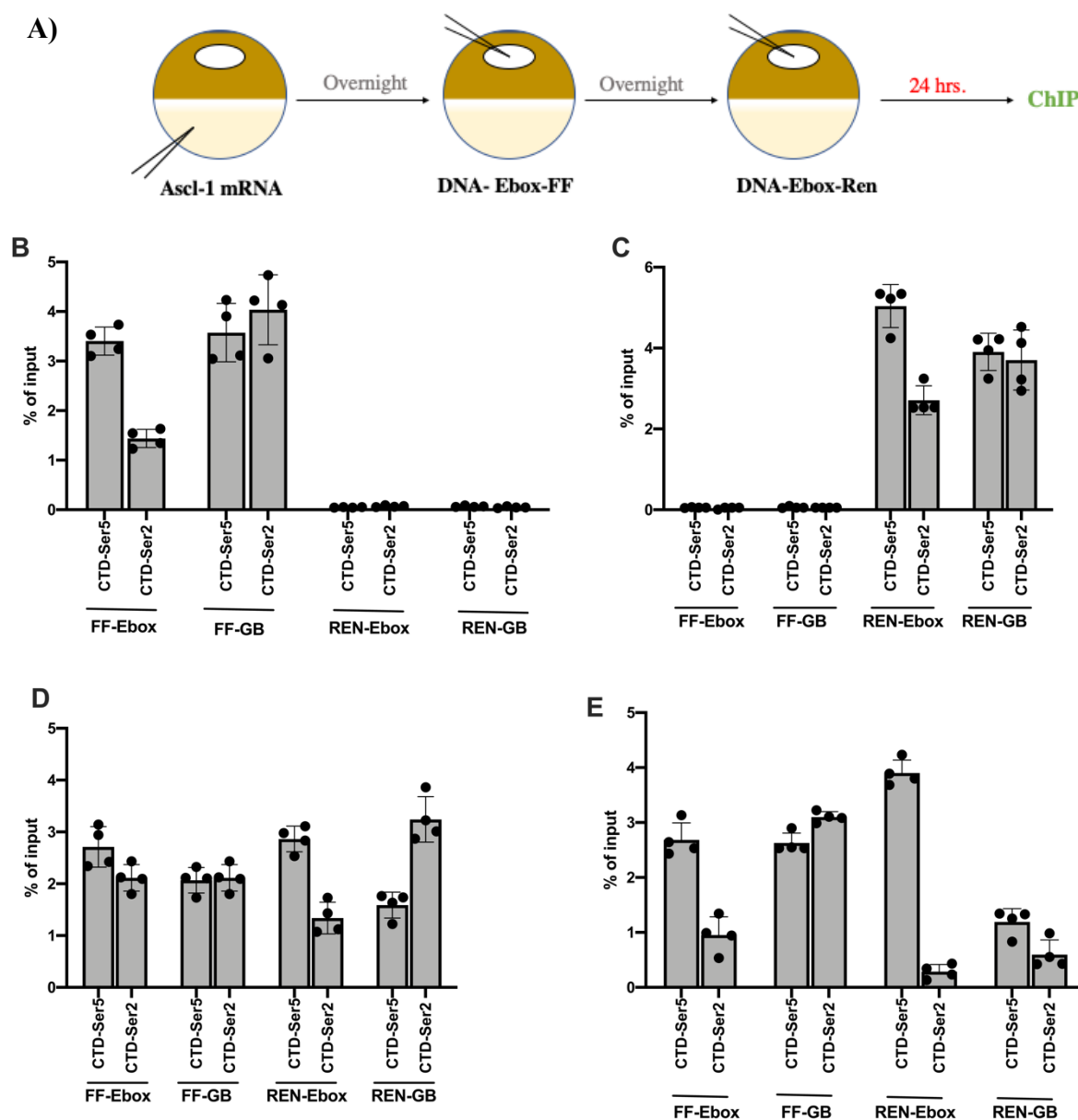


Figure 6.3: *2nd Injected DNA into the oocyte is deprived of Pol-II elongation factor*

A) Schematic diagram of injection of DNA competition. Each injection is separated by at least an overnight incubation. B) Anti-Pol-II serine2 and Anti-pl-II ser5 ChIP of 1st Injected DNA into the oocyte (Firefly-reporter). qPCR mediated amplification of associated DNA shows the enrichment of Pol-II on TSS (Transcription start site) and on the gene body. C) Anti-Pol-II serine2 and Anti-pl-II ser5 ChIP of 2nd Injected DNA into the oocyte (Renilla reporter). qPCR mediated amplification shows the presence of pol-II on the gene body and on TSS. D) Anti-Pol-II serine2 and Anti-pl-II ser5 ChIP of DNA-I and DNA II injected together. qPCR amplification of associated DNA shows the presence of Pol -II on both of the co-injected DNA(s). E) Anti-Pol-II serine2 and Anti-pl-II ser5 ChIP of sequentially injected DNA into the oocyte. qPCR amplification shows the presence of active pol -IIser2 on DNA-I, but the absence of active pol-II on 2nd injected DNA.

Error bars represent SEM of $n=4$ with each experiment containing 3 technical replicates with 20 oocytes in each.

6.3.2. Results

In order to properly analyse the dynamics of the transcriptional machinery, it was necessary to design the primers which not only determine the Pol-II activity on the promoter but also on the gene body. Following are the set of primers that are given with their target sequence, which they amplify.

Primer Name	Target amplification
FF-Ebox	Ebox-PoU on DNA-FF
FF-GB	Firefly reporter Gene Body
REN-Ebox	Ebox-PoU on DNA-Ren
REN-GB	Renilla reporter Gene Body

Table 6.1. Shows the primers used in ChIP and their target sequence, which they amplify.

The RNA-Pol-II dynamics in the DNA-FF only plasmid condition show the presence of both initiation (CTD-Ser5) and elongation (CTD-Ser2) on DNA-FF specific Ebox sequence as well as elongation complex also can be seen in the gene body (Figure 6.3B). In a similar situation, when DNA-Ren was injected alone, the initiation and elongation complex can be seen on both DNA-Ren specific Ebox and on the Ren-GB (Figure 6.3D). The presence of the elongation complex (Ser2) on the gene body shows that the plasmid is transcriptionally active. However, when the DNA-FF and DNA-Ren were co-injected in the oocytes, the strength of Pol-II Ser2 and Ser5 signals on each plasmid decreased. This may be because of the distribution of transcription factors among the two templates. However, both initiation and elongation complexes can be seen on REN-GB and FF-GB. It means that both of the injected DNAs are transcriptionally active when co-injected.

Surprisingly, when the state of initiation and elongation complex was analysed on the DNA-FF and DNA-Ren when they are injected sequentially, it became evident that the initiation complex (CTD-Ser5) is abundant on both under-expressed DNA-Ren plasmid and on

transcriptionally active DNA-FF. However, the elongation complex (CTD-Ser 2) was seen only on the DNA-FF gene body, and it is missing in the REN-GB (Figure 6.3E).

6.3.3. Conclusion.

Analysis of transcription initiation and transcription elongation complexes on the sequentially injected DNA-FF and DNA-Ren show that the major limiting factor which contributes to the phenotype of 2nd DNA under-expression could be a one which regulates the transition of Paused Pol-II to elongation complex.

6.4: Auxin mediated degradation also degrades the associated complexes with the protein of interest

The analysis of initiation and elongation complex of Pol-II has shown that some co-factor molecules which dictate the paused to active pol-II transition in the oocyte are missing or limiting on the 2nd injected DNA-Ren plasmid. I asked a question; Is it possible to relocate the transcription factors from DNA-FF to DNA-Ren? If yes, then it may provide us a chance to change the transcriptional state of 2nd injected DNA-Ren.

To do this experiment, I decided to use the Ascl-1, which is tagged with AID. The AID domain leads to the degradation of the tagged protein upon addition of Auxin in the medium. The hypothesis is that; Auxin mediated degradation will remove all the active transcriptional complexes from the DNA-FF, and removal of Auxin from the medium will cause the redistribution of the co-factors equally between 1st and 2nd injected plasmids.

6.4.1. Experimental procedure.

A limiting amount of Ascl-1-AID at 1.4 ng/ oocytes was injected into the cytoplasm of the *Xenopus* oocytes on Day 0. On Day1, the same injected oocytes were injected with DNA-FF at 300pg/oocyte in the GVs. On Day 2, the preinjected oocytes were again injected with 3ng/oocyte of DNA-Ren. After 6 hours of competition, the medium of the oocytes was supplemented with 1mM Auxin and incubated for 8 hours. After incubation, the medium was

replaced by the normal medium, and oocytes were incubated overnight before the firefly and Renilla assays (Figure 6.4A).

6.4.2. Results

It is important to mention that “R” denotes a condition in which Auxin mediated degradation is recovered through the change of medium overnight. The Firefly and Renilla reporter assays show that upon addition of Auxin, the Firefly values have decreased almost a 90-100 folds (Figure 6.4B Compare, Ascl-1 AID, to Ascl-1 AID+ AUX). However, Firefly reporter expression indicated that after degradation by Auxin, the transcription never gets back to the normal level, even after the removal of Auxin from the medium for overnight (Figure 6.4B See Differences between Ascl-1 AID R and Ascl-1 AID).

However, Renilla reporter expression shows that under-expressed DNA-Ren had shown about 5% of recovery when transcription on DNA-FF was halted through Auxin (Figure 6.4 C comparison of Ascl-1 AID, and Ascl-1 AID AUX R). This experiment tells us that Auxin mediated degradation not only disrupts the tagged protein such as Ascl-1, it may also degrade the co-factors which interact with Ascl-1.

6.4.3. Conclusion

Recovery of transcription after Auxin mediated degradation shows that once Ascl-1 AID is degraded through Auxin, the transcription never returns to normal even after the removal of Auxin from the medium. However, the small extent of Renilla expression supports the hypothesis that if the transcription factor can be displaced from DNA-FF, they can eventually induce some level of DNA-Ren expression.

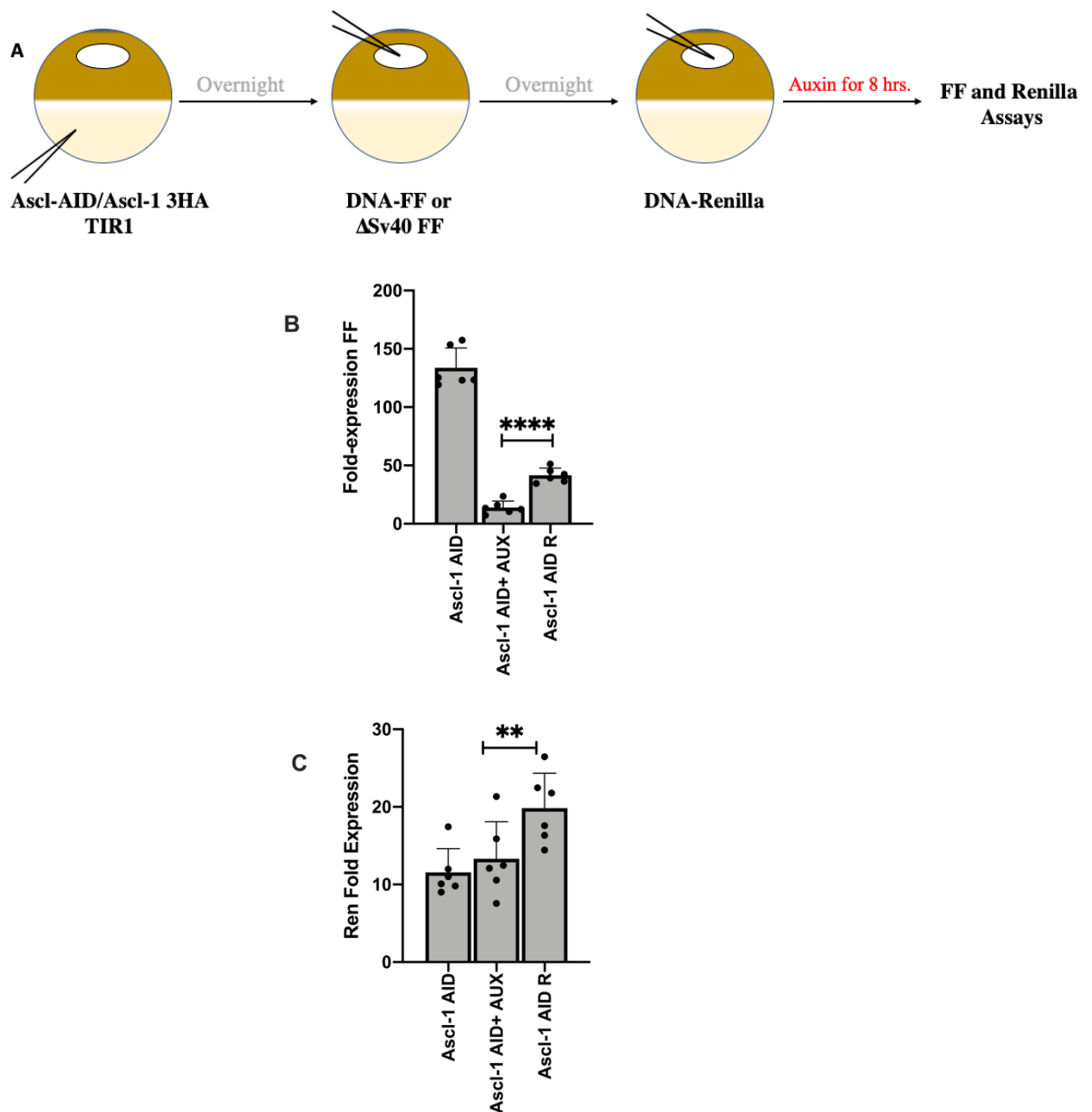


Figure 6.4: Auxin degrades Pol-II CTD with Ascl-1 in an injected oocyte.

A) Schematic diagram of injection of a mutant version of Firefly (DNA-I) plasmid into the oocytes, which is further injected with WT DNA-II. B) Firefly expression assays shows the values of expression after Auxin mediated degradation, and Ascl-1 AID R shows some Firefly expression values after the overnight recovery. C) Renilla expression assays values show the rate of recovery of Renilla expression when the Auxin was removed from the system. The values shown here are fold expression of Renilla+Ascl/Renilla only.

Error bars show SEM of $n=6$ each with 3 technical replicates, with each replicate containing 8 oocytes. ** ($P<0.001$) **** ($P<0.002$).

6.5. DRB mediated blockage of transcription does not disassemble transcription on the injected plasmid in *Xenopus* oocyte

The results from Auxin mediated reconstitution of transcription in the oocyte reveals that the Auxin may degrade the general transcription machinery or some necessary co-factor molecules in the oocyte. However, a small increase in DNA-Ren expression compared to its under-expressed form supports the hypothesis that if factors can somehow transfer from one plasmid to another, they could rescue 2nd DNA underexpression. Hence another method had been applied to reconstitute the transcriptional complexes from one plasmid to another. This method involves 5,6-Dichloro-1- β -d-ribofuranosylbenzimidazole (DRB) (Bensaude, 2011) is a drug that blocks transcriptional elongation by interacting with a DRB sensitive factor in Pol-II elongation complex (Zandomeni et al., 1983). DRB mediated transcriptional elongation has been shown to be effective in 2 hours of treatment in cultured cells. Moreover, the removal of DRB results in the reconstitution of transcription within 6-8 hours. So, there is a possibility that the blockage of transcription on injected plasmid releases the transcriptional complexes, so that removal of DRB allows the redistribution of those onto both DNA-FF and DNA-Ren.

6.5.1. Experimental procedure.

Since DRB has been used in cultured cells, it was a need to determine the effective dose and the effective treatment time in the *Xenopus* oocyte. First, I decided to use different doses of DRB to block the transcription in *Xenopus* oocytes (Figure 6.5 A-II). For this purpose, a limiting amount of mRNA (1.4ng)/oocyte was injected into the cytoplasm of the oocytes at Day 0. On Day1, the preinjected oocytes were injected with 300 pg of DNA-FF. On Day 2, the oocytes were supplemented with a medium containing variable amounts of DRB ranging from 10mM-70 mM at final concentrations. The drug treatment was carried on overnight. After the treatment, the oocytes were washed three times with 1X MBS and then incubated in normal 1X MBS for 2 hours before processing them for Reporter assay.

6.5.2. Results

To understand the effect of DRB on transcriptional elongation on DNA-FF plasmid, a T=0 sample was taken out before the time of DRB addition to the medium. The Firefly values of DRB treated and T=0 oocytes suggest the transcription blockage through DRB can be seen at all concentrations of 10 mM to 70 mM (Figure 6.5B **green line**). However, in a condition, where instead of overnight, the DRB was given to oocyte for 4 hours, showed the transcriptional recovery (Figure 6.5B **Red line**). It can be seen evidently that oocytes treated with a high dose of 70mM of DRB could not recover back to the absolute normal when compared to T=0 (Figure 6.5 B, comparison of **Blue line** and **Red line**).

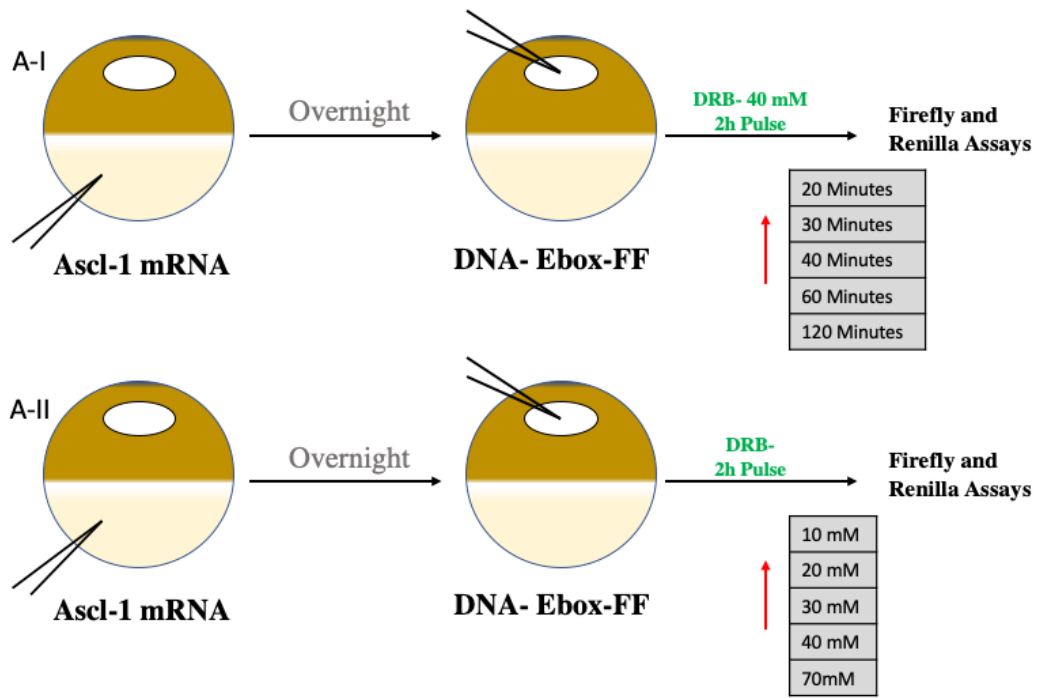
Next, I asked what is the amount of time at which 40 mM DRB (shown to work in *Xenopus* oocyte) can block the transcriptional elongation at the DNA-FF? To do this, a limiting amount of mRNA (1.4ng)/oocyte was injected into the cytoplasm of the oocytes at Day 0. On Day1, the preinjected oocytes were injected with 300 pg of DNA-FF. On the next day, the same oocytes were supplemented with 40 mM of DRB for different time-points, ranging from 20 minutes-120 minutes (Figure 6.5A-I). The Firefly reporter has shown that the 30 minutes treatment of *Xenopus* oocyte at the concentration of 40mM of DRB can effectively block the transcriptional elongation (Figure 6.5C **Blue line**).

After the optimisation of the DRB dose and time, I asked whether the blockage of transcription on DNA-FF will allow the redistribution of transcription factors onto sequentially injected DNAs? To do this, a limiting amount of *Ascl-1* mRNA (1.4ng)/oocyte was injected into the cytoplasm of the oocytes at Day 0. On Day1, the preinjected oocytes were injected with 300 pg of DNA-FF. On Day 2, the oocytes were injected with 3ng of Renilla plasmid. After 6 hours of competition, 40 mM of DRB was added to the medium for 2 hrs. After 2 hrs of DRB treatment, the oocytes were washed with 1X MBS and then incubated in normal 1XMBS overnight (Figure 6.5A-III). Firefly and Renilla expression of DRB treated oocytes

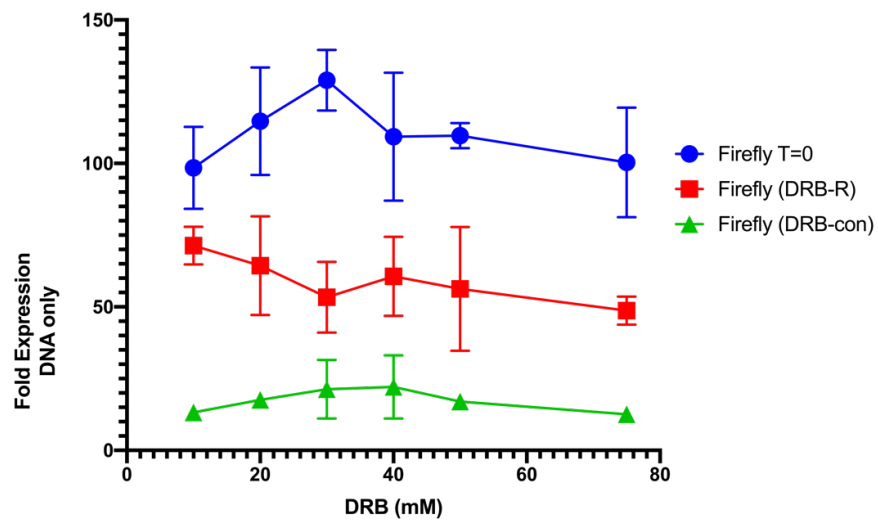
showed the reconstitution of transcription happened only on DNA-FF (Figure 6.5D see DRB 2 hrs condition) when compared to DRB continuous denoted as (DRB Conti). However, there was no activation of Renilla observed in DRB- treated oocytes even after overnight recovery (See Renilla in Figure 6.5D, in condition DRB-2h).

6.5.3. Conclusion

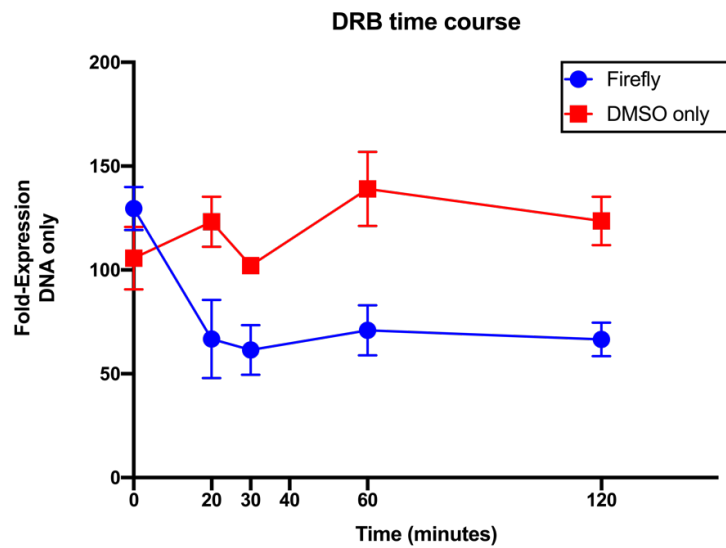
The DRB mediated transcriptional blockage has shown that recovery of DRB blocked transcription only occurs at the DNA-FF. This result can be explained by the following possibility; The DRB treatment may block the transcriptional elongation, but it does not dissociate it from its binding sites. So, when DRB is removed, transcription starts from the same sites where the transcription factors must be still attached.



B)



C)



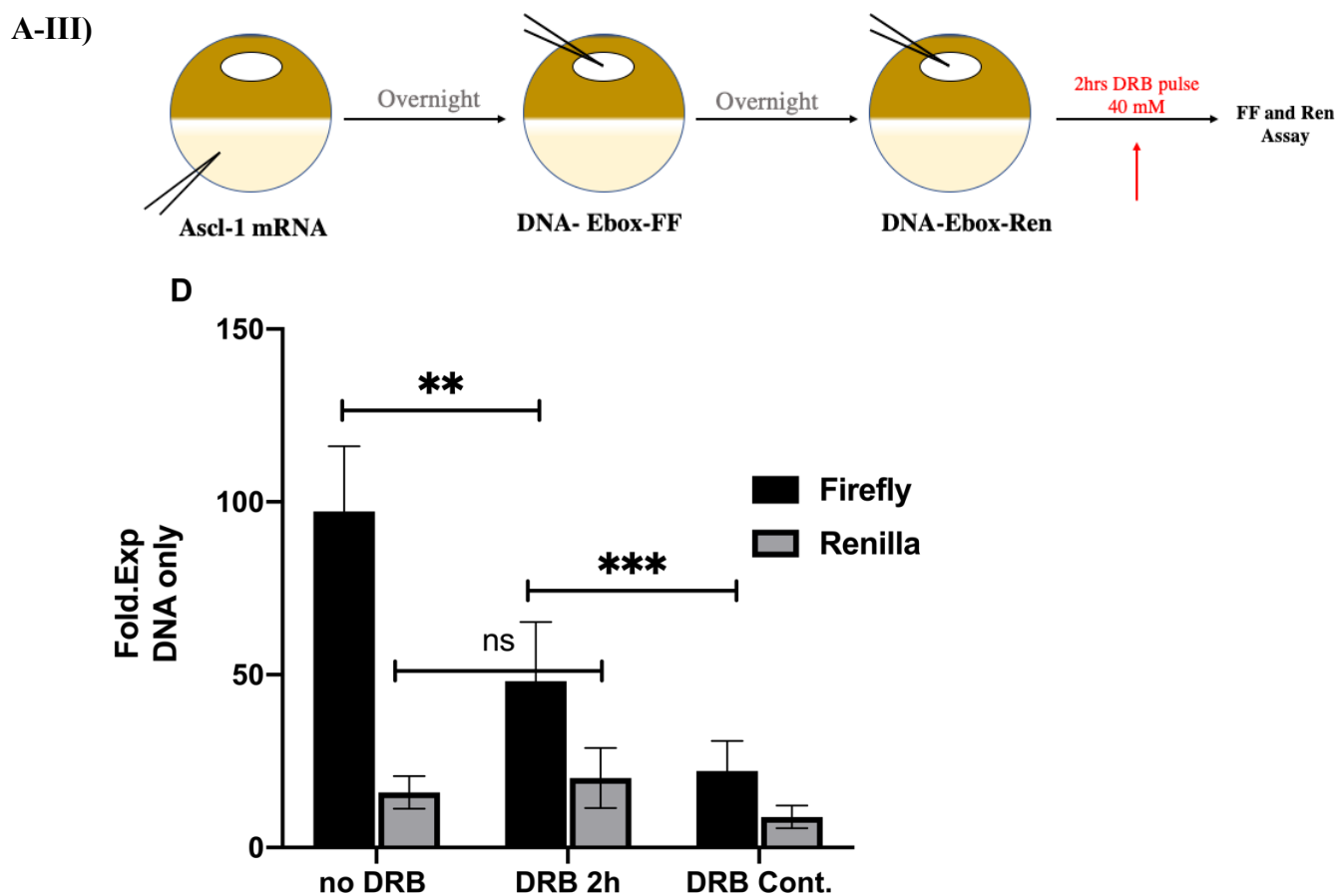


Figure 6.5: DRB does not dissociate preassembled transcriptional complexes in *Xenopus* oocyte.

A) Schematic diagram of injection of *Ascl-1* mRNA and DNA into the oocyte in a sequential manner. The diagram also shows the doses of DRB administered during the experiment and indicated by Red arrow. B) Firefly expression values normalised to DNA only with no *Ascl-1* show the effect of DRB doses in the inhibition of transcriptional elongation. C) Firefly expression values normalised to the DNA-I only show the time-course of DRB administration to the oocyte followed by overnight incubation. D) Shows the Firefly and Renilla values before and after the administration of DRB for 40 mM for 2 hrs in the MBS medium.

Error bars represent SEM $n=4$, while each experiment contained 8 individual oocytes
 ** $P < 0.005$. **** $P < 0.05$ (Two-way ANOVA)

6.6. Conclusion:

In this chapter, a detailed analysis of general transcription factors of *Xenopus* oocyte with respect to the DNA competition experiment has been performed. This analysis included the binding of transcription factor Ascl-1 to under-expressed DNA as well as the dynamics of TATA-binding protein. Moreover, two methods, namely (Ascl-1 AID degradation and DRB mediated blockage of transcriptional elongation), have been used to study the reversible assembly of transcriptional complexes in the oocyte. The following conclusions have been derived from the experiments.

- 1) Ascl-1 does have a longer occupancy time in the oocyte, but it is available to the 2nd DNA when the second DNA is still under-expressed.
- 2) The injected, TATA-binding protein 2 (TBP2) is distributed to both active and inactive plasmids.
- 3) Auxin mediated Ascl-1 degradation also degrades the interacting co-factors in the oocytes.
- 4) 2nd under-expressed DNA-Ren is deprived of Pol-II elongation complex while exhibiting the Pol-II Ser5 complex.
- 5) DRB mediated inhibition of transcriptional elongation does not dissociate the preassembled transcriptional complexes on 1st injected DNA in *Xenopus*.
- 6) A second DNA is not activated in transcription by inhibiting transcription elongation of 1st DNA.

Results in this chapter suggest that there is a need for further investigation of the mechanism of 1st DNA dominance in *Xenopus* oocyte. To know it in further detail, a new method of nuclear protein transfer has been developed. In this method, the whole nuclear content of an oocyte can be transferred from one GV to another one in a living oocyte.

In the next chapter, the rescue experiment of 2nd DNA has been studied in more detail.

Rescue of 2nd DNA expression by nuclear content transfer in the competition state oocytes.

Introduction:

In the previous chapter, it has been investigated that one of the reasons for the under-expression of a 2nd injected DNA is the limited availability of RNA-Po-II elongation complex. A couple of direct methods were used to redistribute the transcriptional complexes between the sequentially injected DNA-FF and DNA-Ren have been used. First, the Auxin mediated degradation of Asc1 was used to free the transcription factors bound to DNA-FF. In the second method, DRB had been used to reversibly block the transcription of the injected DNA-FF to promoter the dissociation of bound factor from the 1st DNA and so make them available to 2nd DNA. Section 6.4, and 6.5 of Chapter 6, show that Auxin mediated disruption of transcription never recovers back to normal. However, in the second case of DRB, the drug cannot dissociate the factors from DNA-FF. This method revealed that blockage of transcriptional elongation in the oocyte does not let the transcriptional complexes to dissociate from their binding sites. Rescue experiment revealed the activation of the first DNA-FF only instead of redistribution of factors in the oocytes. The question arises, whether there any possibility that within the same oocyte, we can express the under-expressed DNA-Ren plasmid? And so identify the component that prevent the expression of 2nd DNA.

In this chapter, a variety of alternative methods are used to supply the transcription factors in various forms to study the expression of the 2nd DNA, which is in the under-expressed form. First, an abundant amount of Asc1-1 mRNA is injected to rescue the 2nd DNA, which also ensures if the Asc1-1 transcription factor is a limiting factor. Second, a new method has been developed to transfer the nuclear factors of one oocyte to another, which is undergoing DNA competition. Third, *Xenopus* oocyte extract is used to supply the factors which might be limiting for the expression of 2nd injected DNA-Ren in the oocyte.

7.1: Injection of a non-limiting amount of Ascl-1 mRNA rescues the under-expressed DNA to a small extent.

DNA and protein competition experiments have suggested the slow turn-over rate of Ascl-1 on DNA-FF (Chapter 5, and Chapter 6, section 6.1). However, binding of Ascl-1 has been detected on DNA-Ren when it is still in the under-expressed form. The Ascl-1 injected in the oocytes undergoing DNA competition is limiting (1.4ng/oocyte). There might be possible to rescue some phenotype of 2nd DNA under-expression by the addition of a non-limited amount of Ascl-1 after the competition condition has been established in the oocyte.

7.1.1 Experimental design:

The oocytes were injected with a limiting 1.4 ng of Ascl -1 mRNA into the cytoplasm on Day 0 and incubated overnight to allow the expression of the protein. On Day 1, DNA-FF was injected into the Ascl-1 injected oocytes and incubated overnight to allow the formation of a transcriptional complex. On Day 2, the preinjected oocytes were again injected with the 10 X more concentration of DNA-Ren and allowed the two DNAs to compete with one another for 3 hours. An extra amount of Ascl-1 at 3ng/oocyte was injected in the cytoplasm of the oocyte, where the DNAs are competing for transcription factors. The illustration of the injection plan is shown in (Figure 7.1 A).

7.1.2 Results:

Analysis of the Firefly expression values suggests that the injection of a non-limiting mRNA does substantially increase the Firefly values (Figure 7.1B compare Firefly values in FF> Ren and FF>Ren+Ascl conditions). However, the expression of Renilla reporter, before and after 3ng of additional Ascl-1 injection, shows the increase of 10% (Figure 7.1B compare Renilla values in FF> Ren and FF>Ren+Ascl conditions).

7.1.3. Conclusion:

This experiment thus, concludes that the DNA which is repressed can be somewhat rescued by the injection of Ascl-1. It also has been suggested by this experiment that a general transcription factor (s) may be a large limiting factor that contributes to the under-expression of DNA-Ren plasmid.

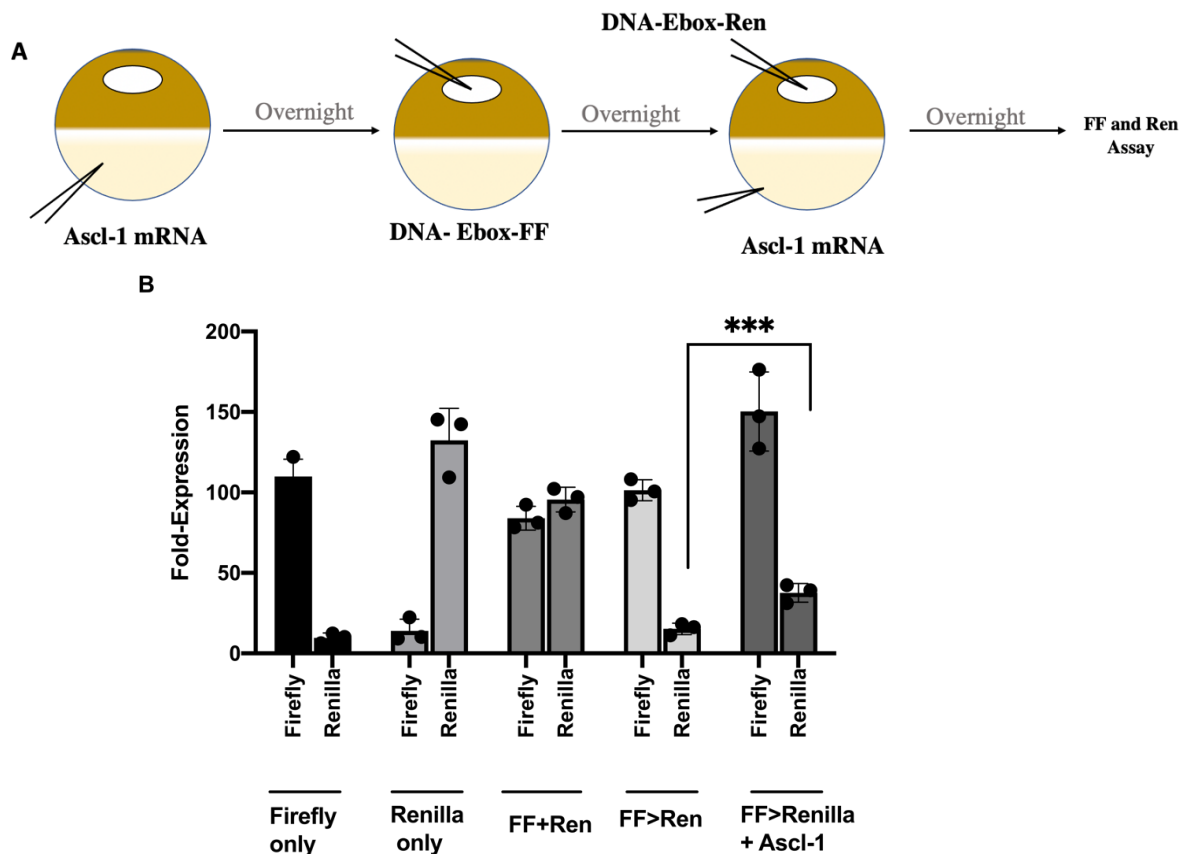


Figure 7.1: The minute rescue of DNA II by the non-limiting amount of Ascl-1 mRNA injection in the oocyte.

A) Schematic diagram of mRNA rescue experiment. The oocytes were injected with mRNA for Ascl-1, followed by the sequential injection of DNA-I and DNA-II. Additional 3ng of Ascl-1 mRNA has been injected 3hrs post DNA-II injection. B) Normalised values of DNA-FF and DNA-R show the effect of the addition of Ascl-1 mRNA on the injected DNA(s) in the *Xenopus* oocyte. *** $P<0.05$)

7.2. Injection of oocyte extract rescues the expression of DNA II in a dose-dependent way

The rescue of DNA II to a substantial amount has not been achieved by the injection of a surplus amount of Ascl-1 in the oocytes, which are in the competition condition. This result points toward the limitation of an oocyte-specific co-factor or the general transcription factor.

I asked a question that; is there a possibility to rescue the expression of 2nd injected DNA-Ren by injecting the *Xenopus* oocyte extract? This will enable us to understand the role of Ascl-1 and oocyte-specific factor in the rescue of 2nd DNA expression

7.2.1: Experimental design:

There are two types of experimental designs mentioned as A and design B (Figure 7.2A and B). Design A describes the preparation of *Xenopus* oocyte extracts. There are two versions of oocyte extract have been prepared. In the first version, the un-injected oocytes were used, while in the second, the oocytes were injected with Ascl-1. The reason for this is to study the effect of oocyte factors alone and oocyte factor and Ascl-1's combinatorial effect on the rescue of 2nd DNA expression. Similarly, the design B of the experiment describes the injection plan of the mRNA and DNAs in the oocyte, which are then injected with the oocyte extract prepared as mentioned in design A.

- A) Oocytes were injected with 23 ng of mRNA of Ascl-1 and incubated overnight at 18 °C incubator. On the next day, the 400 oocytes were then lysed by vertexing for 5 minutes and then centrifuged at (15000 rpm at 4 degrees). The colourless phase was transferred to a new tube and immediately stored at -80. A variable amount of Extract ranging from 23nl-73nl was injected into the oocyte pre-injected with FF DNA. The Extract injected oocytes were then processed for Firefly Assay (Figure 7.2 A).
- B) Oocytes were injected with the limiting 1 ng of mRNA encoding for Ascl-1 in the cytoplasm of oocytes. The oocytes were incubated overnight, followed by the injection of DNA I 300pg/ oocyte. After overnight incubation, the oocytes were injected with DNA II with a Renilla reporter. After 3 hours of 2nd DNA injection, the oocytes were injected with the whole oocyte extract in the cytoplasm of the oocyte undergoing the competition (Figure 7.2B). The oocytes were then incubated for 24 hours before processing for Firefly and Renilla Assays.

7.2.2: Results

The results of this experiment are explained in two different parts. In the first part, the efficiency of *Xenopus* oocyte extract (With and without Ascl-1) has been tested. Different doses of *Xenopus* oocyte Extract with Ascl-1 (Figure 7.2 C Blue Line) and without Ascl-1 (Figure 7.2C Red Line) were injected in the oocytes that are already injected with DNA-FF. After the extract injection, the activation of Firefly reporter was analysed by Firefly expression assay. Figure 7.2C shows that 43 nl- 73 nl of oocytes extract is sufficient to activate the Firefly to ~ 80% (See Figure 7.2C Blue Line). It means that 73 nl of oocyte extract has a sufficient co-factors, which can activate the transcription of plasmid DNA.

In the second part, the rescue effect of *Xenopus* oocyte extract has been studied. For this, injections were performed, as mentioned in design B in section 7.2.1. Renilla reporter expression showed a significant amount (40%) rescue effect of DNA-Ren expression when compared to its under-expressed form (Figure 7.2D Compare FF>Ren with FF>Renilla Extract). Surprisingly, the rescue effect of Extract, originating from Ascl-1 injected oocytes, showed a similar level of ~40% rescue (Figure 7.2 D compare FF>Ren with FF>Renilla+Ascl oocyte extract). This result shows that the major limiting factor in 2nd DNA under expression belongs to *Xenopus* oocyte-specific co-factors or from the General transcription factors.

7.2.3. Conclusion

Rescue of 2nd DNA expression by the injection of *Xenopus* oocytes extract showed that the major limiting factor which contributes to 2nd DNA under-expression belongs to *Xenopus* oocyte.

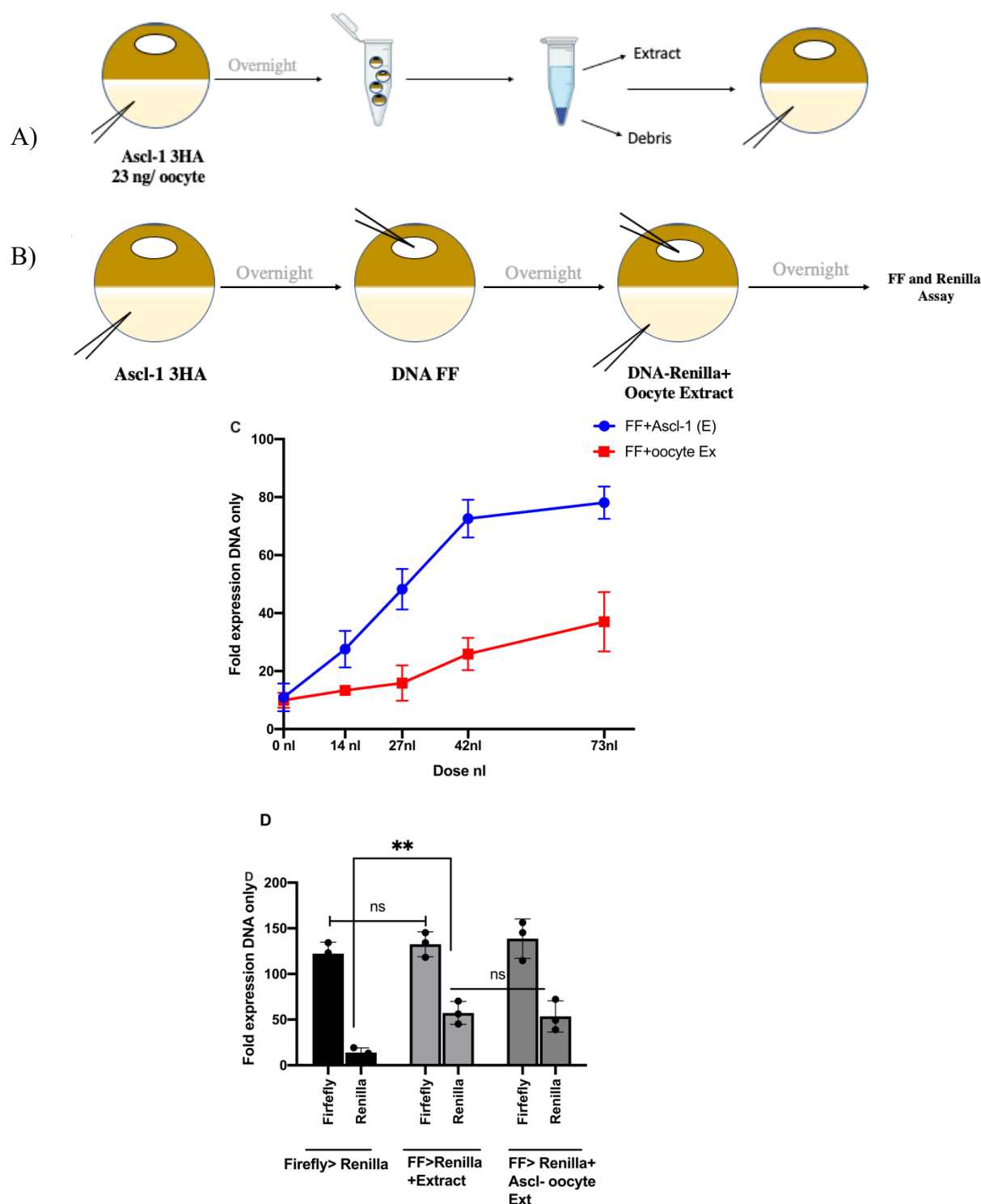


Figure 7.2: Dose-dependent Rescue of DNAILI by injection of oocyte extract (Ascl+/-).

A-B) Schematic diagram of the formation of oocyte extract by injection of 23 ng of mRNA encoding for Ascl-1 protein. 400 oocytes were used for the extract formation. **B)** Schematic of injection of oocyte extract in the oocyte sequentially injected with DNA-I and DNA-II. 73 nl of Extract was injected post 3hrs of DNA-II injection. **C)** Expression of Firefly DNA by Ascl+/- extract injected at different doses ranging from 0-73 nl. **D)** Normalised values of Firefly and Renilla expression before and after the rescue by the oocyte Ascl+/- extract. **** (P<0.01)** Error bars show the SEM of n=4 with each experiment containing 8 individual oocytes per sample.

7.3: Rescue factor oscillates between nuclear and cytoplasmic fractions of the oocyte

Rescue of the 2nd injected DNA -Ren by extract injections reveals that the factor (s) necessary for rescue are present in the un-injected oocytes Extract. However, to understand the mechanism of 2nd DNA under-expression, it is necessary to know further about the nature of those co-factors. I asked a question that, what is the location of the rescue factors? GV or the cytoplasm of the oocytes from where the Extract was made? To answer this question, two separate extracts were made; one is purely cytoplasmic Extract, while for the other, the isolated GVs were used to make a nuclear extract of the *Xenopus* oocytes. The method of making the GV and cytoplasmic extracts of *Xenopus* oocyte is discussed in Chapter 2 Methods section 2.2.14.

7.3.1: Experimental design

A limiting amount of 1.4 ng/oocyte was injected into the cytoplasm of the *Xenopus* oocytes on Day 0. On Day 1, the preinjected oocytes were again injected with the DNA-FF at 300 pg/oocyte in the GV. On Day 2, the same preinjected oocytes were injected with 3ng of DNA-Ren in the GV. After 3 hrs of incubation, the oocytes were injected in two conditions, i) 73nl of cytoplasmic Extract in the cytoplasm of the oocytes. ii) 23 nl of GV extract in the GV of preinjected oocytes (Figure 7.3A). The oocytes were incubated overnight before reporter expression analysis.

7.3.2: Results

Firefly and Renilla reporter expression analyses show that the injections of cytoplasmic or nuclear Extracts have little to no effect on the expression of Firefly reporter, which is already in the active transcriptional state (Figure 7.3B). However, Renilla reporter expression suggests that the rescue effect of nuclear Extract is much higher (2 folds) than the cytoplasmic Extract (Figure 7.3C, compare REN+cyto Extract, to REN+GV content). Rescue effect with both

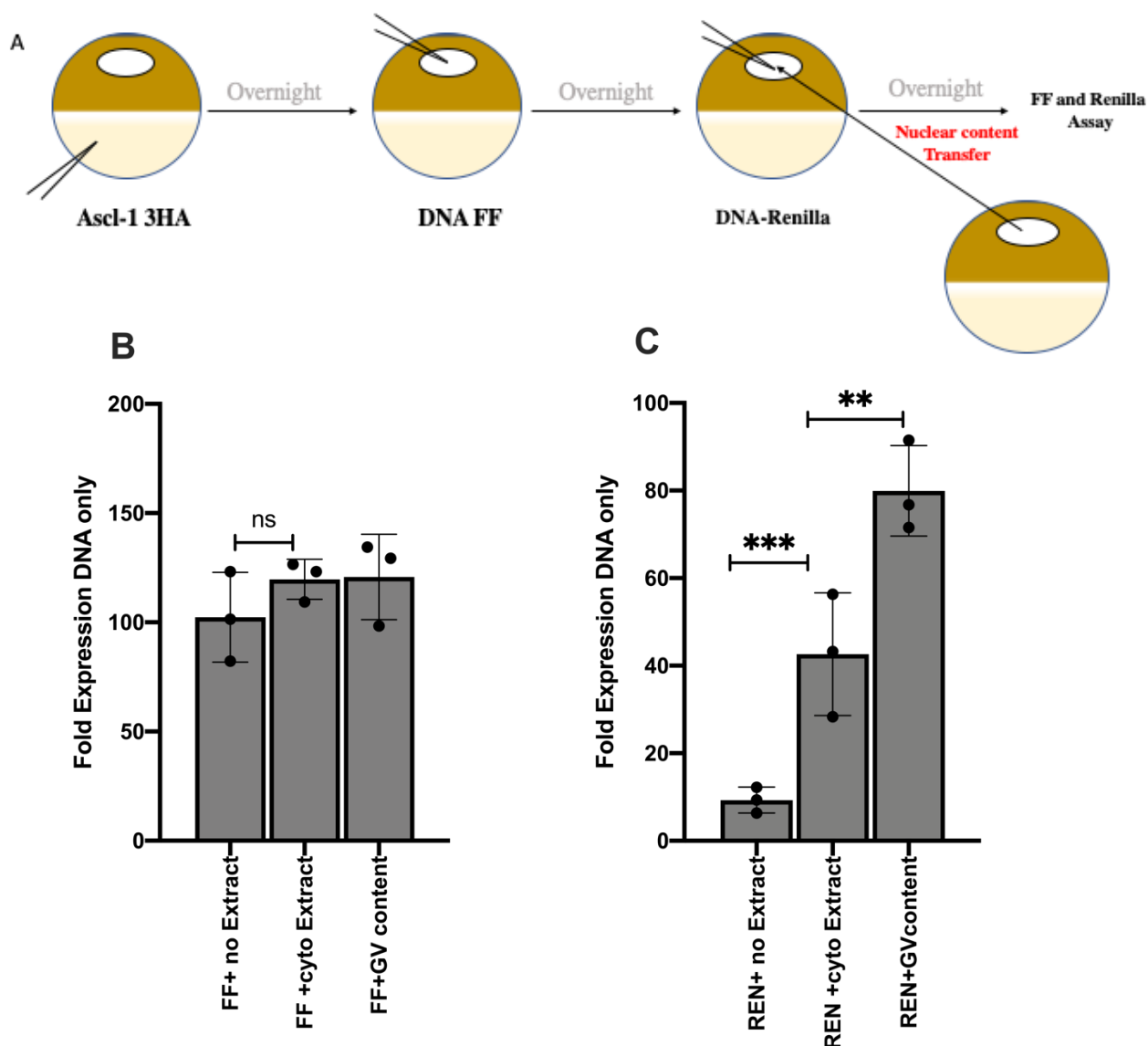


Figure 7.3: Nuclear content transfer increases the Rescue of DNA II expression

A) Schematic of the injection plan for transferring the nuclear content of an uninjected oocyte. The nuclei were isolated in mineral oil, and the content was sucked in by a needle; 23 nl of that content was then injected into the GV of preinjected DNA-I and DNA-II oocyte post 3hrs of DNA-II injections. *B)* Normalised Firefly values showing the effect of nuclear content injection on the transcription of DNA-I. *C)* Normalised Renilla values showing the expression of under-expressed plasmid by the injection of oocyte extract and nuclear transfer. Error bars show the SEM of $n=3$ with each sample containing 8 individual oocytes. *** ($P<0.05$)

cytoplasmic and GV Extract shows that the rescuing factor or factors are present in both cytoplasmic and the GV fractions.

7.3.3. Conclusion

Rescue of the expression of the 2nd injected DNA-Ren by cytoplasmic, and GV extracts show that the rescuing factor(s) are present both in GV and in the cytoplasm of the oocytes. However, the nature of these rescuing factors cannot be determined.

7.4: Pre-incubation of DNA II in GV Extract increases the expression to 40%.

The DNA-Ren was rescued by the oocyte nuclear, and GV extracts raise a question that; are the rescuing factors are oocyte-specific co-factors or are they among the General Transcription Machinery which also exists in other cell types such as Human Embryonic Kidney (HEK 293) cells? The answer to this question will get closer to the nature of rescuing factors.

7.4.1: Experimental design

A limiting amount of Ascl-1 mRNA (1.4 ng/oocytes) was injected into the cytoplasm of the oocytes. On Day 2, the same preinjected oocytes were again injected with DNA-FF at the final concentration of 300pg/oocyte. On Day2, the same injected oocytes were injected with the DNA-Ren, which was already incubated with un-injected GV extracts, and in another condition, the DNA-Ren was preincubated with Extract of HEK 293T cells.

7.4.2. Results

The Firefly and Renilla reporter show the extent of 2nd injected DNA-Ren rescue when it is preincubated with different types of cellular extracts, namely, oocyte cytoplasmic extract, Oocyte GV extract, and HEK293 cells extract. The effect of preincubation of Renilla reporter in the case of all three extracts is explained as follows. When the 2nd injected DNA-Ren was incubated with the oocyte cytoplasmic extract, the Renilla reporter expression was rescued to

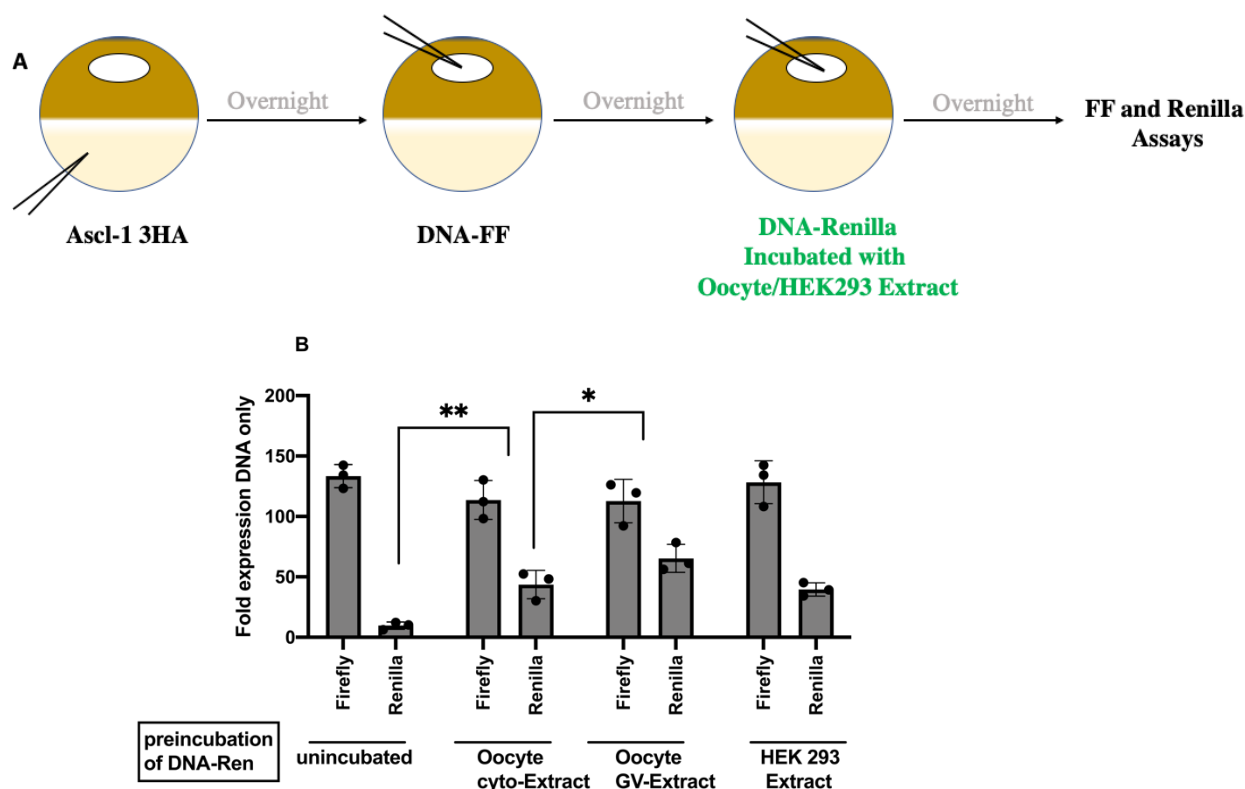


Figure 7.4: Rescue of DNA II by preincubation in GV extract of an oocyte.

A) Schematics of injection plan for Renilla plasmid rescue experiments. DNA-II is injected as preincubated in two types of extracts I) oocyte GV extract and II HEK293T extracts. All of the injections are sequential. B) Normalised Firefly and Renilla values suggest showing the effect of plasmid injection when incubated with GV extract and HEK293T cell nuclear extracts.

Error bars show the SEM of $n=3$ with each sample contains 8 individual oocytes.

** $P < 0.001$ * $p < 0.05$

~15% when compared with un-incubated control. However, when the 2nd injected DNA-Ren was preincubated with the oocyte GV extract, the Renilla expression was rescued to about 50% as compared to its under-expressed form. Surprisingly, the DNA-Ren preincubated with HEK293 cell extract also shown the rescue up to ~13-15% when compared to the under-expressed form. This experiment very interestingly shows that the factor limiting the 2nd DNA expression is divided into two major categories. First, it seems to be a component of the general transcription machinery, and so forth; it is present in the HEK293 cells as well. Second,

determines the major limiting factor is present in the oocyte GV because the expression DNA-Ren preincubated in the GV extract is much higher (50% compared to 15% by the other two), as shown in (Figure 7.4B).

7.4.3. Conclusion

This experiment suggested the two major categories of the limited factors needed for 2nd DNA expression. First, a factor from General transcription machinery might be limiting, as it can be provided by other types of cells. Second, a major limitation of an oocyte-specific co-factor, which is only supplied by the GV extract of the *Xenopus* oocyte.

7.5. Conclusion:

In this chapter, different methods of General transcription factor transfer have been used to observe the rescue of 2nd injected DNA into the oocyte. One novel method of nuclear factor transfer between oocyte has been developed. The following are the main points that have been learned from the general transcription factor transfer experiments.

- 1) DNA-Ren expression can be rescued to a smaller extent by the injection of Ascl-1 mRNA on Day 2 in the oocyte. The possible reason might be that the Ascl-1 injection causes a concentration-dependent displacement of transcription factors from DNA-FF or promotes the synthesis of new ones.
- 2) DNA-Ren expression can be rescued by the injection of an increasing amount of whole oocyte extract.
- 3) The oocyte GV fraction in the oocytes is mainly responsible for the rescue of DNA-Ren from its under-expression. However, the cytoplasmic extract injection also rescues the DNA. This may be due to the distribution of the limiting factor between cytoplasm and GV.
- 4) Pre-incubation of DNA-Ren with Somatic and oocyte extracts reveal that the rescue is due to the multiple factors which may bind through protein-protein interaction.

The main results of this chapter have shown that there is a limitation of transcription factor/co-factor molecules which are not accessible to the other DNA. However, there is also a chance that the injected DNA is converted into semi nuclear bodies like Cajal bodies (Handwerger, Cordero, & Gall, 2005) and sunerposomes (Callan & Gall, 1991), which are granular structures composed of mainly RNA-Pol-II and its related transcription factors. Thus, the conversion of the 1st injected plasmid could then prevent the exchange of transcription factors between two sequentially injected DNA templates.

In the next chapter, this possibility of DNA-FF being converted into a semi-nuclear body has been investigated in detail.

Transcriptionally active DNA makes physically separated complexes in the *Xenopus* oocyte.

Introduction:

In the previous chapter, it has been determined that the under-expression of DNA-Ren can be partly rescued by the addition of general transcription factors either by the administration of nuclear or cytoplasmic extracts. This transfer of nuclear extract has narrowed down the idea to a factor that would be limiting for 2nd injected DNA if preceded by the first DNA.

However, it is also a possibility that the limitation of the factor(s) required for the functional expression of DNA-Ren are confined to some specific nuclear compartments which exist in abundance in the *Xenopus* oocytes. These compartments such as nucleoli, Snurposomes, and Cajal bodies are membrane-less compartments which contain a variety of compounds such as RNA processing machinery in Cajal bodies (Nizami & Gall, 2012), RNA-Pol-II and associated factors in Snurposomes (Callan & Gall, 1991), and ribosomal gene synthesis and RNA-Pol-III machinery in the nucleolus of the oocytes (Mais & Scheer, 2001). Emerging studies have shed some light on the nature of these membrane-less organelles and why they are necessary for the cells. I present some selective examples of the membrane-less subnuclear compartments in *Xenopus* oocytes. The first example is the presence of RNA-protein granules in the Balbiani body of the oocyte, which functions in the regulation of RNA (both coding and non-coding) through the formation of phase-separation (Cabral & Mowry, 2020). The other important example of phase-separation is the nucleolus, in which the extra-chromosomal Ribosomal DNA (rDNA) is assembled to make a transcriptionally active sub-compartment within the GV of an oocyte. Studies suggest that the aggregates of rDNA are surrounding by the dense molecules of Fibrillarin (Mais & Scheer, 2001). Furthermore, it has

been shown that these compartments are mainly associated with the proteins and RNA, which are associated with the regulation of transcription. Similarly, it has been shown that the transcription factors, especially from Pol-III, can remain associated with the DNA after the diffusion of all soluble material from the GV (Hayes, Peuchen, Dovichi, & Weeks, 2018).

The retention of soluble transcription factors with the insoluble granules raises a possibility that the injected DNA-FF entraps the local molecules which are necessary for transcription and then, like fibrillin dense threads, does not allow those molecules to escape from the local space.

In this chapter, I ask whether a variety of visual methods such as confocal and light microscopy to observe the location of sequentially injected DNAs in the GVs of the *Xenopus* oocytes. Additionally, I also use a variety of different methods to dissolve the Phase-separated compartments in the *Xenopus* oocyte to study the rescue of 2nd DNA expression.

8.1. DNA-FF and DNA-Ren retain their transcriptional activity after fluorescent labelling by Cy3 and Cy5 dyes.

Xenopus oocyte GV is a big nucleus that can successfully transcribe several hundred mammalian cultured cells when nuclei are transplanted (Pasque, Jullien, Miyamoto, Halley-Stott, & Gurdon, 2011b). To visualise the DNA injected sequentially into the GVs of the oocytes, it was necessary to develop an approach that can label the plasmid DNAs directly as opposed to the indirect labelling through fluorescent protein binding. Thus, a reliable method of labelling the plasmid DNAs has been demonstrated (Lacroix, Vengut-Climent, De Rochambeau, & Sleiman, 2019) earlier in which DNA plasmids can be covalently labelled with the fluorescent dyes such as Cy3 and Cy5 on the Guanine (G) residues. Surprisingly, unlike other chemical labelling methods, this method retains the transcriptional activities of the labelled plasmid DNAs to as much as 80% of their unlabelled counterparts. I asked the question

of how much transcription activity will be retained by DNA-FF and DNA-Ren if they are labelled with Cy3 and Cy5 dyes? To determine this, an experiment was performed where the transcription activity of labelled and unlabelled DNA -FF and DNA-Ren was determined.

8.1.1: Experimental procedure:

The DNA-FF and DNA-Ren were labelled with Cy3, and Cy5 labelled, as mentioned in the Methods section 2.2.11. To confirm if the labelled plasmids have retained their transcriptional activities, a limiting amount of 1.4ng/oocyte, Ascl-1 mRNA was injected into the oocytes on Day 0. On Day1, the preinjected oocytes were injected with 300 pg/oocytes of DNA-FF-Cy3 and DNA-Ren-Cy5. The injected oocytes were incubated overnight before processing for Firefly and Renilla reporter expression.

8.1.2: Results:

DNA-FF and DNA-Ren plasmids contain two transcriptionally active promoters. One is Ascl-1 specific, and the other promoter is Sv40, which drives the expression of the hygromycin selection marker. I decided to determine the transcriptional activities of both of these promoters after labelling with Cy3 and Cy5. First, the expression of Firefly and Renilla reporter was determined and compared with the unlabelled counterparts, as shown in (Figure 8.1B). Reporter analysis shows that the labelling of DNA with Cy3 or Cy5 does not interfere with their function. However, the reporter's expression suggests that labelled DNA retains about 80% of the efficiency as compared to unlabelled.

A similar trend has been observed on the SV40 promoter. qPCR mediated amplification of cDNA of hygromycin has shown that the labelled DNAs also retained the activity of the SV40 promoter (Figure 8.1C). However, comparison with the unlabelled plasmids suggested the 75-80% of the activity of the SV40 promoter has been retained.

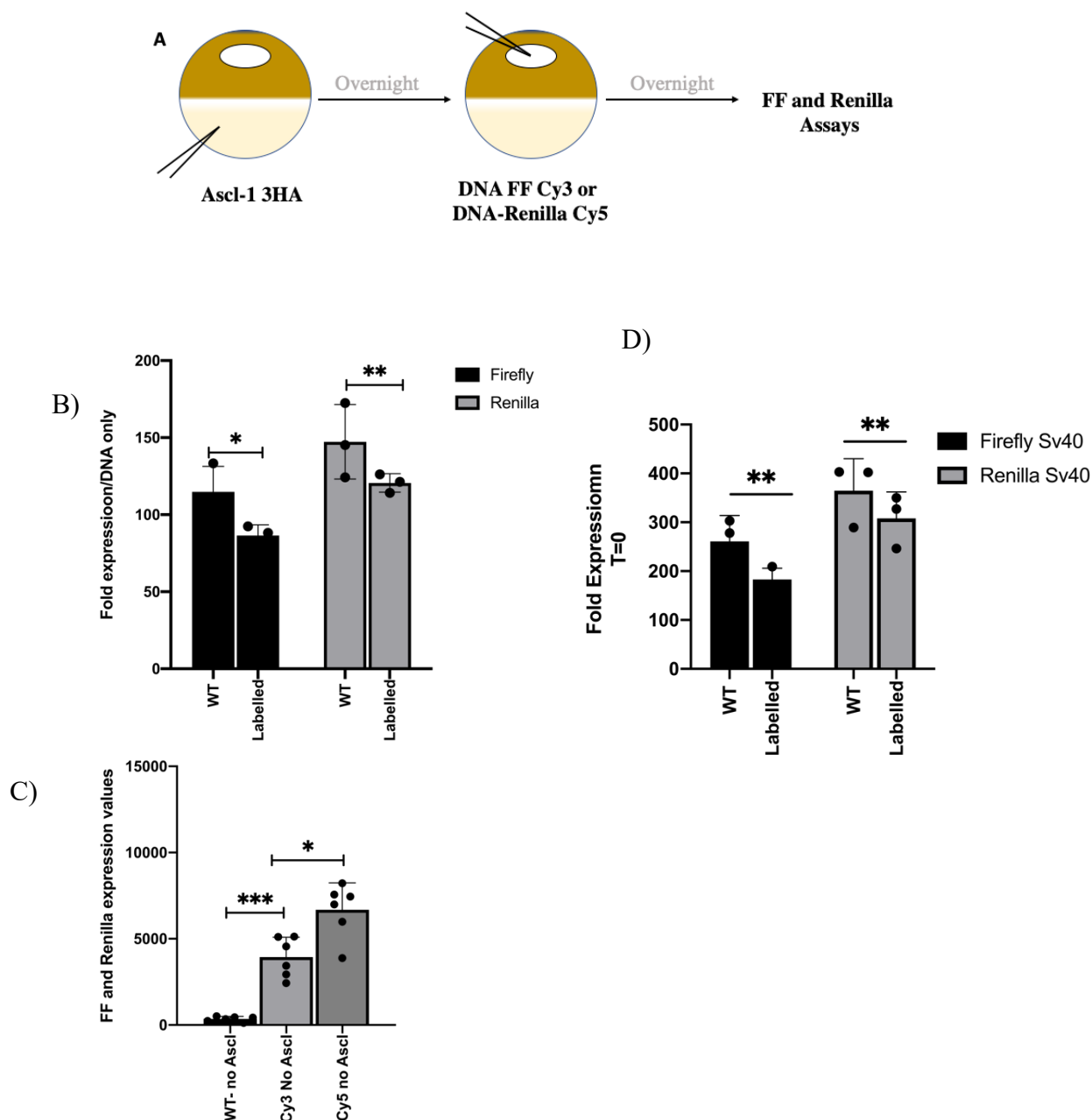


Figure 8.1: Retention of transcriptional activity of the plasmid labelled with Cy3 and Cy5 dyes.

A) Schematic diagram showing the injection plan of labelled plasmids into the oocytes, which are preinjected with Ascl-1. B) Firefly and Renilla plasmid Labelled are plotted to show the comparison of Firefly and Renilla activity differences. Labelled DNA retains transcriptional activity. C) qPCR mediated amplification of the hygromycin gene controlled by Sv40 shows the retention of transcriptional activity while labelled with Cy3 and Cy5. D) Comparison of transcriptional output in FF expression values is labelled and unlabelled plasmid.

*** $P < 0.001$ ** $P < 0.02$ * $P < 0.05$). Error bars show the SEM in $n=3$ for B and C while $n=6$ for D. Each sample contained 8 individual oocytes

8.1.3. Conclusion

Labelling of DNA-FF and DNA-Ren has suggested that, despite having covalent fluorophores, the plasmid DNAs have retained their ~80% of the activity on both promoters present in their sequence, i.e., Ebox and Sv40. This experiment confirms that the labelled DNAs are suitable for studying the Ascl-1 mediated transcription dynamics in the oocytes.

8.2: Cy3 and Cy5 labelled DNAs show the competition for transcription factors in the oocyte

Firefly and Renilla reporter expression have suggested that the labelled versions of DNA-FF and DNA-Ren retain the transcriptional activity 70-80% of unlabelled plasmid DNAs. I asked a question, will these labelled DNAs retain their ability to show resistance to the competition if injected sequentially in the GVs of the *Xenopus* oocytes? To answer this question, I performed a DNA competition experiment with labelled DNA-FF and DNA-Ren.

8.2.1 Experimental procedure:

On Day 0, a limiting amount of 1.4ng of Ascl-1 mRNA was injected into the oocytes. On Day2, 300pg of DNA-FF-Cy3 was injected into the GV of the preinjected oocytes. On Day2, the same oocytes were injected with a 3ng/oocyte of DNA-Ren-Cy5. The triple injected oocytes were incubated for 24 hours before processing for Reporter expression assays.

8.2.2. Results:

Comparison of Firefly and Renilla reporter expression in the sequential injection condition (Figure 8.2B compare Renilla only to FF>Ren) suggests that the DNA injected as 2nd DNA-Ren-Cy5 shows the same trend of under-expression when preceded by a transcriptionally active DNA such as DNA-FF-Cy3. Next, I asked if the under-expression ratio of Renilla plasmid is the same in labelled and unlabelled plasmids? Figure 8.2C shows that when DNA-FF and DNA-Ren are labelled with dyes, the 2nd injected DNA is less under-expressed as compared to the unlabelled control (Figure 8.2C, compare labelled to unlabelled).

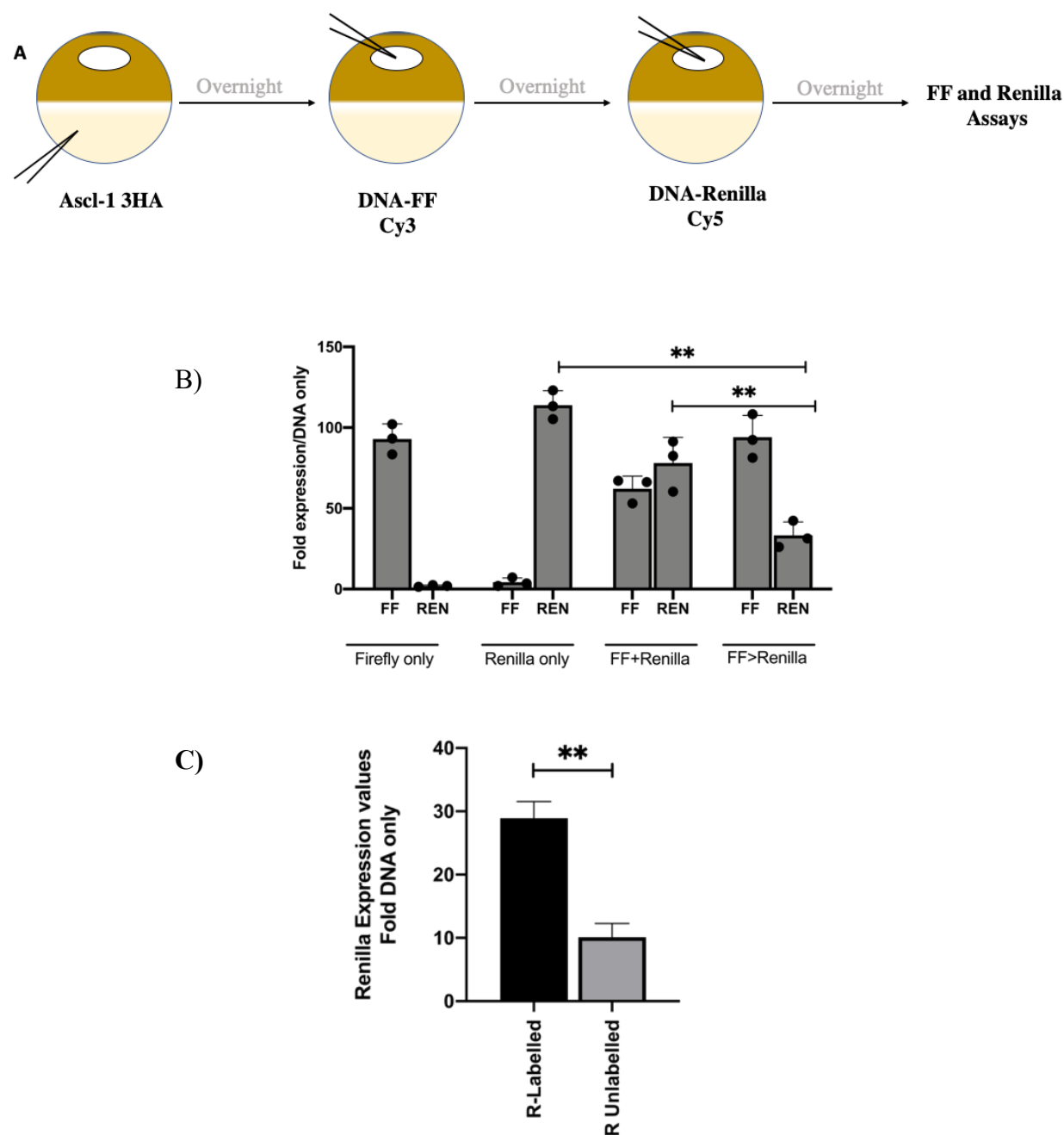


Figure 8.2: Labelled DNA I and DNA II compete for the co-factors when injected sequentially in the oocyte GV.

A) Schematic diagram of DNA competition experiment performed on the Cy3 and Cy5 labelled DNA injected into the oocytes. Ascl-1 preinjected oocytes were injected with DNA-I and DNA II separated by 24 hrs incubation. B) normalised expression value of Firefly and Renilla reporter show the expression pattern of different conditions (Written below in line). C) Bar chart showing the normalised values for Renilla expression with and without the labelling by Cy5.

Error bars show the SEM of $n=3$, while each experiment contained an average of 8 individual oocytes. ** $P<0.05$ ** $P<0.02$

8.2.3. Conclusion

DNA competition experiment with labelled DNA-FF and DNA-Ren suggests that when these plasmids are labelled, the 2nd injected DNA is less under-expressed as compared to its unlabelled counterpart. However, this result still validates that the 2nd injected labelled DNA is not well expressed if preceded by the 1st DNA if compared to the Renilla only expression. This confirms that the labelled DNA-FF and DNA-Ren can be further used for the analysis of dynamics of 2nd DNA under-expression in the *Xenopus* oocytes.

8.3: Labelled DNA can be tracked in isolated GV of *Xenopus* oocyte

Next, I asked if the labelled DNA-FF-Cy3 and DNA-Ren-Cy5 can be visualised in the isolated GVs of the *Xenopus* oocytes. It is important to note here that, amount of DNA-FF and DNA-Ren injected in this experiment is similar, i.e., 300pg/oocyte. This was a necessary alteration for the precise quantification of fluorescent signals.

8.3.1: Experimental procedure:

On Day 0, a limiting amount of Ascl-1 (1.4ng/oocyte) was injected into the cytoplasm of the oocytes. On Day1, DNA-FF- Cy3 or DNA-Ren-Cy5 was injected into the GVs of the same oocytes at the final concentration of 300pg/oocyte. After overnight incubation, the oocytes were dissected in Mineral oil, and GVs were isolated. The confocal analysis was performed by an inverted white light laser confocal microscope at the 458 and 660 nm.

8.3.2: Results

Confocal analysis reveals that the injected DNA can be seen by the Cy3 and Cy5 channel (Figure 8.3B) in the isolated GVs of the *Xenopus* oocytes. It was observed that plasmid is not diffused in the oocyte but makes small aggregates like structures that represent the formation of multi-plasmid aggregates.

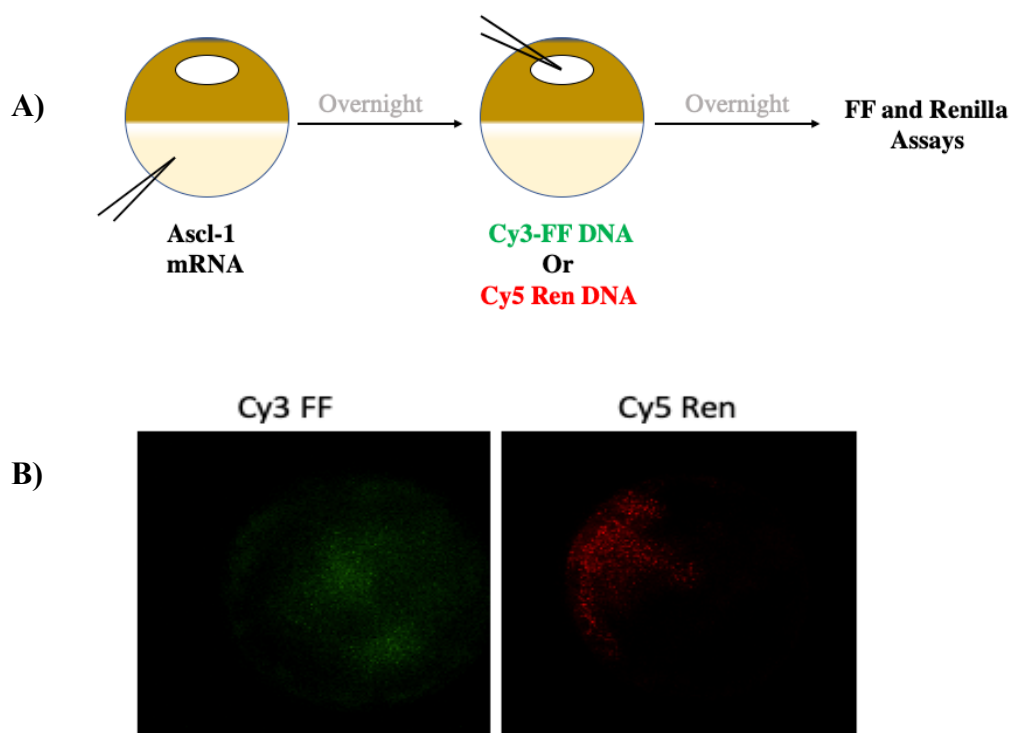


Figure 8.3: Injected plasmids in the oocytes operates through multiple plasmid aggregates

A) Schematic of injection of labelled DNA with cy3 and Cy5 in the GV of the *Xenopus* oocytes. B) Confocal imaging at 20X shows the aggregates of plasmids injected into the GV of *Xenopus* oocyte (Green) Cy3 labelled Firefly plasmid while Red) shows the aggregates of Renilla plasmid labelled with Cy5. The oocytes were imaged in oil under physiological (non-smashed) conditions.

8.3.3. Conclusion:

The Cy3 and Cy5 labelled DNA-FF and DNA-Ren was able to be detected in the isolated GVs in Mineral oil.

8.4: Sequentially injected plasmid DNA do not co-localize in the GV of *Xenopus* oocyte

Next, I asked a question whether the DNAs injected sequentially in the GV of *Xenopus* oocyte are colocalised? For answering this question, I performed a DNA competition experiment by using labelled DNA-FF-Cy3 and DNA-Ren-Cy5. The answer to this question

will allow us to understand if transcriptionally active DNA injected into the oocytes makes rDNA like structure which physically separate themselves from the rest of the nuclear material.

8.4.1: Experimental procedure:

On Day 0, a limiting amount of 1.4ng of Ascl-1 mRNA was injected into the oocytes. On Day2, 300pg of DNA-FF-Cy3 was injected into the GV of the preinjected oocytes. On Day2, the same oocytes were injected with a 350pg/oocyte of DNA-Ren-Cy5. The triple injected oocytes were incubated for 24 hours before processing for confocal analysis.

8.4.2: Results

Cy3 and Cy5 can be distinguished by the wavelength difference. Cy3 emission spectrum is 470-480 nm, while for Cy5 its 650 nm. The confocal analysis suggested that under the physiological conditions i.e., when the isolated GV in the oil is still transcriptionally active, the co-injected Cy3 and Cy5 DNA co-localize (Figure 8.4B See FFCy3+ Cy5 Ren conditions), thus confirming the observation that co-injected DNA-FF and DNA-Ren receive the transcription factors at the same time. However, when the DNA-FF-Cy3 and DNA-Ren-Cy5 were injected sequentially, there was no colocalization (Figure 8.4B see condition) Cy3FF>Cy5Ren, DNA injected 2nd was pushed toward the periphery of the GV. This striking result shows that the 2nd DNA injected does not mix with the 1st injected DNA despite spending 24 hours in the GV.

8.4.3. Conclusion

Sequential injection of DNA-FF-Cy3 and DNA-ren-Cy5 shows that both DNAs are physically separated from each other despite spending 24 hours in the same GV. According to a movie recorded, injected DNAs are dynamically moving in the nucleoplasm of the GV, which suggests that this separation is just not the mere result of a difference in the location of injection in the GV.

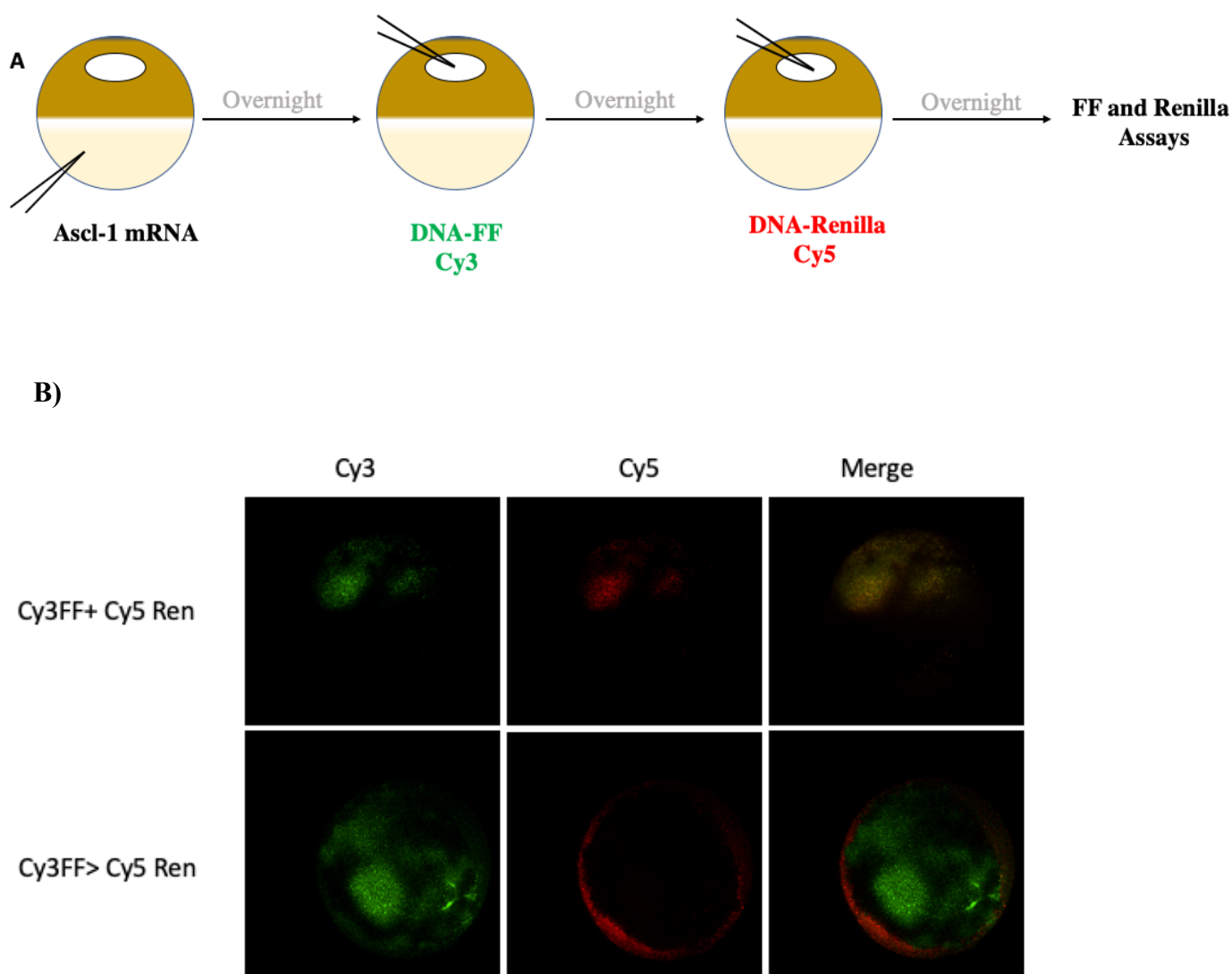


Figure 8.4: Sequentially injected plasmid DNAs do not co-localise in the GV of an oocyte.

A) Schematics of injection of labelled DNA into the *Xenopus* oocyte. The *Ascl-1* injected oocytes were sequentially injected with the DNA labelled with Cy3 and Cy5 and incubated for 24 hours. The GV(s) were isolated in Mineral oil and then analysed under 20X for an Inverted confocal microscope. **B)** competition experiment showing the localization of the injected DNA's into the GV. Row I (Cy3-FF+Cy5 Ren) represent the co-injection of plasmids into the oocytes. Both Cy3 FF and Cy5 Renilla plasmid were mixed 1:1 and then injected into the oocyte. Row II (Cy3FF> Cy5 Ren) shows the sequential injection of the Cy3 and Cy5 labelled DNA into the oocyte. The plasmid was injected in equal amounts, such as 200 pg of Cy3, followed by 300 pg of Cy5 plasmid. The GVs were isolated in sterile mineral oil and observed under a 20X inverted confocal microscope.

8.5: DNA injected to oocyte may operate through liquid-liquid phase separation

Next, I asked, do these aggregates formed by the injected plasmid in the *Xenopus* oocytes show similar behaviour as of nucleolus and Cajal bodies? If this is the case, the aggregates should be reduced in size or completely dissolved by the administration of 25mM ATP or 5% 1,6 Hexanediol. This is because 1,6Hexandiole does not dissolve the protein-protein aggregates nor the structural proteins in the cells. On similar grounds, ATP addition to the medium has also been shown to dissolve the phase-separated sub-nuclear compartments made by the interaction of NPM1 and Fibrillarin ((Brangwynne, Mitchison, & Hyman, 2011; Ying et al., 2005).

8.5.1: Experimental procedure:

Oocytes were injected with a limiting amount of 1.4ng/oocyte mRNA encoding for *Ascl -1* on Day 0., which is followed by the sequential injection of DNA-FF-Cy3 and DNA-Ren-Cy5 on Day 1 and Day 2, respectively. The injected oocytes were incubated overnight. On the next day, the GVs from the injected oocytes were isolated in the Mineral oil. Before the analysis, the GVs were injected with ATP to the final concentration of 25 mM, and another group the oocytes were injected with 5% of 1,6-Hexanediol and waited for 30 minutes before the imaging.

8.5.2: Results

Analysis of the images acquired by a confocal microscope showed that the GVs, which were injected with the 1,6-Hexanediol and ATP, shows the reduction in the size of the aggregates (Figure 8.5B See Hexanediol +). The control GV, which were injected with a similar amount of water, does not show the reduction in the size of aggregates (Figure 8.5B see Hexanediol (-). Surprisingly, the administration of ATP completely dissolved the aggregates and showed the infused green and red colors (Figure 8.5B see ATP 75mM).

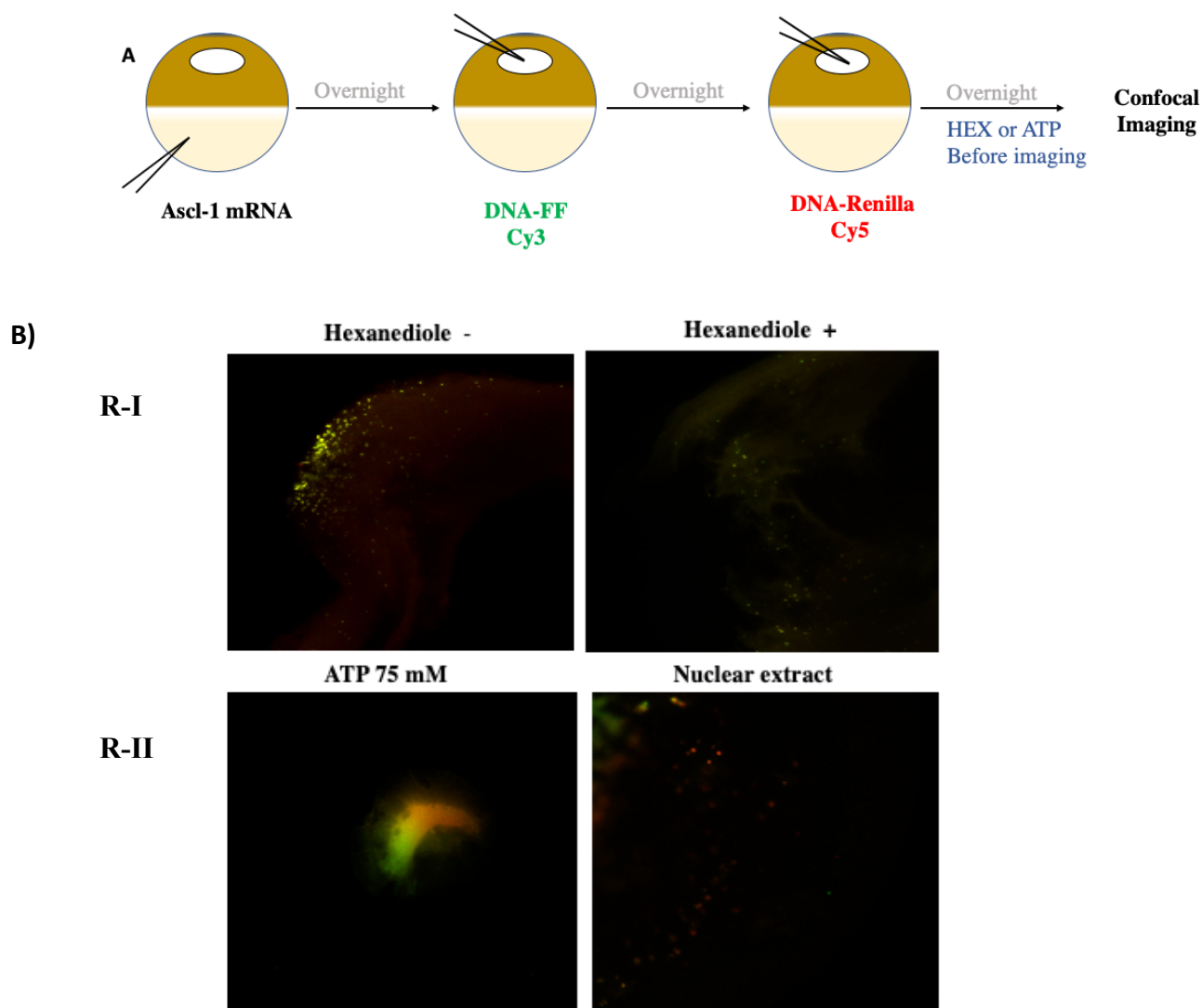


Figure 8.5: DNA injected into the oocyte operates through phase-separation.

A) Schematic of DNA injection in the oocytes, followed by the injection of an LLPS dissolution reagent. 24 hrs post-injection of 2nd DNA, Hexanediol, ATP, and nuclear extract were used to dissolve the plasmid aggregates. B) 20X confocal imaging of the GV injected with the plasmids. Row I shows the injection of Labelled plasmid Column I with distinct puncta, followed by the injection of 5% Hexandiol in the GV isolated in Oil column II. Isolated GVs were injected with 75mM ATP in mineral oil and imaged after 30 minutes of the injection Row II- Column I. In contrast, some rescue has been shown by the nuclear extract injection to the isolated GVs as well.

8.5.3. Conclusion

Injection of Hexanediol and ATP reduced the size of plasmid aggregates in the *Xenopus* oocyte GVs. Therefore, suggesting that the injected DNAs in the oocyte may behave

like a mini compartment. However, a super-resolution imaging technique is required for more consolidated conclusions.

8.6: Injection of ATP with 2nd Injected DNA with ATP and Hexanediol can rescue the phenotype to 10%.

Administration of Hexandiole (HEX) and ATP in the *Xenopus* oocyte GVs has shown that the size of plasmid aggregates can be reduced. This gives rise to a possibility that if the transcription factors required for the 2nd injected DNA-Ren expression are compartmentalised with 1st injected DNA-FF, then administration of ATP or Hexandiole should show some rescue effect on the 2nd injected DNA-Ren. I asked a question does the injection of HEX or ATP rescue the 2nd DNA-Ren expression?

8.6.1. Experimental procedure:

Oocytes were injected with a limiting amount of 1.4ng/oocyte mRNA encoding for Ascl -1 on Day 0., which is followed by the sequential injection of DNA-FF-Cy3 and DNA-Ren-Cy5 on Day 1 and Day 2, respectively. In one group of DNA-FF injected oocytes, the 2nd injected DNA-Ren was mixed with 75 mM ATP at the time of its injection to the GVs.

In the rest of the oocytes, ATP was injected 6 hrs later than 2nd DNA injection on Day 2. In the HEX group, 5% of 1,6-Hexanediol at the final concentration was added to the medium, and the oocytes were again incubated overnight before processing for Reporter assays.

8.6.2: Results:

After the DNA injections and administration of ATP and HEX, the eight individual oocytes were frozen and then analysed for the Firefly and Renilla expression. For the + ve control, an equal number of oocytes were also frozen before the administration of the ATP and HEX. Incubation of the oocyte with HEX overnight resulted in the oocyte death due to toxicity (Figure 8.6B). Furthermore, reporter assays performed on the oocyte, which were injected with

DNA-Ren with ATP, shows a small increase of 15% when compared to the control value (Figure 8.6 C). However, when the oocytes were injected later with the ATP post 6 hours of DNA II injection, only a slight rescue of the expression 9% was observed.

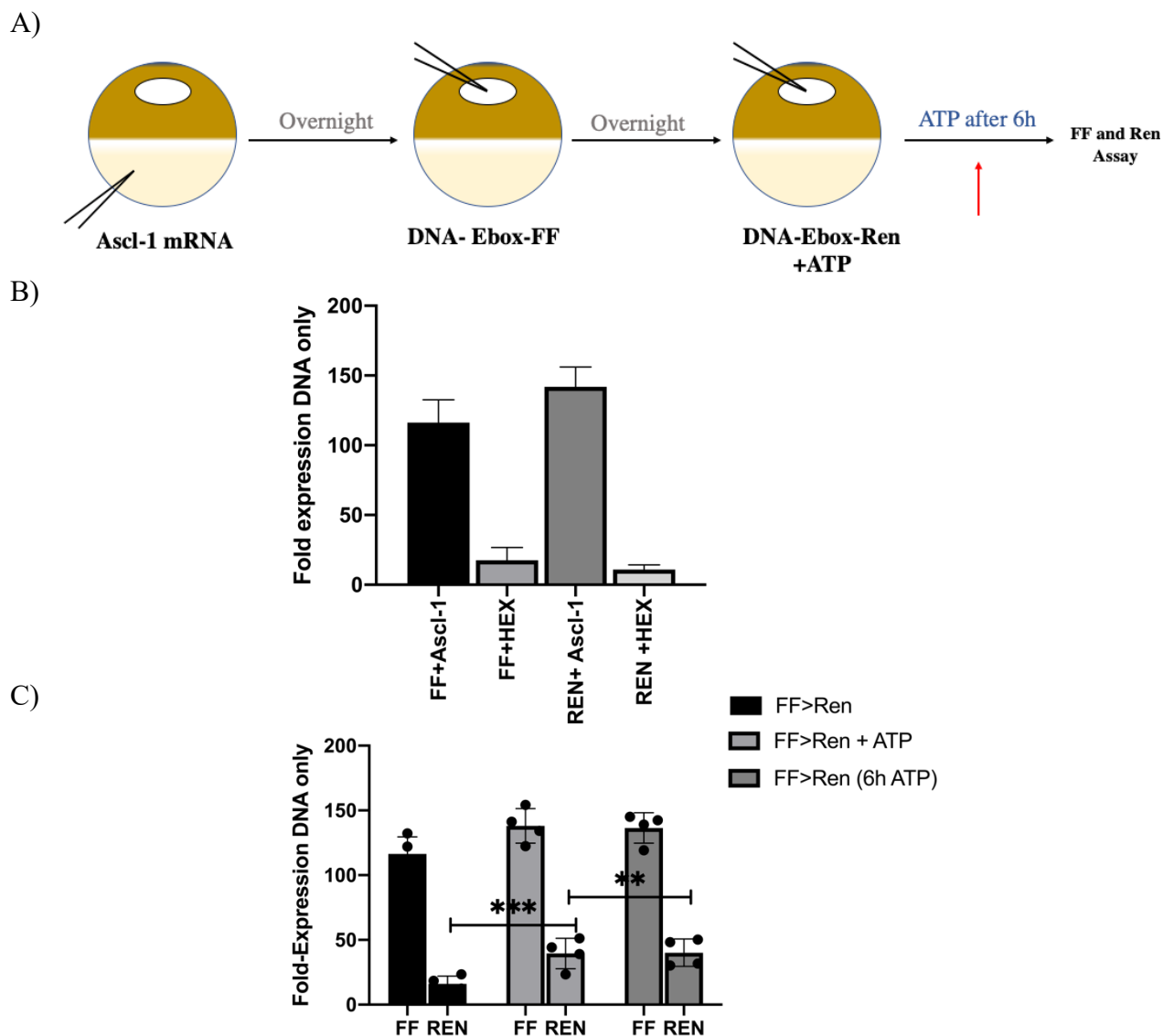


Figure 8.6: ATP injection with DNA II can rescue the competition effect in the GV of *Xenopus* oocyte.

A) Schematics diagram showing the injection plan of the oocytes injected with sequential DNA with and without a rescuing agent. B) Normalised values of Firefly and Renilla plasmid shows the relative activation of Renilla plasmid if the 2nd DNA is injected with (FF>Ren +ATP) at the same time as 2nd DNA and FF>Ren(6h ATP) when ATP is injected 6 hrs later than the 2nd DNA injection in the oocyte. C) Firefly and Renilla normalised values showing the effect of Hexanediol on rescuing the expression of Renilla plasmid.

Error bars represent the SEM $n=4$ with each sample average of 8 individual oocytes. ** $P<0.02$ *** $P<0.001$.

This result, when compared with the ATP injection in the isolated GVs while in the mineral oil, suggests that there must be an immediate decrease in the concentration of ATP in the GV when injected into the intact oocytes. The concentration of the ATP may drop quickly because of the easy diffusion to and from GVs in alive oocytes.

8.6.3. Conclusion.

The rescue experiment by the injection and addition of ATP and HEX, respectively, show that Hexanediol can be toxic to the oocytes and can cause the death of the oocytes if incubated more than 6 hours. However, injection of ATP in the GVs, which contains sequentially injected DNAs, rescues the expression of 2nd DNA to ~15%. Thus, this experiment concludes that the possibility of phase-separation like structures made by 1st injected DNA-FF exists.

8.7: Renilla DNA expression can be rescued by Tris-HCl and through heat shock to the oocytes

Injection of HEX resulted in oocytes' death due to its alcoholic nature; however, ATP showed some rescue of 2nd DNA expression. Apart from ATP and Hexanediol, other components such as high salt (Huang, Thurston, Blankschtein, & Benedek, 1990), and high amino acid concentration have also shown an effect on dissolving the sub-nuclear compartments (Taratuta, Holschbach, Thurston, Blankschtein, & Benedek, 1990). I asked a question, if a similar type of experiment where the 2nd injected DNA-Ren is injected mixed with salt, can re-distribute the factors bound to DNA-FF? To answer this question, I mixed the DNA-Ren with 1M tris pH 8.00 and injected it into the oocytes.

8.7.1: Experimental procedure:

The oocytes were injected with the mRNA encoding for Ascl-1 in a limiting amount on Day 0 and incubated overnight. On Day 1, DNA-FF was injected into the GV of preinjected oocytes at 300pg/oocyte, followed by overnight incubation. After 16-24 hours, the same oocytes with Ascl-1 and DNA-FF were injected with DNA-Ren, which was suspended in 1M

Tris HCl and incubated for another 16 hours before processing them for Firefly and Renilla assay (Figure 8.7A).

8.7.2. Results

Firefly and Renilla reporter assays show a trend in the activation of 2nd injected DNA-Ren with and without 1M Tris injection. First, I will discuss the effect of high salt on the overall health of the oocytes. The comparison of uniform pigments on the oocyte showed that overnight incubation of = oocyte in high salt injected GVs are viable. Second, I will discuss that Firefly expression increased to about 10% when the 2nd DNA-Ren was injected as diluted in 1M Tris (Figure 8.7B compare Firefly only to FF>REN+1M Tris HCl). Third, a 25% increase in the expression value of 2nd DNA-Ren was observed when it was injected as diluted in 1M Tris (Figure compare Renilla values in condition FF>REN and FF>REN+1M Tris HCl).

8.7.3. Conclusion.

Injection of 2nd injected DNA-Ren as diluted in 1M Tris has shown about 25% rescue of its expression when compared to its under-expressed form. This experiment concludes that high salt concentration, such as Tris, can re-distribute the transcription factors in between injected templates. This experiment also includes that the phase-separation could be a possible mechanism which causes the under-expression of 2nd injected DNA.

more than 25% of Renilla reporter expressions. Additionally, oocytes require the injection or administration of the non-physiological concentrations of the compounds such as ATP and Hexanediol, or even 1M Tris, which along with dissolving the phase-separated aggregates, also can disrupt normal physiological functions of the cells. So, the question arises that is there a possible way to rescue the expression of 2nd injected DNA without any alteration or injections of the compounds like HEX? Surprisingly a recent study has shown the *Xenopus* oocyte in response to heat form mini Cajal bodies, which were originally part of the B-Snurposomes (Handwerger, Wu, Murphy, & Gall, 2002), this redistribution of the transcription related components raised a point that is it possible that Heat-shock to the oocyte can re-distribute the transcription factor from DNA-FF to DNA-Ren and rescue its expression?

To investigate this question, I performed a DNA competition experiment, in which I heat-shocked the oocytes 4 hours after 2nd DNA-injections.

8.8.1. Experimental procedure:

The oocytes were injected with a limiting amount of mRNA encoding for *Ascl-1* on Day 0, and the oocytes were incubated overnight. On Day1, the same oocytes were injected with DNA-FF at the concentration of 300pg/oocyte. On Day 2, the preinjected oocytes were again injected with DNA-Ren and incubated for 4 hours. After 4 hours of DNA-Ren injection to the GV of the oocytes, a temperature shock was given to the eight oocytes (Figure 8.8A) for 30 minutes at 33 degrees. The oocytes were then incubated in an 18-degree incubator overnight before processing them for Firefly and Renilla reporter assay.

8.8.2. Results:

Analysis of Firefly and Renilla expression by reporter assay suggests that heat shock reduced the expression of Firefly to a small extent (Figure 8.8B compare firefly values in FF>REN with FF>REN(HS) and increased the expression of Renilla plasmid in the presence

of *Ascl-1* (Figure 8.8B compare Renilla values FF>REN with FF>REN(HS)). Firefly values suggest that the incubation of oocytes at 33 degrees decreases the Firefly (Already established transcription) and may have displaced the transcription factors from it. While Renilla reporter assay suggests that the Renilla values have risen up to 35% when compared to its under-expressed form without treatment (Figure 8.8B compare FF>REN with FF>REN(HS)).

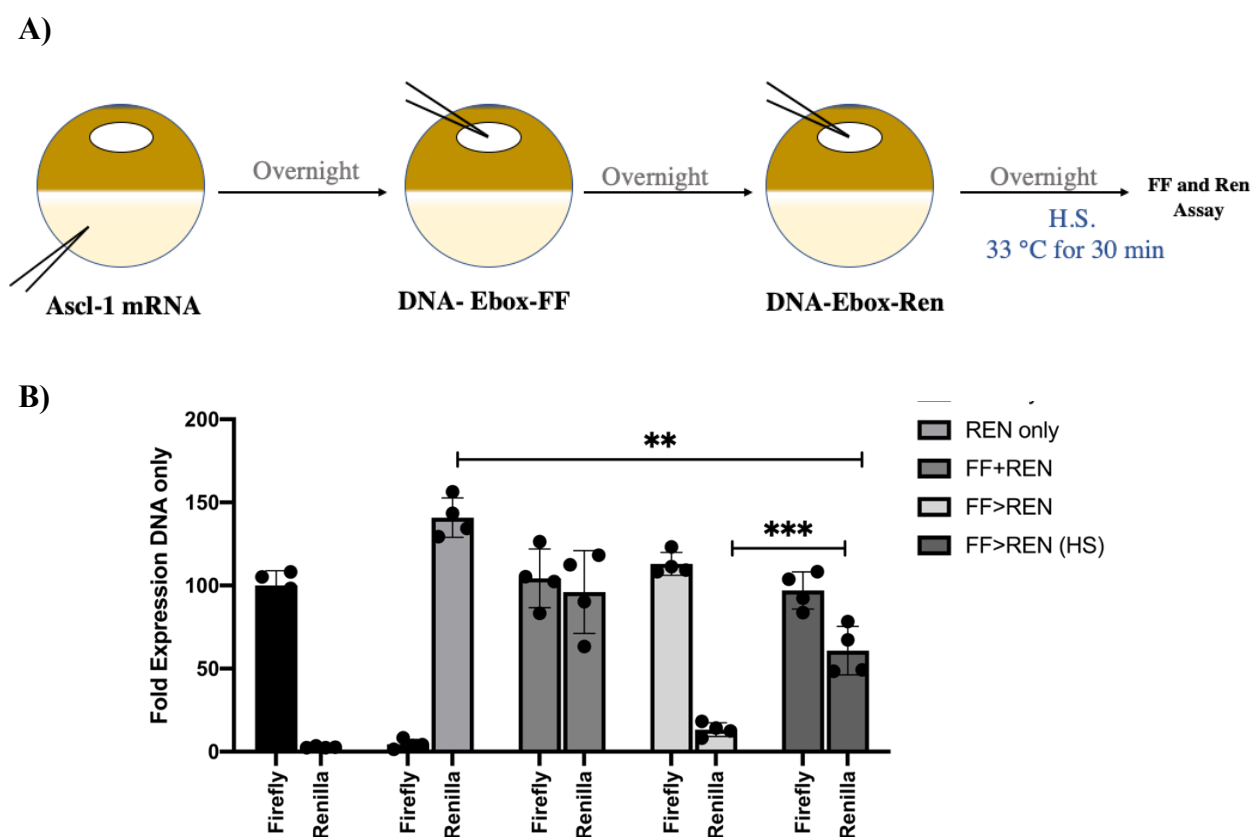


Figure 8.8. Rescue of DNA-II by heat-shock during the DNA competition condition

A) Schematic diagram representing the injection sequence for DNA competition experiment in the oocyte. B) Normalised values for DNA FF and DNA Renilla with their *Ascl-1* (-) controls are plotted to show the relative expression. Different colours show the relative expression of either Firefly or Renilla. FF>REN (HS) represents the experiment condition in which oocytes have been subjected to heat-shock at 33 degrees for 30 minutes and then re-incubated at 18 degrees overnight.

Error bars show the SEM $n=4$ with each sample containing the average of 8 individual oocytes.

** $P<0.05$ *** $P<0.001$

8.8.3. Conclusion

The Heat-shock response has surprisingly rescued the 35% rescue of the DNA-Ren, which was in its under-expressed form. It means that the phase-separated complexes can be redistributed by a cell's natural response to a changed stimulus. This experiment has provided the first of its kind evidence that the macro-molecules assemblies can redistribute under physiological conditions. Non-expression of 2nd DNA could be because of liquid phase separation.

8.9. Chapter Conclusion:

In this chapter, it has been determined that several factors can affect the expression of DNA-Ren when it is in its under-expressed form. These factors include Hexanediol and ATP, which are shown to dissociate the complexes formed by the weak or transient interactions such as Liquid-Liquid Phase Separation (LLPs). The following are the main conclusions derived from this chapter.

- 1) DNA injected sequentially to the *Xenopus* oocyte may form physically separated aggregates, which may not allow the exchange of transcription factors.
- 2) DNA-Ren injected sequentially into the oocyte can be rescued to about 35% by administration of ATP, 1M Tris, and Heat-shock.

In summary, in this chapter, it has been discovered that the DNA injected into the oocyte may form a physically separated boundary, which may entrap the transcription factors and hence not making them available for the expression of 2nd DNA. In the next chapter, I have presented a model that summarizes the whole findings and illustrates the current and more advanced depiction of DNA competition in *Xenopus* oocyte.

A mechanistic model of DNA competition in *Xenopus* oocyte

Introduction:

In the last chapter, it has been shown that stable transcription complexes in the *Xenopus* oocytes can be disassembled by the addition of some compounds like high salt, high ATP concentration, and by the injection of HEX. It has also been shown that incubation of oocyte at an elevated temperature of 33 Degrees can dissociate those transcription factor complexes from the oocytes. However, the exact nature of this complex was not clearly stated.

In this chapter, an experiment has been performed to analyse whether the transcriptional complexes formed on the DNA injected first in *Xenopus* oocyte are stable in nature or they entrap factors and localise them to a particular space in the GV? To investigate this, an experiment was performed in which both reporter DNAs were injected into the oocyte (Separated by overnight incubation) prior to Ascl-1 mRNA injection into the cytoplasm of the oocytes. This experiment will allow us to understand whether the DNA injected 1st (if transcriptionally active, with or without Ascl-1) can entrap the transcription factors and does not allow any competition if preceded by another DNA template. It is very important to note that the DNA-FF and DNA-Ren both contain another promoter, Sv40, which is active independently of the Ascl-1 injection. It means, when DNA-FF is injected in the GV of the oocytes, it already starts the transcription of hygromycin, which is driven by the Sv40 promoter present on the plasmid. Injection of Ascl-1 thus starts the transcription of Firefly reporter; the expression of Firefly is controlled by the Ebox-poU-Ebox promoter.

9.1.1. Experimental procedure:

Oocytes were injected with 300pg/oocyte DNA-FF on Day 0 and incubated overnight. On Day1, the same injected oocytes were injected with DNA-Ren at 3ng/oocyte and incubated for another overnight. On Day 2, a limiting amount of 1.4 ng/oocyte mRNA encoding for Ascl-

1 was injected into the preinjected oocytes (figure 9.1 A). The oocytes were then incubated overnight and processed for the Firefly and Renilla expression on the next day.

9.1.2. Results

During this experimental procedure, two types of DNA-I were injected into the oocyte. The first type of DNA is Wild type DNA-FF, which already forms a transcriptional complex because of the expression of the Sv40 promoter. The second version is a mutated version of DNA-FF, which does not contain an SV40 Promoter and hence unable to form a transcriptional complex unless Ascl-1 is injected.

First, I asked how much of Firefly reporter expression varies if the sequence of DNA injection is changed, i.e., the DNA-FF injected a day before Ascl-1 or a day after Ascl-1. Reporter expression assay showed that Firefly activation is independent of the sequence of addition of the plasmid. There was a non-significant difference between the two conditions (Figure 9.1B). Next, I asked which DNA will be activated when Ascl-1 is injected on Day2 and has an equal opportunity to activate both DNA-FF and DNA-Ren because they already are present in the GV. Surprisingly, Renilla and Firefly reporter assay showed that Ascl-1 only activated WT-DNA-FF even when both DNA were present (Figure 9.1 C compare FF+REN to FF>REN). However, when a transcriptionally inactive DNA such as pUC-19 was injected in place of DNA-FF, the Ascl-1 activates DNA-Renilla (Figure 9.1C compare FF>REN to pUC>REN). In another condition, when a mutated version of DNA-FF was injected as 1st, the later injected Ascl-1 activated both DNA-FF and DNA-REN to an equal extent (Figure 9.1D).

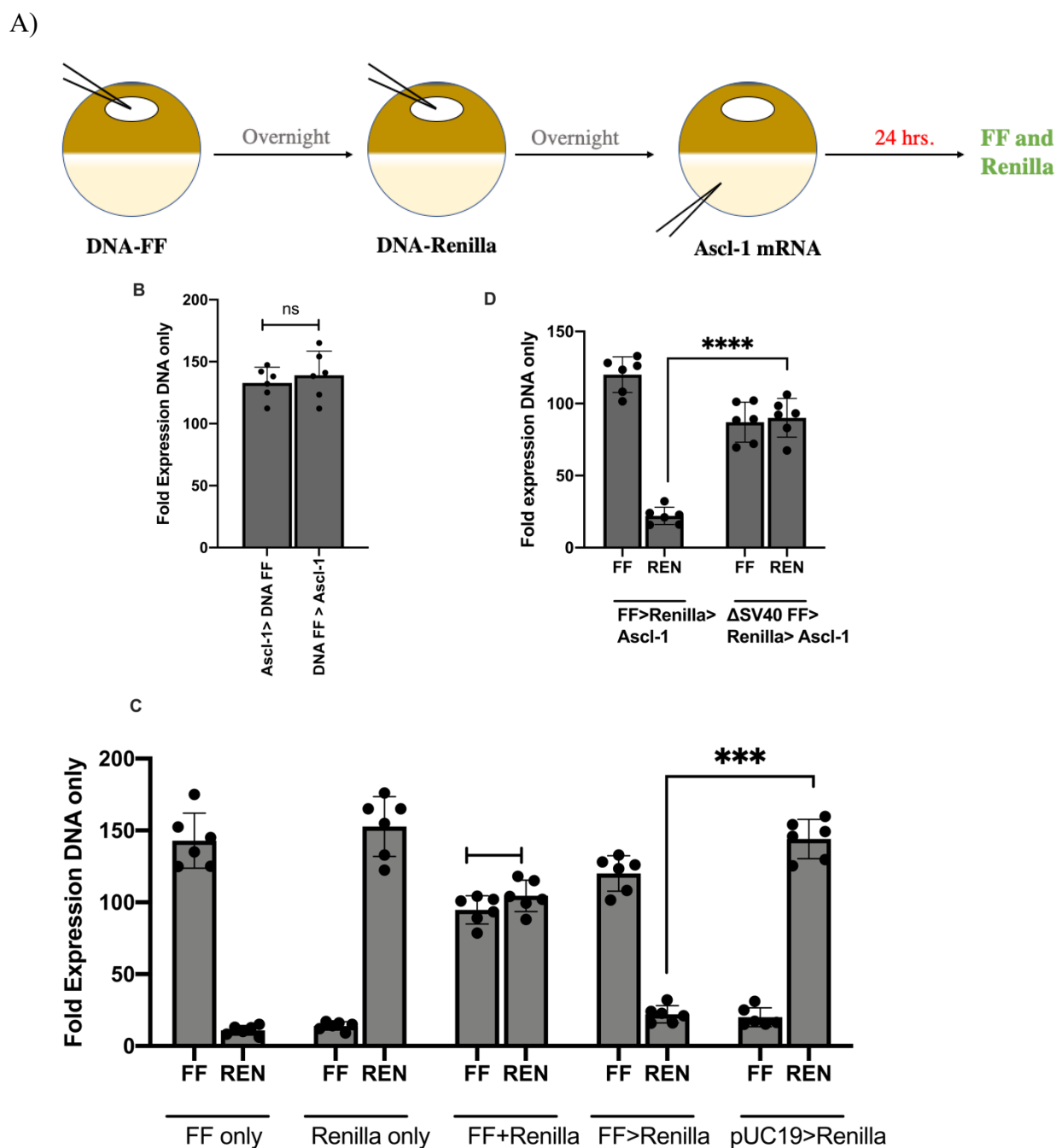


Figure 9.1: Transcriptionally active DNAI entraps the transcription factor machinery in a physically separated environment.

A) Schematic of DNA injection into the oocytes. The oocytes were injected with DNA-I and DNA-II into the oocyte. A day later, *Ascl-1* mRNA encoding for the protein has been injected into the oocyte cytoplasm. B) Firefly expression values comparison between *FF>Ascl-1* and *Ascl-1>FF*. C) Normalised values of Firefly and Renilla expression showing the relative expression of the reporter in different conditions (Written beneath the graph). D) Normalised values of Firefly and Renilla show the expression of Renilla when preceded by Δ SV40 DNA-I.

Error bars show SEM of $n=6$, with each experiment having an average of 8 individual oocytes. *** $P < 0.05$ **** $P < 0.001$.

9.1.3. Conclusion.

I have used the above results of Figure 9.1 to test old and new proposed model of 2nd under-expressed DNA in *Xenopus* oocyte. According to old model (A), the injected DNA-FF makes stable complex on the specific DNA sequence such as Ebox or Sv40. The factors associated with sequence are hence not available to 2nd injected DNA. If this hypothesis was true, the outcome of the above experiment (section 9.1) should have been as represented in model (Figure 9.2 model A) where FF-HYG is (hygromycin gene on DNA-FF) while FF-Ebox and REN-Ebox present the Ebox sequences present on respective plasmids. The Red colour of box

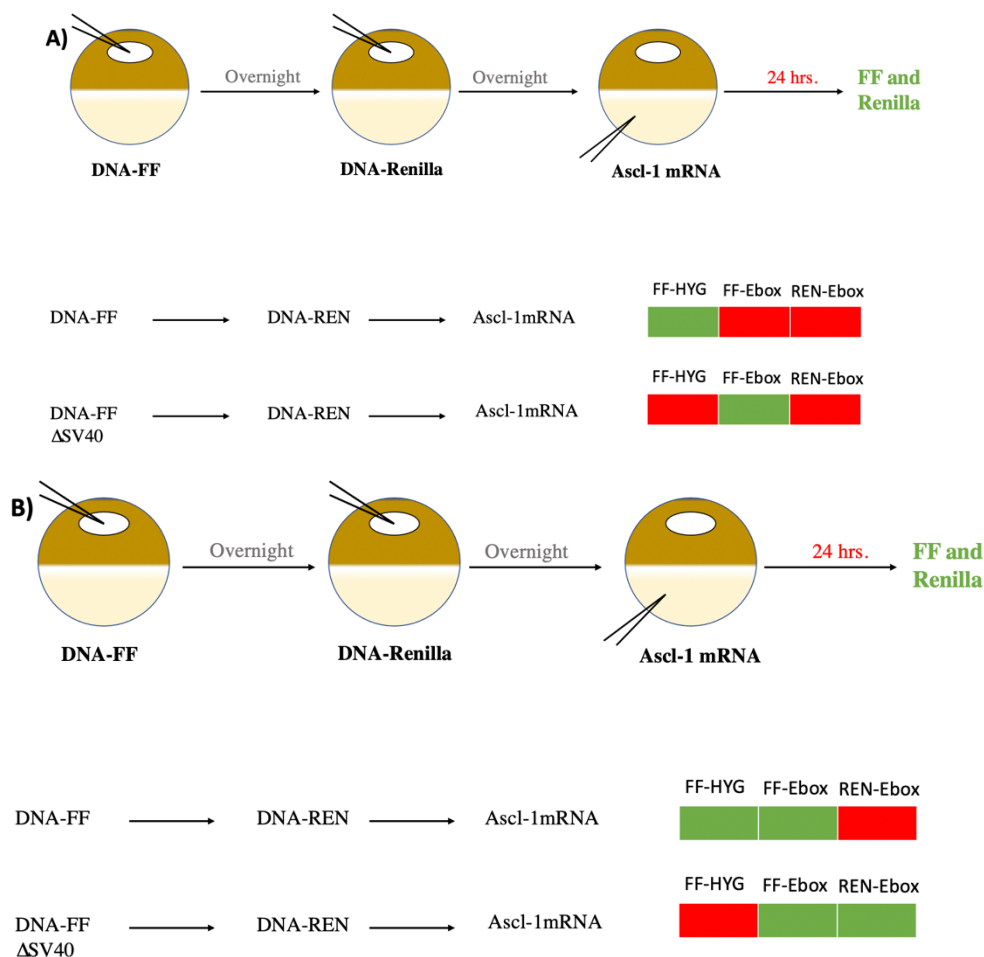


Figure 9.2: confirmation of Physical separation as the cause of 2nd DNA under-expression
A) represents how the outcome of the experiment 9.1 would look like according to Model A, which favours in stable/tight binding. **B)** Represent how the outcome of experiment 9.1 look like if Phase-separation is the cause of 2nd DNA under-expression. The tested Results exactly resembles Model B, hence confirming phase-separation to be the cause of 2nd DNA under-expression.

means that the gene is not expressed, while **Green** colour means that Gene is expressed. When the experimental results obtained (in Figure 9.1) were tested on both Model A and B, the results exactly matched to Model B which, represents that the first injected plasmid makes a physically separated boundary where all the molecules are free within its local space and can activate transcription on another binding site present on DNA-FF (Figure 9.2B). The illustration of both models is given in (Figure 9.3).

9.2. Model of 2nd DNA under-expression injected in the *Xenopus* oocyte GV

Transcriptionally active DNA injected in *Xenopus* oocyte associates itself into a complex which entraps the general transcription factors and co-factors associated with the expression of genes. By extensive analysis of transcription factor Ascl-1 dwell time, it has been determined that in an oocyte transcription factor, either related to cell fate regulation such as Ascl -1 or ER, which is associated with cell signalling and proliferation, both have a slow turnover rate. First, it was hypothesized that there is a stabilization factor in the *Xenopus* oocyte, which stabilises the gene expression (Figure 9.2A). However, experiments performed in this study suggest that DNA injected first in *Xenopus* oocyte attracts the transcription factors and make a physically separated boundary in which all those factors are accessible to different regions of the same plasmid(s) in that aggregate (Figure 9.2B). The ability of Ascl-1 to activate the DNA, which is otherwise in an enclosed compartment, suggests that the phase-separated complexes are selectively permeable to certain molecules like some transcription factors and mRNA while other molecules such as DNA plasmids cannot enter those compartments. This mode of gene regulation has not yet been observed in any other species and may explain how a non-dividing cell can maintain its gene expression for prolonged periods of time.

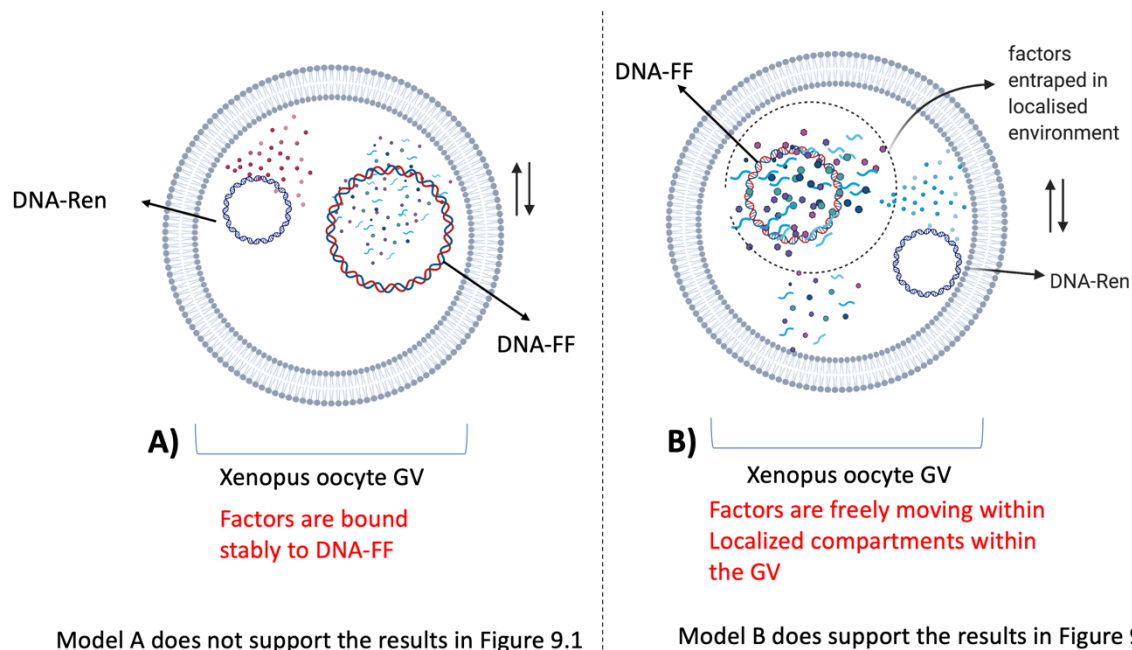


Figure 9.3: Model for 2nd DNA under-expression in a Xenopus oocyte. A) shows that upon injection of DNA-FF all the molecules essential for *Ascl-1* mediate transcription of FF gets associated stably with DNA-FF and hence not available to a 2nd injected DNA. B) Model B presents an alternative idea that injection of a transcriptionally active promoter in Xenopus oocyte GV causes the local entrapment of transcription factors which are not stably bound to a particular DNA sequence, but however, remains in a confined environment and are not available to 2nd injected DNA.

9.3. The new model of transcriptional regulation in Xenopus and its implications in the stability of a cell's fate

At the beginning of this thesis, a major question asked was, how does a terminally differentiated cell can maintain its gene expression for such a long time? The model presented here could be applied to answer that question. I hypothesize, that a cell then goes into a terminally differentiated state, makes those mini compartments in which it concentrates the necessary factors which control the expression of genes, which are essential for the control of cell identity and its survival. This strategy enables the cell to maintain a high expression of relevant genes. The more interesting finding is that the pioneer factors such as *Ascl-1* could cross this barrier and hence interfere with the transcription by using entrapped factors. That is

why some transcription factors, when over-expressed, can bring a dramatic change such as nuclear reprogramming or cell fate change.

10.0. Discussion:

In this thesis, two major problems have been addressed using a non-dividing *Xenopus* oocyte. First, the prospect of a lineage determination factor being able to stay longer than thought on its binding site, which is supported by the oocyte specific factors. This result has been supported by a newly developed Pioneer transcription factor competition, ChIP. Second, through DNA competition ChIP and direct visualisation of the sequentially injected DNAs in the oocytes, I have postulated a new model of transcription in the *Xenopus* oocyte. According to this model, the *Xenopus* oocyte, which is an example of a non-dividing cell, support its transcription by making small subnuclear compartments. These compartments have a nature like liquid-liquid phase separation and allow only selective molecules to cross those barriers. These compartments form when a transcriptionally active DNA plasmid or chromatin prepared from nuclei of a mouse or Human origin are used. The mechanistic analysis has shown that the Pol-II elongation factor contributes to this sort of interaction.

The major significance is of the work done in this thesis is the presentation of a new model for the transcription of non-dividing cells. The finding in the thesis leads a path to investigate further if similar strategies of transcription are valid in other non-dividing cells such as Neurons and muscle cells.

A systematic and critical analysis of all findings, their broad implication, and their limitation has been discussed in a stepwise manner.

10.1 Expression of a mouse transcription factor in the *Xenopus* oocytes for the study of dwell time

Xenopus oocyte has been used to study the regulation of transcription (COLMAN, 1975) because of its unmatched properties of injection of a precise amount of the molecules, i.e., protein, DNA, RNA, and other chemical components in the cytoplasm or to the nucleus of

the cell. In this study, a transcription factor, *Ascl-1*, which is present in the developing nervous system of vertebrates, is used to study the dynamics of transcription because of the following reasons. First, *Ascl-1* is a transcription factor which is alone capable of converting the somatic cells like MEFs into neuron without any help of other transcription factors such as *Brn2* and *Myt1l* (Wapinski et al., 2017), *Ascl-1* has a pioneer transcription factor function which enables it to bind to previously inaccessible chromatin regions (Lee et al., 2020). Binding of *Ascl-1* recruits other chromatin modifiers such as HDAC and other transcription factors, which then further regulate the fate of the host cell (Gao et al., 2016). Secondly, unlike many other transcription factors such as *Oct4*, which require distal enhancer regions for their function and activation of their target genes (King & Klose, 2017), *Ascl-1* requires a canonical Ebox sequence (Yutzey & Konieczny, 1992), which is sufficient enough to induce the gene expression when cloned upstream to a reporter gene such a Firefly Luciferase (Gurdon et al., 2020). Thirdly, the dynamics of *Ascl-1* expression in mouse neural stem cells govern the fate decisions into mature neurons or self-renewal of stem cells (Roychoudhury et al., 2020). The expression of *Ascl-1* persists after the terminal differentiation of neurons, which are among the most stable cell fate in the organism (Imayoshi et al., 2013).

(Hardwick & Philpott, 2018) show that the expression of mouse *Ascl-1* activates the neuronal gene pattern in a more efficient way than its *Xenopus* counterparts. Therefore, as the mouse *Ascl-1* in *Xenopus* embryo has shown a similar function, I have used the *Ascl-1* and its canonical binding site cloned on a plasmid to study the dynamics of *Ascl-1* in the *Xenopus* oocyte. Moreover, the study of transcription factor binding dynamics in a homogeneous pool of co-factor facilitates more specificity and temporal regulation.

Despite the *Xenopus* oocyte being a very reliable source of studying the transcriptional regulation of *Ascl-1*, it has some limitations as well. *Ascl-1* binds to a canonical Ebox sequence, which may or may not depend upon the enhancer regions for activation of productive

transcription. Cloning a minimal binding site does not fully recapitulate the process, which may be happening during physiologically relevant conditions, such as neural differentiation. One way is to clone the whole 5' UTR region of the genes such as Dll1 or Dll3 and to clone them on a reporter DNA, which can further be analysed by the pattern of reporter expression in *Xenopus* oocyte.

10.2. Use of DNA and competition ChIP to study the dwell time of transcription factors in *Xenopus* oocyte

I have used a peculiar strategy of competition experiments (DNA vs. DNA) and (Protein vs. Protein) to analyse the dwell time of a single transcription factor in the *Xenopus* oocyte. In this case, by a relatively simple approach, a model was used in which a single factor on/off rate from its binding site is measured by its ability to activate a different reporter (Renilla) by the same binding site. This approach provides an advantage over the previous counterparts, which contain a lot of more complicated binding sites such as on a long chromosomal DNA. On the contrary, the DNA injected has a single binding site. Mutation analysis of the Ascl-1 binding site shows that the Ebox-PoU-Ebox is essential for the activation of reporter DNA, while mutations in them show several folds transcriptional suppression (Figure 5.5). Jumping of Ascl-1 from Firefly DNA to Renilla DNA does not clearly define the dwell time of the transcription factor. As the activation of 2nd Reporter also relies on the availability of other general transcription factors that are present in the oocytes. Protein competition, however, utilises an approach in which two transcription factors (Same Ascl-1 with different epitope tags) are used to compete for a single binding site, that does answer the question to a limited extent (Figure 5.4). The protein competition experiments done in the oocyte suggest that Ascl-1 can occupy the binding sites for a day or more than that. This idea is unique and adds additional information on the transcription factor binding dynamics in terminally differentiated cells. However, Protein competition does not differentiate if the

binding of Ascl-1 is happening in homo-dimeric form, it means that if Ascl-1-GFP is making a dimer with Ascl-1 HA. A further investigation is needed in which the immunoprecipitated Ascl-1 in competition ChIP can be further analysed by the Mass-spec or specific antibodies to confirm the existence of Ascl-1 homodimers.

10.3. 1st DNA wins model is not unique to Ascl-1 only

DNA competition experiments performed by using non-lineage determination factors such as oestrogen receptor yields similar results as Ascl-1 in the *Xenopus* oocyte. In the reporter DNA, there is another promoter apart from the binding motifs of the transcription factor. The SV40 promoter in the Plasmid is transcriptionally active without Ascl-1. The deletion of an SV40 promoter from the Reporter Plasmid significantly reduces the level of competition between the sequentially injected Plasmid. This result suggests that the competition phenomenon may not be solely dependent on the concentration of Ascl-1; it may involve transcription factors and co-factors from general transcription machinery. (Mancebo & Etkin, 1988) have done several experiments showing the existence of competition for co-factors in the *Xenopus* oocyte with injected plasmids containing the promoter. Most other experiments were done using the GV extract and by using the promoters, which are constitutive (Bogenghagen, Michael Wormington, & Brown, 1982). This is the first time I showed that the competition exists in the process of induced transcription in the oocyte by using multiple transcription factors. This finding supports the idea of the existence of a transcription complex(s) that stabilise the transcription for many rounds in the oocytes and may use a set of transcription factors that is unique to the oocytes hence allows the formation of a network of factors, which is stable for hours.

10.4. Dynamics of transcriptional complexes rely on transcriptional elongation factors

Despite the identification of the DNA competition phenomenon in the 1980s, there was very little known about the dynamics of the stable transcriptional complexes in the oocytes.

Several were made to elucidate the formation of complex and to find a single transcription factor responsible for under-expression of 2nd injected DNA. (Park, Hatfield, & Lee, 1997) shows that TBP2 can rescue the injected DNA expression to a small extent. Secondly, Mattaj and colleagues have suggested that Octamer binding sequences may be playing an important role in the establishment of transcription (Mattaj, Lienhard, Jiricny, & De Robertis, 1985). However, none of the rescue procedures they used completely rescued the expression of 2nd DNA. This suggested that the limiting factor for 2nd DNA expression is not just one factor, but a class or variety of transcription factors involved in this. Moreover, the studies done several decades ago did not use any procedure such as ChIP, which could directly detect the binding of transcription factors to the injected DNA.

In this thesis, I have shown that about 50~60% percent of the 2nd DNA expression can be rescued by the nuclear content transfer between the oocyte. Furthermore, I have shown through DNA competition ChIP that the Plasmid injected as 2nd received the RNA-Pol-II, which is phosphorylated at Ser5 but not on Ser2. Comparison of 1st and 2nd Injected Plasmid narrowed down the possibility of a limitation of elongation factor(s) being the limiting factors in 2nd DNA under-expression.

The DRB mediated halting of transcription has been shown to work perfectly in other cells (Bensaude, 2011). In the *Xenopus* oocyte, DRB has been used to identify TFIIF as one of the major components involved in transcription elongation, especially the transition from Pol-Ser5 to Pol-II Ser2. However, reactivation of transcription to its fullest potential has been a limiting cause to deduce a result whether blockage by DRB actually disassembles the transcription factors from 1st Plasmid and relocates them to both after DRB removal (Figure 6.5). Despite the identification of DNA- elongation factors being a limiting factor on the 2nd injected DNA-Ren; a full rescue, just by the injection of defined factors, could not yet be achieved. For the continuation of this work, an experiment is underway, in which both active

and inactive plasmids will be pulled down, and proteins associated with them will be identified with Mass-spectrometry.

10.5. The peculiar way of transcriptional regulation in the non-dividing cells

Xenopus oocyte depicts the transcription program of non-dividing specialised cells, such as terminally differentiated cells. Dynamics of general transcription factors such as TFIID and TRF3 TAF complexes have been studied in some systems such as Muscle cell differentiation into myotubes (Kabadi et al., 2015) and dynamics of SWI2/SNF (Deato & Tjian, 2007b) complexes in the neurons. However, it's not known how the master regulators of the cells fate behave when the cells undergo terminal differentiation during development and in the reprogramming? In this thesis competition, ChIP for Ascl-1 and oestrogen suggests that a master regulator transcription factor can occupy its binding site for hours in a non-dividing cell, like Xenopus oocyte. The long dwell time of transcription factors has also been observed in other organisms such as yeast (in the case of Rap1) (Lickwar, Mueller, Hanlon, McNally, & Lieb, 2012b) and in the Human (TBP in HEK293 cells) (Hasegawa & Struhl, 2019a). Unlike these studies, the dwell time of Ascl-1 and ER has only been observed on a single binding site, which provides the precision and dynamics of a transcription factor in more detail.

However, the usage of a single site for a competition experiment also has its cons. In general, the expression of a transcription factor in the cells binds to the different binding sites and activates thousands of genes by a direct or an indirect process. e.g., Ascl-1 over-expression in MEFs regulates the expression of more than 7000 genes (Wapinski et al., 2013b). Moreover, the competition experiment performed in the oocyte uses the oocyte-specific general transcription factors, which may or may not be present in the natural system of neuronal differentiation. Furthermore, a lineage determination factor binding to its site also changes the chromosomal configuration. Hi-C analysis shows that MyoD binding during trans-differentiation of fibroblasts to myotubes involves a comprehensive change in chromatin

organisation (Dall'Agnesse et al., 2019), in the contrary, Plasmid injected in the *Xenopus* oocyte do not show such a behaviour. However, as far as stability of transcription complexes is concerned, sequentially injected nuclei in the *Xenopus* oocyte show similar competition as plasmids. In another scenario, to understand the role of dwell time in reprogramming and in terminal differentiation, one should also consider performing a genome-wide competition ChIP, which would draw a broader picture of how a pioneer factor behaves on different binding sites on DNA.

10.6.A mechanistic model of 2nd DNA under-expression in *Xenopus* oocytes

For almost half a century, it has been thought that transcription complexes on injected plasmids into the *Xenopus* oocyte are very stable, and they stick to one promoter sequence and resist the competition experiment (Wolffe, 1988). This has also been proposed for the transcription-dependent on RNA-Polymerase III (Bogehagen et al., 1982). Most of these experiments have been performed using *Xenopus* oocyte or egg extracts. However, the dynamics of transcription on their binding site is best studied by the procedures like immunoprecipitation of protein complexes and Chromatin Immunoprecipitation (ChIP-IP). In this thesis, I have investigated the exclusion of 2nd DNA template by ChIP and direct visualisation techniques like confocal microscopy. Meticulous analysis of results originated by transcription factor competition ChIP and DNA competition ChIP revealed that transcription complexes show a slow turn-over from their binding sites. However, the turn-over rate is enough to provide factors for the functional expression of 2nd DNA. However, direct visualisation of sequentially injected DNA into the oocytes under physiological conditions revealed a physical separation of plasmid DNAs when injected sequentially. Moreover, it was found that the Plasmid injected into the GV of an oocyte makes small aggregates, with one aggregate containing multiple plasmid molecules. Administration of 1,6-Hexandiole and ATP in the GV suggested that physical separation of Plasmid shows a Liquid-Liquid Phase

Separation (LLPS) like behaviour. Cajal bodies and nucleolus of the *Xenopus* oocyte have been shown to form LLPs like structures (Hayes et al., 2018), which can be disassembled by the 75 mM ATP. In this thesis, I have shown that an under-expressed DNA can be rescued by Heat-shock response, which fits on an earlier finding by (Handwerger et al., 2002), which shows that heat-shock given to *Xenopus* oocytes makes mini Cajal bodies, which essentially contain the transcriptional machinery. Further, I have shown by an experiment (Figure 9.1) that instead of tight binding, a transcriptionally active DNA in *Xenopus* oocyte entraps general transcription factors to a localised environment and do not let any other DNA compete for these factors. It's been shown that multiple promoters in the 1st injected DNA can still be activated when the 2nd injected DNA is under-expressed. This finding confirms the presence of transcription factors in a localised environment instead of being tightly bound to a specific sequence.

10.7. Future work

Work done in this thesis considerably advances the understanding of 2nd DNA under-expression in *Xenopus* oocytes and sheds some light on the general strategy of a non-dividing germ cell to regulate the transcription of genes. However, a complete understanding of this phenomenon requires a detailed proteomics study, which will allow us to decipher the molecules attached to the DNA, either its active or inactive in the oocyte. A comprehensive set of experiments in this regard is ongoing in the lab, where DNA injected sequentially are labelled with Biotin and have shown some promise to pull down associated proteins.

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Long-term association of a transcription factor with its chromatin binding site can stabilize gene expression and cell fate commitment

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Some lineage-determining transcription factors are overwhelmingly important in directing embryonic cells to a particular differentiation pathway, such as *Ascl1* for nerve. They also have an exceptionally strong ability to force cells to change from an unrelated pathway to one preferred by their action. Transcription factors are believed to have a very short residence time of only a few seconds on their specific DNA or chromatin-binding sites. We have developed a procedure in which DNA containing one copy of the binding site for the neural-inducing factor *Ascl1* is injected directly into a *Xenopus* oocyte nucleus which has been preloaded with a limiting amount of the *Ascl1* transcription factor protein. This is followed by a further injection of DNA as a competitor, either in a plasmid or in chromosomal DNA, containing the same binding site but with a different reporter. Importantly, expression of the reporter provides a measure of the function of the transcription factor in addition to its residence time. The same long residence time and resistance to competition are seen with the estrogen receptor and its DNA response elements. We find that in this nondividing oocyte, the nerve-inducing factor *Ascl1* can remain bound to a specific chromatin site for hours or days and thereby help to stabilize gene expression. This stability of transcription factor binding to chromatin is a necessary part of its action because removal of this factor causes discontinuation of its effect on gene expression. Stable transcription factor binding may be a characteristic of nondividing cells.

Xenopus | transcription factor | oocytes | dwell time | *Ascl1* gene transcription

Biochemical experiments have concluded that the dwell time of a transcription factor on its specific DNA site is remarkably short, on the order of seconds (1–5), and may involve oscillation of binding. It would be of considerable interest if the dwell time for some kinds of transcription factors were found to be enormously longer under certain circumstances. This is especially so if the duration of transcription factor binding to DNA is required for some kinds of normal cell lineage progressions and for the stability of cell differentiation. The results described here give evidence of a very long dwell time by the same molecule for a cell lineage-determining factor. This helps us to understand the mechanism of action of a transcription factor that guides cell fate and stabilizes cell differentiation.

We distinguish the dwell time of a factor at its site on DNA or chromatin from the site occupation time by a factor. The dwell time means the length of time for which the same molecule of a factor remains bound to its specific binding site. The site occupation time is the time for which a binding site is occupied by the same kind of transcription factor but not necessarily by the same actual molecule.

Some transcription factors are of great importance in directing embryo cells into their intended differentiation fate. The first example of this was MyoD, which has the ability, when overexpressed, to switch the fate of most kinds of cells into muscle (6). Subsequently, other examples of lineage-determining factors

were and include *Ascl1*, a major neurogenic inducer in embryonic cells and one which causes cells to follow a neural differentiation pathway (7, 8). It can also make adult cells of different kinds become neural (9), like MyoD does for muscle (10). *Ascl1* is known to bind to a specific DNA sequence, CANNTG (11, 12), and is assumed, like the glucocorticoid receptor, to continually bind and dissociate from its DNA binding site, raising the question of its mechanism of action. We find here that the site occupation and dwell time of *Ascl1* can be much longer than has been generally believed. This may enable this factor to both initiate and stabilize a differentiated state and so give new understanding of the mode of action of a transcription factor in some kinds of cells.

Results

Experimental Design. This design needs explanation and validation. The residence time of a transcription factor determining cell fate is tested by induced gene response to a transcription factor and hence by function. The procedure for this use of oocytes is as follows (Fig. 1A). We inject messenger RNA (mRNA) encoding a transcription factor, in this case the neurogenic factor *Ascl1* (14), into the cytoplasm of a *Xenopus* oocyte, allowing the encoded protein to reach a desired concentration in the oocyte nucleus or germinal vesicle (GV). We then inject plasmid DNA, which has binding sites for the factor and an expression reporter, directly into the oocyte GV, so that it is immediately delivered to the nuclear environment of the

Significance

Some kinds of transcription factor proteins are very important in initiating and guiding cell fate differentiation. Overexpression of these factors can force many other kinds of cells to become muscle or nerve. Examples are MyoD for muscle and *Ascl1* for nerve. It is not known how long such a factor must remain bound to its binding site for it to have its function; this could be seconds, minutes, hours, or days. We have developed a procedure to determine the required residence time for the *Ascl1* nerve factor to have its function. This factor remains closely associated with its chromatin binding site for hours or days. This may be a general characteristic of such factors in nondividing (adult) cells.

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The authors declare no competing interest.

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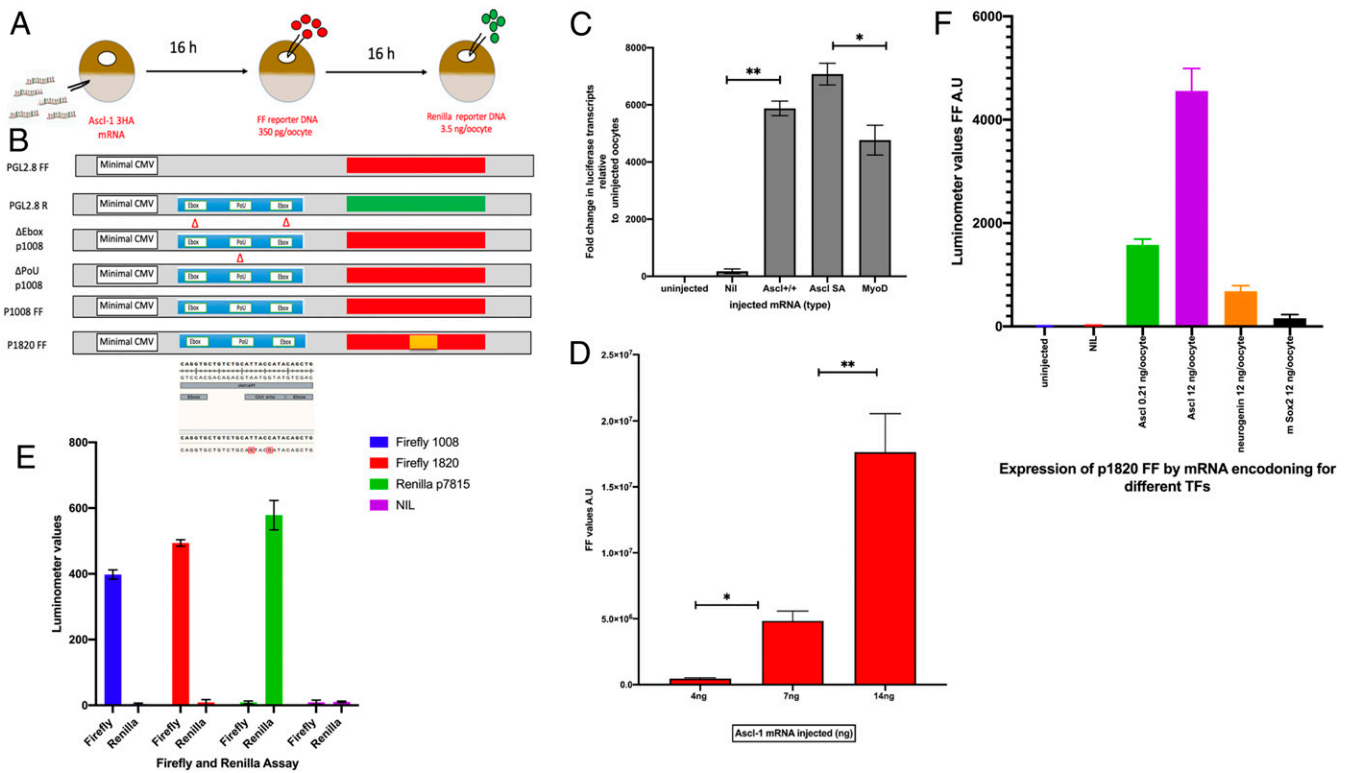


Fig. 1. Experimental design. (A) The sequence of injections on living oocytes of *Xenopus*. The normal injection volume is 14 nL, aimed for the cytoplasm (mRNA) or the nucleus (GV). Nucleic acids were made up in water for injection. (B) DNA plasmid composition. The various plasmids used are derived from PGL4.28 (Promega). P1008 was provided by A. Philpott (Jeffrey Cheah Biomedical Centre Cambridge Biomedical Campus, Cambridge, UK) (ref. 25). The base pairs marked in red had been mutated as shown for the E-box and Pou sequences. (C) The mRNAs shown cause a substantial increase in the abundance of transcripts from the E-box–Pou–E-box domain of p1008 DNA. *Ascl1* SA is a variant of mouse *Ascl1* in which alanines have been mutated to serines. **P* = 0.001; ***P* = 0.009. (D) Different amounts and kinds of *Ascl1* mRNA induce a huge increase in Firefly expression of p1008 DNA. **P* = 0.005; ***P* = 0.007. (E) The reporters FF for p1008 and p1820 respond strongly and independently to *Ascl1* mRNA. The reporter Renilla responds similarly to DNA p7815 R. (F) Different kinds of mRNA induce specific reporter responses. Neurogenin (orange) has almost no effect and Sox2 (black) of mice and no effect at all on Firefly expression (red). Error bars represent the SEM in three independent experiments (*n* = 3).

Ascl1 protein. We also use mRNA for a membrane-bound GFP, coinjected with the *Ascl1*-binding reporter DNA (p1008), to identify successfully injected oocytes (15, 16). The next day we assay each DNA-injected oocyte for expression of its reporter as an indication of the functional binding of the transcription factor to its specific DNA binding sequence. The next step is to inject plasmid DNA into the same already injected oocytes so that it can compete for any of the same factor that may have been released from the first plasmid DNA. If the residence time of the factor is short, the second DNA should compete for it and so cause expression of the second DNA. The extent to which the second DNA is expressed should therefore provide a measure of the residence time of the factor on the first DNA.

To what extent does this experimental procedure represent normal transcription in oocytes and in other cells? The injected DNAs are soon converted into nucleosomal chromatin, using the large store of oocyte histones (17–20) to which the factor binds, but are not integrated into the oocyte’s chromosomal DNA. Successful injection of template DNA into the oocyte nucleus is routinely accomplished in about 80% of attempts. We consider the injected nonintegrated plasmid DNA/chromatin to be the equivalent of the extrachromosomal nucleolar DNA always contained in a normal *Xenopus* oocyte GV (21). A typical injection of plasmid DNA (p1008) at 100 pg/oocyte amounts to injecting 10⁷ molecules, and hence, this number of test genes is not much more than the 10⁶ endogenous extrachromosomal ribosomal DNA genes always present and transcriptionally very active in uninjected *Xenopus* oocytes. We know that both the

E-box and Pou domains in p1008 DNAs are important for the transcription and translation of *Ascl1* and its reporter (22) (Fig. 1B). In typical trials, plasmid transcription is controlled by the *Ascl1* binding sites (E-box and Pou domains), so that assayable luciferase proteins are expressed. Plasmids lacking the E-box and Pou domains have a background expression of nearly 1,000-fold lower.

For many experiments we use the *Ascl1* binding reporter DNA plasmid p1008 FF, which causes a strong expression of a stable Firefly luciferase. Therefore, the luciferase activity that we record is the one which has accumulated from the time of DNA injection to the time when samples are frozen for analysis. In most cases this time is between 1 and 3 d. In other experiments we use the same plasmid with a very short half-life luciferase, namely, p1820 FF, but with exchanged reporter and regulatory sequences. Using cycloheximide to arrest protein synthesis, we find that the luciferase encoded by p1820 FF has a half-life of about 1.5 h as found by others (23) in oocytes, compared to about 50 h for an average protein (24). Using this DNA, we therefore see only the last 1 to 2 h of luciferase expression, even if the whole oocyte incubation period is up to 3 d. The half-life of luciferase mRNA synthesized in oocytes is about 5 h at 16 °C (α-amanitin assay). DNA p1820 and its reporter FF are not transcriptionally activated and expressed unless mRNA encoding *Ascl1* is present. The reporters FF and R (see Fig. 1B) are easily distinguishable and not overlapping.

A very important aspect of this oocyte assay is that the binding of *Ascl1* is assessed by its functional effect on gene expression.

Most other assays for transcription factor association with a binding site measure binding visually or biochemically rather than by function.

Characterization of the System. The composition and expression of our p1008 and p1820 plasmid DNA are particularly important for the work described here. The DNA sequence to which the transcription factor *Ascl1* binds is an E-box–Pou–E–box motif, this CANNTG sequence being characteristic of several transcription factors, including *Ascl1*. The rest of the plasmid DNA (Fig. 1B) contains 12 CANNTG sequences (13, 25). Moreover, mutations in these two E-box parts of our p1008 plasmid reduce expression of the FF reporter down to about 4%, while the mutation of its Pou domain alone reduces its *Ascl1*-induced expression down to 80% (Fig. 1B). This means that this single DNA region is almost wholly responsible for the *Ascl1*-induced expression which we see. This is in contrast to many other transcription factor binding sites in DNA, where there are many such sequences in the genomic DNA, at least several of which may be important for expression induced in normal development (26). In p1008 and p7815, there are 12 CANNTG sequences, and we do not know whether one or many of these are normally required for the downstream effects of *Ascl1* to be seen.

When we assay injected oocytes for transcripts from plasmid DNA p1008, we see an abundance of FF luciferase transcript within a day of DNA injection (Fig. 1C), and most of this RNA is dependent on a previous cytoplasmic injection of *Ascl1* mRNA, as well as being sensitive to α -amanitin. *Ascl1* and MyoD share a very similar binding sequence (22). In subsequent experiments we have chosen to assess our results by the amount of induced Firefly-tagged activity because this gives a measure of *Ascl1*-induced gene expression at the level of protein. The injection of mRNA encoding a transcription factor and plasmid DNA containing its binding region is followed by a very large increase in reporter luciferase activity of 10 to 100 times above the level in oocytes not injected with mRNA (Fig. 1D). Both FF and R reporter expression give a strong response, and there is no overlap between these two reporters (Fig. 1E). These reporters do not respond to injection of mRNAs encoding unrelated transcription factors, including neurogenin (Fig. 1F). In the absence of mRNA, there is a low level of FF luciferase expression from injected DNA, but this is usually only 1 to 10% of the level of luciferase seen after *Ascl1* mRNA when large amounts of mRNA are injected (Fig. 2A).

With large amounts of *Ascl1* mRNA both reporters, FF and R, are well expressed (Fig. 2B). Enough *Ascl1* protein is generated to allow transcription of sequentially injected reporter plasmids. The induced *Ascl1* protein concentrates in the GVs of recipient oocytes when it settles to a steady concentration for a few days (Fig. 2C). The localization of *Ascl1* protein in the GV is enhanced by the use of a nuclear localizations sequence (NLS) signal in the *Ascl1* mRNA (Fig. 2D). To confirm previous work, we have checked that injected DNA becomes quickly associated with histones (Fig. 2E). The order of injection of the two DNAs (FF or R) does not affect the strong response of the DNA to mRNA injection (Fig. 2F). In the absence of mRNA injection there is a low level of FF luciferase from the injected DNA, but this is usually only 1 to 10% of the level of luciferase seen after *Ascl1* mRNA when large amounts of mRNA are injected (Fig. 2A). After mRNA injection into oocytes, *Ascl1* protein soon accumulates in oocytes, where it settles to a steady concentration for a few days. Western analysis of isolated GVs has also enabled us to show a steady concentration of *Ascl1* protein in the GV of injected oocytes over 3 d and that the concentration of *Ascl1* protein in the GV is 10 to 20 times above that in the cytoplasm (Fig. 2C).

To determine the duration of DNA site occupation by a transcription factor, we subsequently inject a second plasmid

DNA with the same transcription factor binding site to act as a competitor, but with a different reporter. This is p7815 with a *Renilla* reporter (Fig. 1B). If the *Ascl1* factor is not stably bound to the first plasmid DNA, it will be released and then bound by the second (competitor) DNA, especially if a competitor DNA is in a high concentration and if the concentration of *Ascl1* mRNA is limiting. We need to be sure that both the test reporter DNA plasmid and the competitor plasmid respond similarly to over-expression of the mRNA that encodes *Ascl1*. To further characterize this experimental system, we also want to be sure that oocytes that have already received a cytoplasmic injection of mRNA and a GV injection of DNA can, nevertheless, transcribe and express a second GV injection of another DNA a day later. We initially tested this with a first injection of competitor DNA, followed by reporter DNA, to see if, as expected, the reporter DNA expression is not precluded by the first injected DNA. In these experiments, we used a large amount (2 ng or more per oocyte) of *Ascl1*-encoding mRNA. We see that expression of the second DNA (p1008 or p1820) increases substantially as a high level of mRNA is supplied (Fig. 2B) to give these conditions of excess mRNA. This excludes the possibility that under these conditions, a second DNA is either degraded or converted into a nonfunctional form.

The conclusions from many experiments, which gave very similar results, are as follows: 1) Two sequential injections of DNA into the same GV can result in meaningful transcription of two plasmid DNAs, as long as there is a large amount of *Ascl1* protein from injected mRNA so that the two DNAs are not in competition. 2) The second DNA injection in these experiments normally includes a GFP membrane-associated plasmid DNA which shows that the success of GV injection is generally over 80% and enables injections that miss the GV to be excluded. 3) The overall conclusion from these experiments is that oocyte injection provides a valid assay for a functional transcription factor effect on its DNA binding site and on the consequential gene expression. It is important to appreciate that the amount of mRNA in these initial experiments was large enough for this not to be limiting (Fig. 2B). Therefore, even when all *Ascl1* binding sites on the first DNA are occupied by *Ascl1*, there will still be enough of the *Ascl1* factor in the test oocytes to permit some transcription and expression of the second injected reporter DNA.

Competition Experiments.

Limiting mRNA. We now need to make the amount of *Ascl1* protein limiting to create competition between the two DNAs for the *Ascl1* factor derived from injected mRNA. At low amounts of injected *Ascl1* mRNA, reporter Firefly luciferase activity increases in proportion to the amount of mRNA injected (Fig. 3A), and a further increase in the amount of *Ascl1* mRNA makes very little difference to the induced FF expression. In most batches of oocytes, 1 ng of *Ascl1* mRNA or less behaves as a limiting amount and saturates subsequently injected DNA. Increasing amounts of injected DNA give a small, but not proportionate, increase in the amount of induced luciferase reporter expression. The amount of mRNA needed for a limiting level varies between batches of oocytes but is generally <4 ng/oocyte (Fig. 3A).

Expression DNA first. Under the conditions of 490 pg mRNA/oocyte, we injected p1820 FF as the first DNA at 120 pg/oocyte and then, 1 d later, injected a second DNA, p7815 R, at increasing concentrations (Fig. 3B). In further tests, we increased the duration of incubation after the second DNA to up to 48 h from the time of the first DNA (Fig. 3C). In Fig. 3D, we summarize the results of 17 independent experiments. Note that the scale in Fig. 3B–D is logarithmic to draw attention to the magnitude of the difference between control and experimental samples. In all cases, we see an extraordinary stability of *Ascl1* binding to its DNA or chromatin region and no evidence of its dissociation of

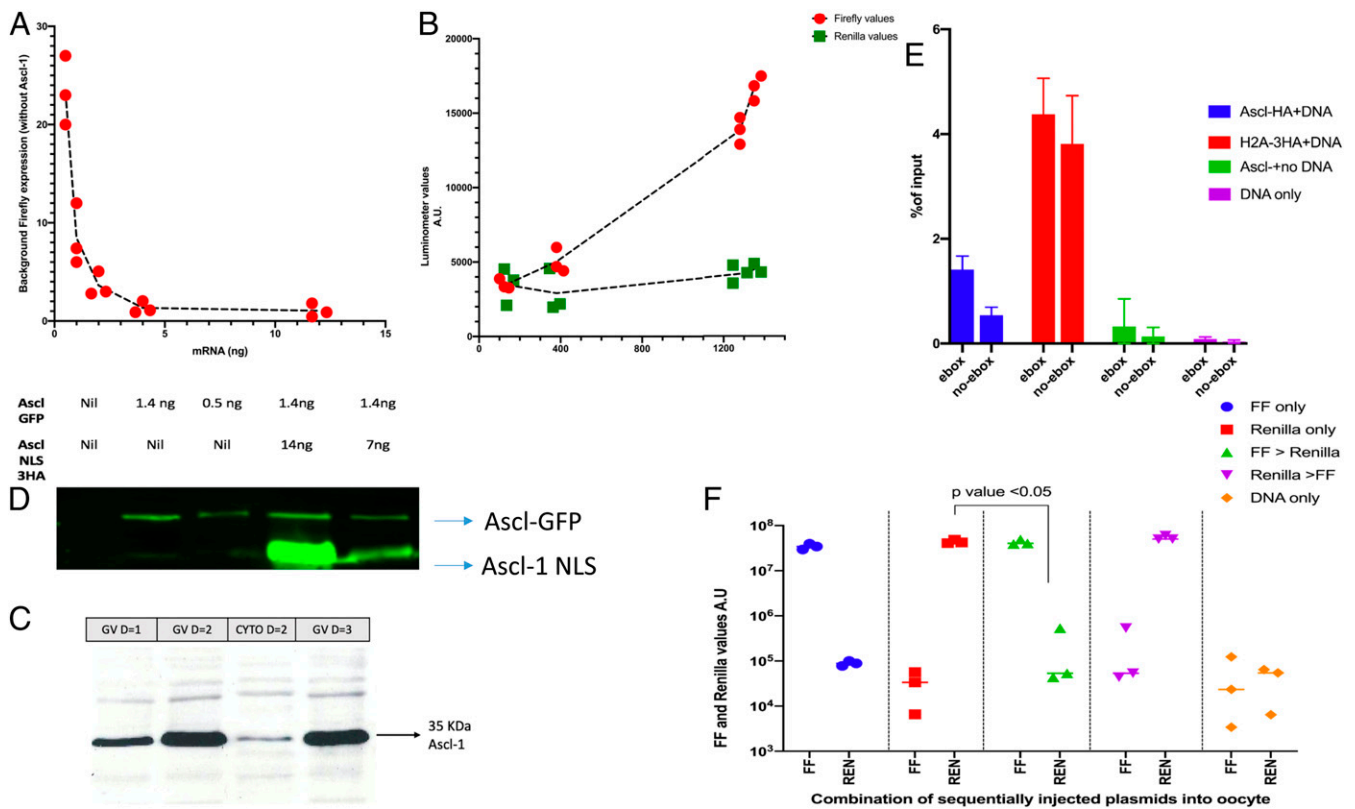


Fig. 2. Characterization of gene expression induced by *Ascl1* mRNA in DNA-injected oocytes. (A) The background level of luciferase in oocytes that received DNA but no mRNA was 1 to 10% of the mRNA-induced level. (B) High levels of injected *Ascl1* mRNA are sufficient to induce expression of a second injected DNA. (C) *Ascl1* mRNA-encoded protein concentrates in the GV of injected oocytes and remains at a high level for 3 d. (D) A nuclear localization signal in *Ascl1* mRNA helps to increase GV localization injected oocytes. (E) Injected DNA soon becomes associated with histone from injected histone mRNA. (F) The order of addition of DNA (FF or R first or sequential) gives the same conclusion: The first DNA dominates over the second. Error bars shows SEM for ($n = 3$) where applicable.

Ascl1 from DNA or chromatin within 2 d and under conditions of very large competition.

Confirmation by inverted order of injected DNAs. To verify the overwhelmingly dominant effect of the first DNA over a subsequent DNA, we inverted the order of addition of DNAs (Fig. 3E). We injected a first DNA (tagged with *Renilla*) followed by increasing amounts of a second DNA FF. We saw no change in expression of the first DNA. This is true even with a huge excess of the second DNA concentration (10,360 pg/oocyte) and an incubation time of 24 h after the supply of competing DNA (Fig. 3F). Therefore, again, the second DNA (p1008 FF) in excess is not able to increase its own expression or to change expression of the first DNA (p7815 R). This means that the *Ascl1* transcription factor did not dissociate to any extent from the p1008 DNA since any that had been released would have been taken up by the first DNA and increased its expression.

We emphasize that in the design of these experiments, we do not require expression of the second competitor DNA; it has only to be able to bind any available *Ascl1* protein, including that which might be released by a short dwell time on the first injected DNA. Nevertheless, we want to be sure that a second injected DNA is in a functional state. It could be imagined that when it does not compete with a first DNA, a second DNA could be converted, directly or indirectly, to a noncompetitive state. We have tried therefore to rescue expression of the non-competing (second) DNA as follows (see Fig. 4A).

Rescued expression of the second DNA. We prepared oocytes with limiting *Ascl1* mRNA to 210 pg/oocyte. This was followed by injection of a first DNA, *Renilla* (p7815 R), which was expressed.

This was then followed by a second DNA injection of p1820 FF. As found before, this second DNA was poorly expressed because of the preceding (p7815 R) DNA, in this example, FF values of 1,346 compared to 6,900 for no first DNA (Fig. 4A, second column). However, when we supplied a second injection of *Ascl1* mRNA (2.2 ng/oocyte) at the same time as the second DNA so that *Ascl1* protein was no longer limiting, this increased expression of the second (repressed) DNA p1820 FF from 1,346 to 13,427, an increase of 10 times (Fig. 4A, second column versus fourth column). An independent repeat of this experiment with different amounts of injection gave a similar result (Fig. 4B). Therefore, we have been able to enforce expression of a second DNA that is repressed by competition, using an increased supply of *Ascl1* mRNA. Two further such experiments gave a similar result. Thus, we find that when a second DNA is not well expressed, following an injection of the first DNA, it is able, with high doses of extra *Ascl1* mRNA, to be bound by expressed *Ascl1* protein.

The rescuing effect of the second *Ascl1* mRNA injection is not complete. It reaches about half the maximum level that can be reached by *Ascl1*-induced FF expression if there had been no competitor DNA at all. If we try an RNA rescue experiment using a complete GV extract including all of the numerous transcription factors present in a normal oocyte GV, this can also have some rescuing effect.

DNA Response Element Integrated into Chromosomal DNA. We now ask whether the stable occupation of a binding site on DNA or chromatin by a transcription factor as observed in our

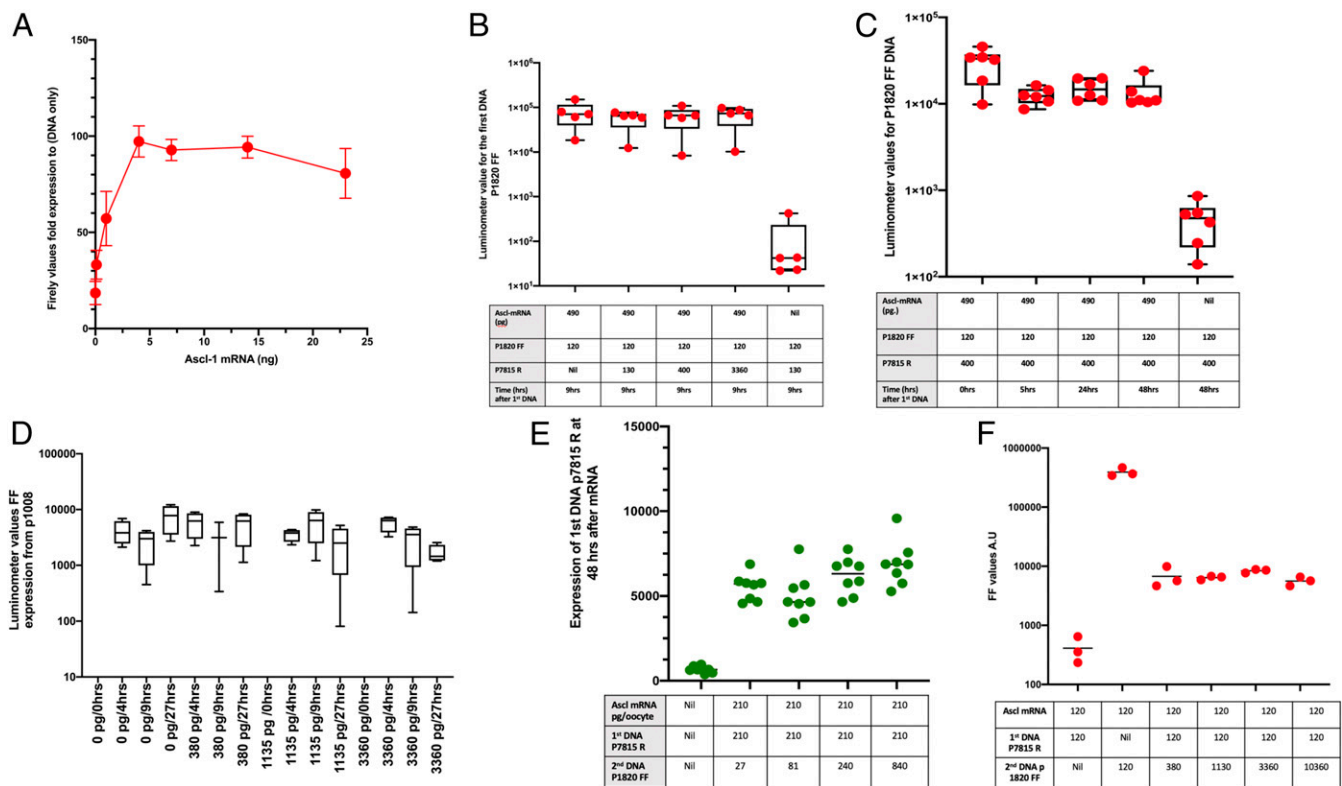


Fig. 3. Lack of competition for sequentially introduced DNA binding sites. (A) Fifty picograms of plasmid DNA are nearly saturated by 4 ng of Ascl1 mRNA. In most samples of oocytes, 500 pg to 1 ng of Ascl1 mRNA per oocyte containing 210 pg of p1820 DNA was sufficient to ensure that the synthesized protein limits the amount of luciferase reporter seen. Beyond this level, no further reporter expression is obtained by injecting a further amount of DNA or RNA. (B) Increasing the amount of a second competitor DNA up to 3,360 pg/oocyte does not increase the amount of the first DNA's reporter (p1820 FF). (C) A lack of competition is seen by a second DNA if the duration of incubation is prolonged for 2 d after the first injection. (D) Summary of results from 17 frogs in different experiments, showing that no significant reporter expression is obtained from a second injection of DNA. The synthesized DNA was injected at 0.2 to 12 ng/oocyte. (E) After Ascl1 mRNA at 210 pg/oocyte, we inject competitor DNA 7815R, also at 210 pg/oocyte. The next day, expression p1820 FF is injected at concentrations from nil to 840 pg/oocyte. (F) The amount of expression DNA p 1820 FF is the same in all samples, but the amount of p7815R as first DNA, as a competitor, shows the expected increase.

extrachromosomal plasmid DNA experiments above is also true of the same DNA binding sequence integrated into chromosomal DNA. The experimental design is similar to that used in our previous plasmid experiments (Fig. 3B) where DNA was transfected. We transfected the whole E-box-Pou-E-box response element from plasmid DNA p1008 FF into chromosomal DNA of a mouse embryonic fibroblast (MEF) cell line commonly used in our other oocyte experiments. We transplanted these MEF nuclei carrying a transfected E-box-Pou-E-box DNA for *Ascl1* binding into oocytes already expressing the *Ascl1* transcription factor after mRNA injection, as in our plasmid experiments. The enhancement of p1008 FF expression in transplanted nuclei shows the same 25-fold increase over non-mRNA-injected samples, as is seen in equivalent plasmid DNA experiments. We then carried out competition experiments as described above for plasmid DNA. The same conclusions are reached. The competitor plasmid DNA p7815 causes no detectable reduction in Firefly luciferase encoded by the DNA-transfected injected nuclei (Fig. 4C). Remarkably this is even true if the amount of competing DNA is increased from 10^2 to 10^6 (Fig. 4C). This result was seen at both 24 and 48 h after injection of competitor DNA (Fig. 4C). We conclude that a chromosomally integrated *Ascl1* binding site behaves the same as in our extrachromosomal plasmid DNA experiments; in each case the duration of the transcription factor binding site occupation is remarkably long and resistant to a large excess of competitor DNA. Again, there is no evidence for the release of

bound *Ascl1* protein from DNA or chromatin DNA, even after many hours.

Protein Competition. Since we have reached an unexpected conclusion different from that of previous work, using an unconventional assay, we have sought to test our conclusion by another procedure (Fig. 4D). This involves a protein competition experiment. We cannot use the same DNA-encoded FF reporter assay as above because a competing *Ascl1* protein would also induce expression of the same DNA Firefly reporter and so obscure any competition effect that might exist. We have therefore used chromatin immunoprecipitation (ChIP) analysis to determine the binding of *Ascl1* protein to its DNA binding site. We start with an injection of *Ascl1*-GFP mRNA, which is soon translated into *Ascl1* protein. After nuclear injection of DNA p7815R into the GV, we then inject mRNA for an *Ascl1* tagged with HA (Fig. 4D) to compete for binding with the first injected *Ascl1*-GFP mRNA and ask whether, by ChIP, an excess of *Ascl1*-HA can displace the other already bound *Ascl1*-GFP. This would happen only if the first bound *Ascl1*-GFP protein were to have a short dwell time on its DNA. We see a reduction in *Ascl1*-GFP binding compared to no competition at all, but only by 30%, and this is with a 10-fold excess of competing RNA and for a 23 h incubation period (Fig. 4E). However, this could be due to an unspecific effect of a large amount (10 ng) of competing RNA. To test this, we have competed the first *Ascl1*-GFP with an mRNA encoding the completely unrelated *xklf2*-

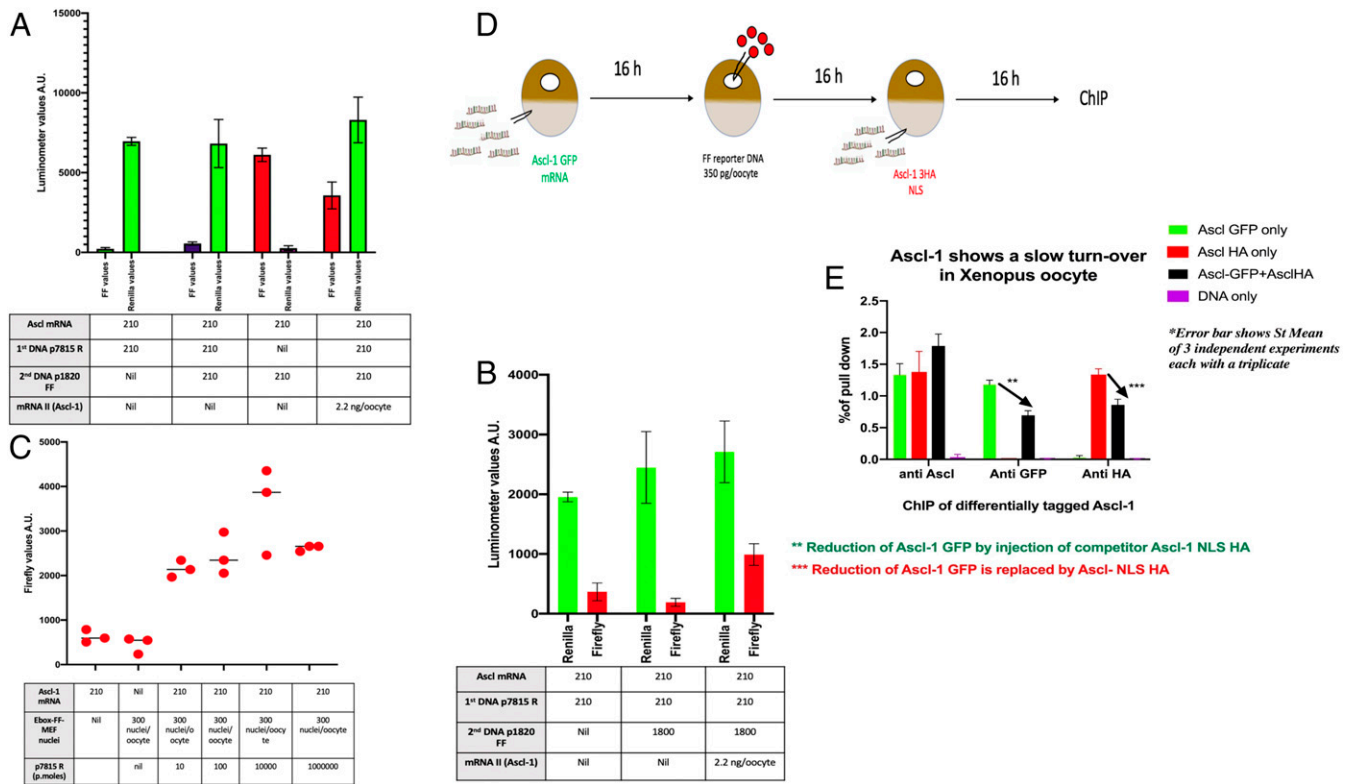


Fig. 4. Rescue of nonexpressing second DNA. (A) It starts with the usual injection of Ascl1 mRNA at 210 pg/oocyte. After 8 h, a GV injection of the first DNA (p7815R) is made. Twelve hours later, another GV injection of DNA is made, this time of p1820 FF at 210 pg/oocyte, as the second DNA. As expected, this second DNA is poorly expressed because of the earlier injection of the first DNA. However, if a second injection of Ascl1 is now made with 2.2 ng/oocyte to raise the level of Ascl1 protein above the limiting amount, it now causes a strong expression of the second DNA. This increased FF values from 1,346 to 6,900. This compares to the high initial level (13,427) of expression of p1820 FF DNA if the reexpression of the previously expressed second DNA (p1820 FF) has now been raised by five times had been no initial expression of it by 7815R. (B) Confirmation of second DNA rescue due to a further supply of Ascl1 mRNA. The experimental design is similar to that in A, except that a greatly increased amount of p1820 FF was introduced. Even now there is no indication of release of Ascl1 from the first DNA, to which it seems to be stably bound. The second injection of mRNA II causes a large increase in expression of the second (repressed) DNA. After Ascl1 mRNA and subsequent competitor p7815R DNA, both at 210 pg/oocyte, p1820 FF was injected 20 h later at the increasing picogram amounts shown. FF was analyzed 24 or 26 h later (the two time point values have been combined). The increasing amount of the second (p1820) DNA does not reduce the response of the initial competitor DNA expression (red). (C) Transplanted nuclei with an integrated E-box-Pou-E-box DNA sequence. One day after nuclei injection, p7815 Renilla competitor DNA was injected into the GV in different amounts. Thirty-six hours after that luciferase, FF or R, was scored. The figure combined with the experimental design shows the luciferase values for FF were about the same independent of additional competitor DNA ranging from 0 to 106 molecules per oocyte GV (Fig. 5A). (D) Protein competition design; two differentially tagged Ascl1 proteins compete for the same binding site. (E) Using two kinds of proteins together with ChIP analysis shows occupation of the Ascl1 binding site in DNA by each kind of protein competitor. Using p1008 FF as a competitor, we see a reduced binding of it to DNA down to about 70% of the high competitor level. If we use Xklf2 mRNA to compete with the Ascl1 mRNA, we see a reduction of binding from 100 to 80%. Therefore, the binding of Xklf2 competes almost as well as Ascl1 for the binding site on DNA. The competitive binding of Xklf2 is therefore not competing directly with Ascl1 mRNA.

HA, which has shown strong protein expression in our other unrelated experiments. We now see a reduction in *Ascl1*-GFP binding, but only by 20% (Fig. 4E), and this is only 10% different from competition with the specific competitor *Ascl1*-HA. Therefore, the main effect of this DNA binding experiment results from a high amount of mRNA. We have checked that the amount of competitor *Ascl1*-HA protein in this experiment was as high as expected from its high mRNA injection.

We point out that even if there is any real difference between the DNA and protein competition results, the main conclusion is the same. It is that a dwell time of *Ascl1* protein in these living cell experiments is very long indeed compared to previous results of others with the glucocorticoid receptor.

A Long Residence Time Is Also Seen with the Estrogen Receptor. We pointed out in the introduction above that previous work in this field has been done with receptors which govern gene expression and that need to change frequently according to conditions;

these include the estrogen receptor (3, 27, 28). We now ask whether the long residence time reported for *Ascl1* in oocytes is also true of the estrogen receptor when tested in oocytes by a competition assay.

We used mRNA for an estrogen receptor ER1 and estrogen response elements tagged with Firefly or *Renilla* (Fig. 5A). The first DNA was injected 20 h after the mRNA; the second (competitor) DNA was supplied in excess 1 d after the first DNA, and samples were collected 24 to 48 h after the second DNA. We also find that in an experimental design similar to that carried out for *Ascl1*, the provision of an estrogen reporter element elicits an enormous enhancement of estrogen reporter element expression (Fig. 5B). Estrogen does not need to be added. We find that a second DNA is minimally expressed if it is induced a day after the equivalent, but differently tagged, first DNA (Fig. 5C). This result is seen whether the Firefly- or *Renilla*-tagged DNA is injected first (Fig. 5C). We find that a large amount of competitor DNA does not reduce expression of

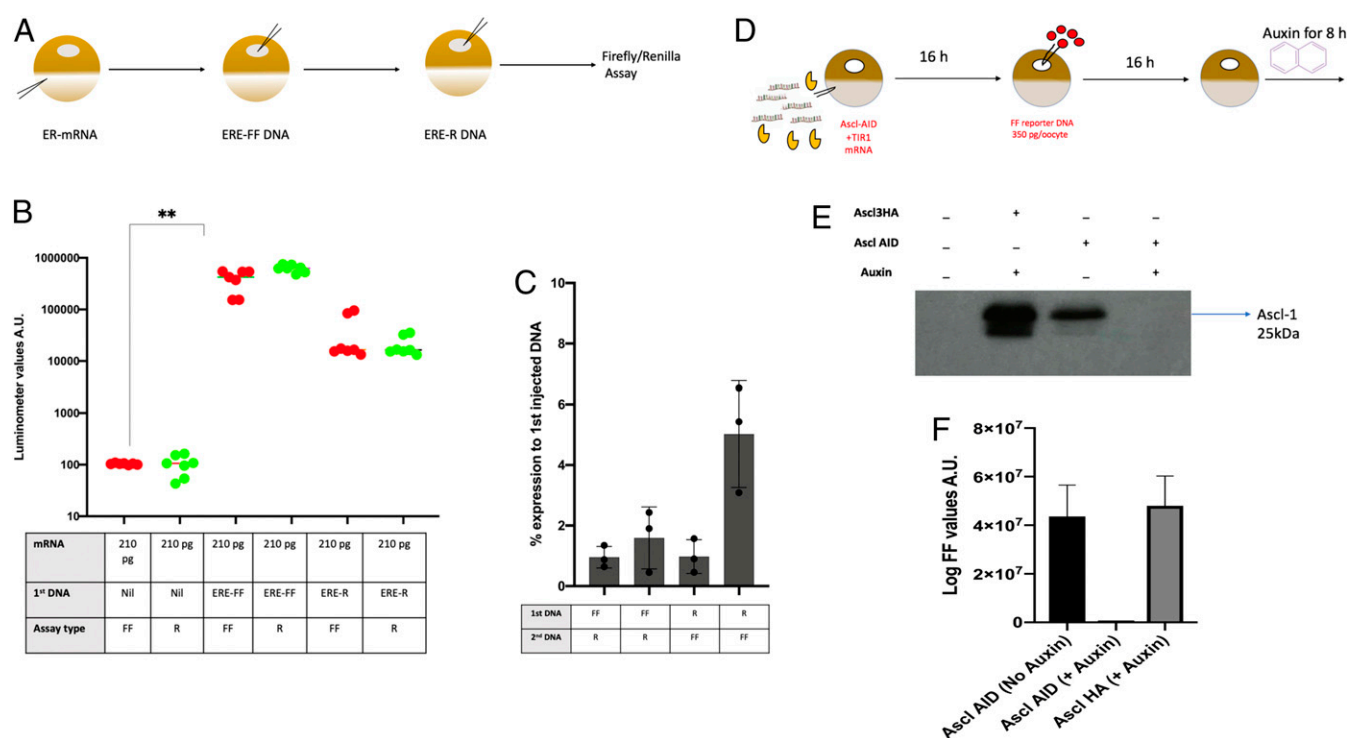


Fig. 5. An estrogen receptor induces a very strong and stable expression of estrogen reporter gene expression. (A) Design of experiment. Estrogen response mRNA was injected at 210 pg/oocyte. One day later, estrogen response element DNA at 210 pg/oocyte, tagged with either FF or R, was injected into the GV. One day after that single oocytes were assayed for FF or R fluorescence. (B) Estrogen mRNA gives a very strong response if followed by estrogen response element DNA. Thus, estrogen response element tagged with FF increased by 106-fold (red), and that tagged with R increased by about 100-fold (green). The first and second columns show incubations for 48 h between mRNA injection and assay; for the fifth and sixth columns, the incubation period was 72 h. (C) An estrogen receptor bound to its estrogen reporter DNA resists competition by DNA with a differently tagged reporter. The percentage values given show that the second DNA (whether tagged with FF or R) gives only a 5% expression compared to the same DNA injected first. The same effect as for Ascl1 is seen: Whichever DNA is injected first dominates over the second DNA. (D) A functional necessity for persistent Ascl1 protein on E-box chromatin: auxin depletion of Ascl1 protein. The design of the experiment is as follows: Oocytes were injected with 1.0 ng Ascl1-3HA mRNA. The next day, 210 pg of p1008 DNA were injected, and 1 mM auxin was added to the medium. (As a negative control, Ascl1-HA mRNA [no AID] was included.) Oocytes were processed for Firefly analysis. The results show that both Ascl1-HA and Ascl1 AID were strongly expressed 72 h after the first mRNA. (E) Western analysis to show auxin depletion of protein. Injected oocytes were separated in oil into GVs and cytoplasm; groups of 10 oocytes were then frozen and analyzed for Western blot procedure. (F) Complete removal of protein by auxin is seen. Injected oocytes were analyzed as in previous samples for FF values.

the first DNA even though it could do so without its own expression. Thus, the reporter expression of the first DNA is not reduced by the coinjection or not of a second DNA (Fig. 5C).

We conclude that the long residence time and long duration of resistance to DNA competition for an estrogen receptor are similar to those for the *Ascl1* receptor. There is therefore some special characteristic of an oocyte or its components which stabilizes the binding of a receptor to give a very long duration of induced gene expression.

The Continuing Presence of *Ascl1* Is Required for Its Induced Stable Gene Expression. Following the conclusion that *Ascl1* has an unexpectedly long dwell time on its chromatin, the next key question is whether this is of functional importance. We need to ask if the continuing presence of *Ascl1* is required for its long-term stable effect on induced gene expression. We consider the possibility that *Ascl1* is no longer present and that its stable effect on chromatin is taken over by another mechanism. The most direct test of this question is to remove *Ascl1* after its stabilizing effect has been established and to assess the binding of *Ascl1* protein by ChIP analysis to check that it has been removed. If so, does this reduce the suppression of a second DNA and hence show that the long dwell time that we find is part of the function of the transcription factor? We have been able to do this by making use of the auxin-dependent degradation procedure of Tan et al. (29) and Natsume et al. (30). Oocytes were prepared by the injection

of *Ascl1*-HA-AID mRNA, followed the next day by p1008 FF DNA (Fig. 5D). On the following day, auxin was added to the oocyte medium. Soon after that, competition plasmid DNA (7815R) was injected into the GV, and a few hours later samples were collected for ChIP analysis. The Western results, in Fig. 5E, show that the *Ascl1* protein was eliminated after auxin treatment. In the same samples, Firefly expression was also reduced to a negligible level compared to the nil-effect no-auxin controls and was not reduced at all when a nondegradable *Ascl1* was used (Fig. 5F). We therefore see that the induced expression of DNA + FF is dependent on the continuing presence of *Ascl1* and that its stabilizing effect, resistant to competition, is not replaced by another mechanism.

We conclude that the presence of the *Ascl1* transcription factor is required for its long-term and stable gene-inducing effect. This provides a functional test for the long-term and site occupation stability of the *Ascl1* transcription factor bound to chromatin.

Discussion

The bottom line of this work is that the early bird catches the worm (31). We find that in a nondividing cell, two transcription factors that bind to their specific DNA sites can remain for several hours or days and that this largely resists competition from similarly specific DNA sequence (or chromatin). This gives insight into the mode of action of a transcription factor. This

result is very different from the common view that transcription factors have very short residence times of seconds or minutes.

For a number of years, Hager and colleagues have used many different methods to estimate the time the glucocorticoid receptor remains bound to its DNA binding sequence. The dwell times they have found are surprisingly short, namely, from less than 1 s to a few minutes (2, 27, 28, 32–37). Work with the estrogen receptor has given similarly short times. In accord with this finding is the oscillatory behavior of *Ascl1* expression in proliferating neural progenitor cells (38). Most recently, very short dwell times were found for factors that regulate gene expression in early rapidly dividing *Drosophila* embryos. In these cases genes need to be able to respond rapidly to changing metabolic conditions as in embryos (37). Using a gel shift dissociation assay, Fong et al. (12) found a binding time of 2 to 3 min for MyoD (CAGGTG) and NeuroD (CAGATG) in cultured mammalian cells. One of the longest dwell times described is that of the binding to the heat shock gene in differentiated *Drosophila* cells, where a replacement time of 1 h was seen (39). Individual transcription factor components can also exchange very rapidly, such as TFIID (40). The results reported here are clearly not in accord with this previous work, which was mostly done with dividing and proliferating cells. The dwell time in our experiment is hours, or even days, and is seen even when a huge excess of a competitor is directly introduced into a nucleus.

The idea of a long residence time for a transcription factor and resistance to competition was already discussed many years ago in the widely appreciated work of Brown and colleagues (41–43) using an entirely different system from what we describe here. The system used by Brown and colleagues was an in vitro transcription of 5S ribosomal genes by RNA polymerase III. In vitro transcription is successful using RNA pol III, but no in vitro reinitiating transcription by pol II has been described, though it does take place in living, injected *Xenopus* oocytes, which also give accurate transcription with RNA pol III (44). In *Xenopus* oocytes, there are 20,000 genes that encode the oocyte-specific 5S ribosomal gene, and these are actively transcribed in oocytes but not at all in somatic cells, whose 5S ribosomal gene transcription is undertaken by 600 somatic genes (45). It was suggested that the nontranscription of the oocyte 5S genes could be explained by a repression effect of histone with a differential binding affinity of the transcription factor TF IIIA for oocyte versus somatic genes (43).

How can we account for this difference between our results and those described before? DNA injection in *Xenopus* oocytes was used, long ago, to find some competition between two different kinds of DNA in induced gene expression (25, 46, 47). But this work does not relate to the mode of action of identified transcription factors. The most obvious difference is in the assays made. Previous work concentrated on transcription factor binding, whereas our results measure expression induced by the transcription factor. Expression seems to us most relevant for cell lineage and cell fate decisions. This difference does not, of course, explain the very short dwell times seen in previous work.

The most likely explanation is by cofactors which could bind close to *Ascl1* (Fig. 6A). However, another possible explanation for this (unexpectedly) long dwell time and resistance to competition in our DNA injection experiments would be for the first injected DNA to become associated with another nearby non-chromosomal complex, where it could take up the limited supply of *Ascl1* protein, so that there would not be enough of this protein to give transcription of a second injection of DNA (Fig. 6B). For example, transcription complexes in amphibian oocytes are believed to be in liquid phase-separated complexes (48, 49). Support for the idea that an *Ascl1* chromatin could be subject to phase separation comes from our finding that the injection of adenosine triphosphate (ATP) with the second DNA has the strong effect of making it well expressed in a competition

test (Fig. 6C). Hayes et al. (49) pointed out that ATP has both hydrophilic and hydrophobic properties and could therefore interfere with phase separation. Future work will explore the likely role of a cofactor, phase separation, or both in stabilizing *Ascl1* in chromatin.

A very interesting possibility is that there are two modes of transcription factor binding, namely, one for short-term binding in dividing and proliferating cells, including oscillation (5, 38, 50), and the other for stable gene expression in nondividing (including oocytes and adult) cells. Stable binding could help to secure long-term gene expression and the differentiated state of cells. It is an attractive aim to try to identify any cofactors that can stabilize transcription factor binding. An important early paper (51) discussed some general transcription factor principles.

Materials and Methods

***Xenopus* oocytes.** *Xenopus* oocytes experiments were approved by the University Biomedical Services at the University of Cambridge and complied with UK Home Office guidelines (Animal Act 1986). The frogs used to supply oocytes were reared from fertilized eggs in our laboratory. The oocytes are taken from the ovary under terminal anesthesia. After subcutaneous (s.c.) injections of MS222 (methane sulfonate) frogs are kept on ice while fully anesthetized, and the ovary is then removed. The frogs do not recover from the terminal anesthesia.

Defolliculation of oocytes is required before they can be injected. This is done by incubating small groups of oocytes in Liberase (research grade supplied by Roche) for 2 h at room temperature. The individual oocytes retain one inner layer of follicle cells but no blood vessels or blood cells. They are then incubated in modified Barth saline solution (52) overnight with 0.1% bovine serum albumin (BSA) in the medium. The BSA helps to reduce the potentially damaging effect of the Liberase solution on the oocytes. The few dead oocytes are removed before injection. Before cytoplasmic injections, material is deposited equatorially. For germinal vesicle injections, a needle is introduced at right angles to the surface of the animal pole. The success of injection material into the germinal vesicle of an oocyte is about 80%. To monitor the success, we inject a membrane GFP encoding plasmid DNA together with the rest of the sample into an oocyte GV (2, 37). Oocytes successfully injected into the GV are seen as bright green fluorescence on the whole oocyte the day after injection. For GV injection, see below in *Oocyte GV Injection* (7).

Equipment. Glass needles are pulled out by machine, and the tips are given a sharp point and smooth opening using a microforge constructed in our laboratory (53). The opening of an injection needle is about 5 μ m in diameter.

Culture Medium. We use Modified Barth's Solution (MBS) saline solution (52) for this supplemented with 0.1% BSA. Injected oocytes are cultured at 16 to 18 °C.

DNA Constructs. We inject plasmid DNA at the stated concentrations. The various constructs we use are based on PGL 4.28 (Promega). For most purposes we use p1008 with a C-terminal Firefly reporter. In some cases the Firefly reporter is replaced by a *Renilla* reporter. Plasmid p1008-SA has seven serines in the parental DNA replaced by alanines. The DNA for injection is in a double-stranded circular form. Constructs for the estrogen work were donated by J. Carroll (University of Cambridge, Cambridge, UK), from whom further information can be obtained (Jason.carroll@cruk.cam.ac.uk). The cell line with an E-box–Pou–E-box integration appears to have only one integration site as a result of selection during the preparation of this cell line.

Nucleic Acid and Protein Extraction. Nucleic acid and protein extraction is done by our standard procedures, as described by Halley-Stott et al. (15).

Statistics. In most of this work the effects we see are very large; for example, we see a 100 times larger effect over background than when mRNA or DNA is omitted (so we do not see the need for statistical analysis); see, for example, Fig. 2F. The statistical tests for Fig. 2E and F were Student's *t* test and two-way ANOVA.

Oocyte GV Injection. Since the position of the GV in recipient oocytes is not visible, some practice is required to obtain an 80% or better rate of success in

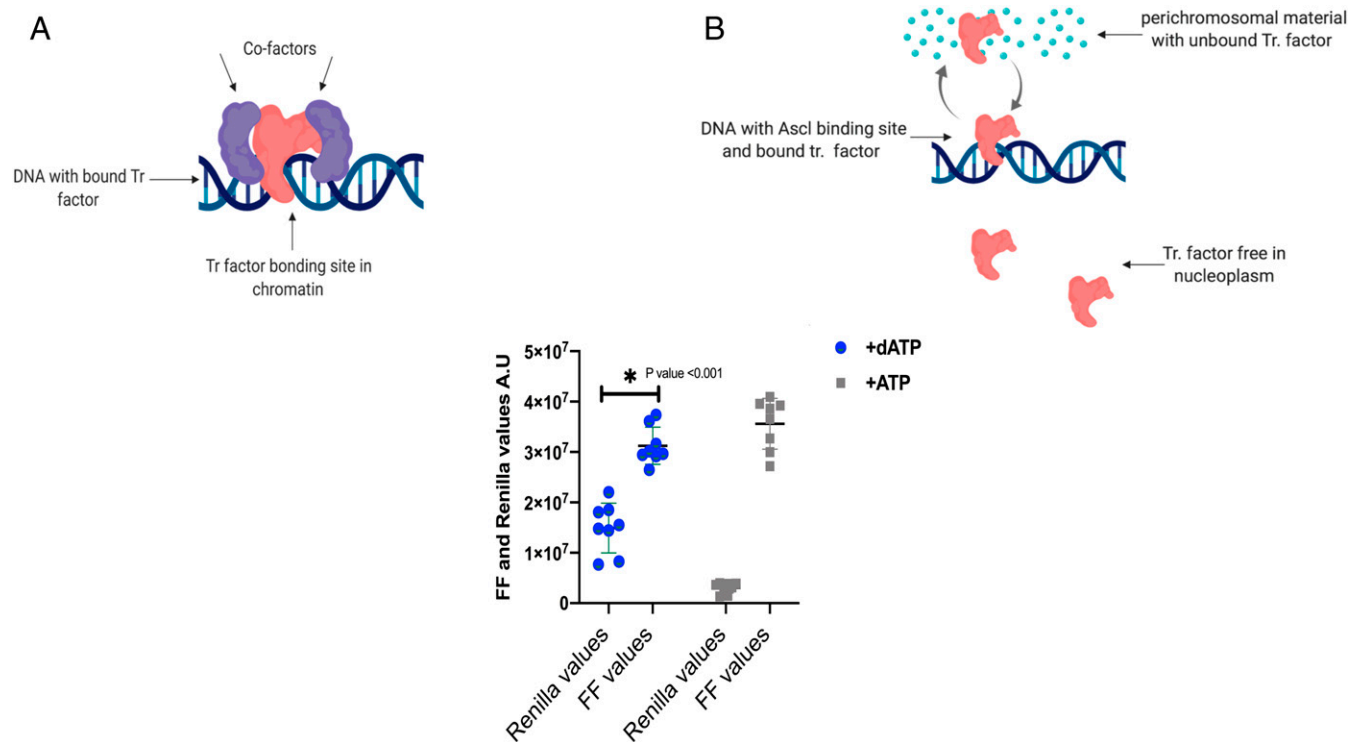


Fig. 6. Hypothesis based on cofactors for stabilization. (A) Cofactors are assumed to bind close to the bound Ascl1 protein and to stabilize Ascl1 binding for several hours. (B) Hypothesis to show the phase separation concept. Bound Ascl1 protein is supposed to dissociate from DNA and to rapidly move between its DNA binding site and nearby liquid phase-separated material. (C) Effect of ATP overexpression. mRNA and subsequent DNA plasmid (p1820) were injected as usual. Then a second DNA (p7815R) and ATP at 25 $\mu\text{g}/\text{mL}$ (15 nL) were injected. The next day FF expression was assayed as done previously. We see that the provision of ATP induces strong expression of the otherwise unexpressed second DNA.

depositing material in the GV of living oocytes. In some experiments, we coinject a plasmid DNA with a GFP tag such that the oocyte's membrane is visibly green under fluorescence, and the oocytes not showing this GFP are excluded from subsequent analysis. The chance of a second DNA being deposited in the same part of the GV as the first DNA is not always achieved, and the gel-like consistency of the GV means that the two DNAs do not always move to the same part of the GV. The variability of luminometer values for the two FF or R fluorescences depends on how effectively the second GV injection was deposited in the same part of the GV as the first injection. Moreover, the values for oocytes with or without a second DNA injection are sufficiently large that the conclusions are not affected by this uncertainty (see Fig. 6).

Data Availability. All the data used in this manuscript has been mentioned in the text. The data that support the findings of this study are available from J.B.G. upon reasonable request.

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Injected cells provide a valuable complement to cell-free systems for analysis of gene expression

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ABSTRACT

The aim of this short review is to comment on the advantages of injecting purified molecules into a normal living cell as a complement to the constitution of a cell-free system for analyzing the function of cell components. We emphasize here that the major difference is that, by injection, most components of a cell are maintained at their normal concentration, which is difficult, even if at all possible, to achieve in a cell-free system. We exemplify the benefits of a cell injection system by the efficiency and long duration of DNA transcription by RNA polymerase II, as used by most genes, and by the widespread success of injecting purified messenger RNA for protein synthesis. The most recent work using cell injection also gives a new understanding of a long lasting transcription factor residence on its DNA or chromatin not shown by other procedures. Lastly, we re-visit an old idea that transcription factors that guide cell fate may be stably bound to DNA or chromatin, except at S-phase or mitosis in the cell cycle, when they can undergo exchange with equivalent molecules in the cell.

1. Introduction

The purpose of this contribution is to review the levels at which gene activity takes place in normal oocytes, and especially after nuclei, chromatin, DNA or RNA is injected into them. For over a century, amphibian eggs have provided a favorite material for experimental embryologists (Boveri, Spemann, Hadorn, Fischberg, Nieuwkoop) on account of their size, ready availability, and ease of culture. In recent time, they have been much used to analyse steps in gene expression during nuclear reprogramming and in other ways. Here, we summarise what is known about amphibian eggs and oocytes under normal and in experimental conditions. We emphasize here the advantages of injecting a purified molecule into normal living cells to complement a reconstituted cell-free system. The major benefit of injection is that the function of a molecule is tested at its normal *in vivo* concentration of other cell components in a way that is not possible with a reconstituted cell-free system.

In amphibian literature, the term “oocyte” refers to the growing germ cells in the ovary of a female; these are at the diplotene stage of first meiosis and remain in this state for weeks or months. In contrast, the term “egg” refers to the fertilizable germ-cell; it is at the metaphase stage

of second meiosis, and, once fertilized or activated, it progresses rapidly through multiple cell divisions to reach the early embryo stage. Oocytes and fertilized or unfertilized eggs share many of the same components, but are dramatically different in the activity of their genes and DNA. An oocyte does not divide or replicate its DNA, but is intensely active in transcription of its lampbrush chromosomes [1–3]. Conversely, the egg, once it is activated or fertilized, is inactive in transcription but intensely active in DNA synthesis and cell division. Most of the components of an oocyte are the same as those of an egg. In normal life, an oocyte is changed under the influence of a pituitary hormone to become an egg in only a few hours.

The ovary of a frog contains thousands of oocytes in various stages of growth. One oocyte contains about one million circles of ribosomal DNA. These are free in the oocyte nucleus (germinal vesicle, GV), and are not integrated into the chromosomal DNA. Oocytes also contain many other components including Cajal bodies [4].

The following special characteristics need to be born in mind when injecting oocytes. Each oocyte is surrounded by about five layers of follicle cells, including blood vessels. These need to be removed before injecting an oocyte [5] because their gene activity is different from that of the oocyte. Injected oocytes can be cultured in a simple salt solution

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[5], changing the medium daily for up to one month. The best temperature for culture is 18 °C. The following sections concern various aspects of gene activity for which oocytes can be used to analyse.

2. Transcription

Soon after the unexpected success of injecting purified mRNA into oocytes for injected mRNA translation, it seemed worth testing the possibility of transcribing DNA in the same recipient cells. It was already known that injected purified DNA is converted into chromatin if injected into the oocyte nucleus [6]. Injected DNA was not transcribed, but broken down, if injected to the oocyte cytoplasm. Using SV40 DNA [7], and then 5 S DNA [8] we found that DNA was efficiently and accurately transcribed in oocytes, as long as the DNA was injected into the GV of an oocyte. The initiation and termination of transcription was more accurate in an oocyte GV than the best that could be done with in vitro cell-free systems [8]). This was especially true of transcription by RNA polymerase III used naturally for 5 S genes. The major advantage of oocytes for transcription has been by RNA polymerase II, as used for most eukaryotic genes. No satisfactory in vitro transcription by pol II has so far been developed.

Under the heading of transcription, we particularly wish to mention the value of oil-isolated GVs, a procedure first discovered by Lund and Dahlberg [9]. An oocyte is first isolated with minimal aqueous medium, and then immersed in aqueous-free oil. The GV of the oocyte is then isolated in oil, wiping away as much of the adherent yolk as possible. The isolated GV can be maintained in oil until required for injection or other purposes. We have used this procedure for injecting DNA and its reporter, and then incubated it, in oil, for 24 h. We were able to conclude that a GV can remain transcriptionally active, in oil, for at least 24 h. We comment (last part of this article) on the likely reason for this.

The main use of injected oocytes for transcription has been to identify the first steps in the reprogramming of transplanted somatic cell nuclei. The aim of nuclear reprogramming is to create new cell types by derivation from fibroblasts or other adult cell-types. The Yamanaka iPS procedure is the best but is very slow and inefficient. Single nuclear transfers of somatic nuclei to unfertilized eggs are more efficient and faster than reprogramming of cultured cells. Nuclear transfer to oocytes is fastest, but not complete (does not produce new cell-types). Comparison between nuclear transfer to oocytes *versus* eggs can tell us whether the 1-day changes produced in oocytes are likely to be the first that eventually take place in both nuclear transfer and iPS procedures. The changes in injected oocytes are primarily of a chromatin composition kind; they involve the uptake of nucleoplasm, linker histone exchange (H1 or B4) replacement, and histone H3.3 uptake [10]. In this way, the use of oocytes complements that of eggs for nuclear reprogramming analysis. For nuclear transfer to oocytes about 300 nuclei (in 35 nl) are injected (transferred into an oocyte GV). It is also possible to do multiple injections into the same GV (see below). The aim of this direction of work is to identify components of oocytes that initiate nuclear reprogramming, and also to provide a way of determining the function of many oocyte components, including those found by Blow and Laskey [11]; as well as by Session *et al.* [12] and Peshkin *et al.* [13].

A particularly interesting result has been described by J. G. Gall and colleagues. This is that the injection of demembrated sperm heads into the GV of *Xenopus* and other species of amphibia causes their conversion within a few hours into a lampbrush-like configuration [14,15]. A dramatic difference is seen when somatic nuclei are injected into eggs (inducing DNA synthesis) as opposed to injection to oocyte GVs (resulting in transcription). Their chromatin dispersion does not depend on transcription or on splicing of transcripts. One technical difference is that nuclei in eggs are not demembrated as they are for GV/oocyte injections, but this is unlikely to explain the oocyte/egg difference. An important discovery by Masui and colleagues is that a minimally diluted (X3) centrifugal extract of activated eggs can cause in vitro DNA replication but not transcription of somatic nuclei [16]. A major difference

between lampbrush chromatin in oocytes and in vitro extracts is the time for these configurations to form, as discussed and illustrated by Gall and Murphy [14]. It is likely that many DNA-inducing components of eggs may overwhelm transcription in egg extracts.

2.1. mRNA translation - Protein synthesis

Eggs and embryos are full of ribonuclease, as seen in homogenized eggs or embryos, and it would make no sense to add purified mRNA to these eggs or oocytes extracts, yet it seemed worth a try. Prof Jean Brachet and Prof Chantrenne from Brussels kindly provided us with globin mRNA. After cleaning all the equipment with chromic acid, the experiment worked amazingly well, and the injected embryos synthesized rabbit globin perfectly [17]. In retrospect, we can appreciate that a needle injected into an egg probably causes no more release of endogenous ribonuclease than would a sperm at fertilization. Nevertheless, this result was a great surprise, and opened the way to a wide range of injection experiments with *Xenopus* oocytes. mRNA injection has now been very widely used in cell and developmental biology in many organisms, including mammals [18]. It is remarkable how stable the oocyte-injected mRNA can be; injected globin mRNA has been continually translated for up to one month, when tested daily [17].

The importance of post-translational modification has become increasingly clear; because of the highly unselective ability of the frog oocyte to translate mRNAs from completely unrelated organisms (above), the question arises whether protein modifications are equally unselective. Laskey *et al.* [19] tested this first, using the expression of encephalocarditis virion protein, and found surprisingly successful translation of viral RNA. This was addressed in more detail by Wickens *et al.* [20] using a chicken ovalbumin gene. The chicken ovalbumin gene requires seven splicing events, compared to one for SV40; if each splicing event for ovalbumin was 50% as efficient as normal, this would explain why ovalbumin was synthesized at only 2% of the rate of SV40 [21]. In conclusion, oocytes can undertake post-translational processes across a wide range of organisms, but at lower efficiency compared to normal. It is possible that the efficiency might be improved if the amount of messenger RNA had been reduced.

3. Stability of a transcription factor

This aspect of transcription is not well understood at present. It is currently believed that the stability of transcription is remarkably short, being only a few seconds before the factor dissociates from its DNA or chromatin [22]. This conclusion depends largely on FRAP (fluorescence recovery after photobleaching) and measures time associated with DNA and not function. By use of oocytes, it is possible to combine dwell time with function (i.e. with the expression induced by the factor) [23]. This is because the factor induces by this route transcription of the DNA and expression of its adjacent reporter. This involves an injection of mRNA, encoding the transcription factor protein followed by expression of the DNA to which the factor binds; this is followed by another injection of a competitor DNA (with a different reporter). The competitor DNA in excess will capture any of the released factor from the first DNA and so cause expression of the second reporter which is not expressed with limiting amounts of the initially injected mRNA.

The result of this procedure is that the dwell time of this factor (*Ascl1*) is hours or days and therefore entirely different from the minutes or seconds conclusion from FRAP. *Xenopus* oocytes can therefore be used for this novel type of experiment because of their resilience to repeated injections of large volumes at desired times.

During the cell cycle, major changes [24] in DNA configuration take place, and so chromosomal protein exchange might be expected. More recently, Halley-Stott *et al.* [25] followed up this idea, linking it to nuclear reprogramming and pointing out that in normal development there is a phase of frequent cell division after fertilization and changes in gene expression, until the adult stage when neither of these commonly occur.

Core histone binding to DNA is restricted to the S phase of the cell cycle, but Palozola et al. [26] summarize several examples where some proteins including hsp70 remain in an open state during mitosis and some of these resume transcriptional activity after mitosis when transcription is markedly reduced. It is, therefore an interesting question whether a long dwell time as illustrated by *Ascl1* could be true of other chromosomal proteins or of parts of the cell cycle.

A potentially interesting further aspect of oocyte activity concerns the possibility that protein interactions are unusually stable in this cell. It was suggested, long ago, that transcription factors (and other proteins) might dissociate from their DNA binding preferentially at mitosis [24], this idea was pursued later [25] and is still of interest.

Mitosis and S-phase are two stages of the cell cycle when a major rearrangement of DNA and associated proteins take place. As cells become adult, they become stabilized in their differentiated state and do not change. In contrast, embryonic cells continually change their differentiation pathway, and generate more cell-types, undergoing frequent cell division that this stage. This concept still seems plausible today (review by Ref. [26]). The long dwell time described above for *Ascl1* and its binding to DNA or chromatin fits this idea well.

4. Concentration and time: *In vivo* or *in vitro*

For a very long time, use has been made of reconstituted cell-free systems to determine the function of purified cell components. Here we have emphasized an alternative, which is to inject purified cell components into normal living embryos or cells which can then be cultured. Transcription by RNA polymerase II has been particularly resistant to the development of efficient cell free systems. In our view, this is primarily a problem of concentration. In a living cell, components operate at extremely high concentrations; protein concentration in a living cell nucleus, for example, is at about 100 mg/ml. It would not be possible to make a cell free system with components at this concentration. Therefore nearly all components in a cell free system have to operate at many times below their normal concentration. In an injected cell, one or a few components can be purified, concentrated, and injected into a cell where other components are still at their normal concentration.

Time can also be important. If a concentration of a molecule is low, it may take time for electrostatic charge interactions, hydrophobic effects, and hydrogen bonds to be established. If many components are needed in a complex, it may take time for the last (limiting) one to find its place. It is not known in living cells how long processes take to be established. In *Drosophila* development, for example, new transcription patterns can be activated in as little as 10 min. On the other hand, the injection of DNA into the GV of an oocyte, where core histones are already present, needs 6 h before that DNA, or its adjacent reporter, is expressed [23]; this much time is required for the DNA to be converted into expressing chromatin. Most cell free transcription systems do not function well for as long as 6 h, but injected cells can give meaningful results for several weeks. Even small cells can be injected, technically, but the large size and resilience of an amphibian oocyte gives it substantial advantages. Furthermore, unlike a cell-free system, injected cells can be cultured for hours, days, or weeks. Therefore, concentration and time can give injected cells advantages over cell free systems, where this is possible. In many ways, a living cell is like a biochemical test tube [27].

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