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Chaperone-mediated chromatin assembly and transcriptional regulation in *Xenopus laevis*

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ABSTRACT Chromatin is the complex of DNA and histone proteins that is the physiological form of the eukaryotic genome. Chromatin is generally repressive for transcription, especially so during early metazoan development when maternal factors are explicitly in control of new zygotic gene expression. In the important model organism *Xenopus laevis,* maturing oocytes are transcriptionally active with reduced rates of chromatin assembly, while laid eggs and fertilized embryos have robust rates of chromatin assembly and are transcriptionally repressed. As the DNA-to-cytoplasmic ratio decreases approaching the mid-blastula transition (MBT) and the onset of zygotic genome activation (ZGA), the chromatin assembly process changes with the concomitant reduction in maternal chromatin components. Chromatin assembly is mediated in part by histone chaperones that store maternal histones and release them into new zygotic chromatin. Here, we review literature on chromatin and transcription in frog embryos and cell-free extracts and highlight key insights demonstrating the roles of maternal and zygotic histone deposition and their relationship with transcriptional regulation. We explore the central historical and recent literature on the use of *Xenopus* embryos and the key contributions provided by experiments in cell-free oocyte and egg extracts for the interplay between histone chaperones, chromatin assembly, and transcriptional regulation. Ongoing and future studies in *Xenopus* cell free extracts will likely contribute essential new insights into the interplay between chromatin assembly and transcriptional regulation.

KEY WORDS: *transcription, histone chaperone, H2A-H2B, H3-H4*

Chromatin and transcription have long been known to be interrelated, especially during the remarkable regulatory transitions that occur during early metazoan embryogenesis. The eukaryotic genome *in vivo* is composed of DNA complexed with histone proteins in an assembly called chromatin (Shechter and Allis, 2007). Histone deposition and chromatin assembly pathways have a long history of discovery in *Xenopus* cell-free extracts, providing key insights with broad application to eukaryotic biology and human health and disease. Here, we highlight the continuing relevance of the unique attributes of *Xenopus* cell-free extracts for crucial insights into the biological pathways performing chromatin assembly and transcriptional regulation. We also describe important discoveries about the function and mechanism of histone chaperones made in *Xenopus*.

Animal development, chromatin and transcription

Animal development is initially programmed by maternal RNAs and proteins deposited in the egg. Zygotic control supersedes the maternal program during the maternal-to-zygotic transition (MZT) when zygotic genome activation (ZGA) occurs (O'Farrell *et al.*, 2004, Schier, 2007, Tadros and Lipshitz, 2009). This transition is frequently concomitant with the substantial cell-cycle and morphological changes at the mid-blastula transition (MBT). The MZT occurs in all metazoans, but not always at the same developmental time as the MBT. Intriguingly, the timing of this transcriptional transition is highly species-dependent (O'Farrell *et al.*, 2004). In zebrafish the transition occurs at the 10th cell cycle, in *Xenopus laevis* the transition occurs at roughly the 12th cell cycle, in fruit flies it occurs at the 13th cell cycle, while in mammals it occurs in the first or second cell cycle (Schier, 2007, Tadros and Lipshitz, 2009). This observation is consistent with the hypothesis that the

Abbreviations used in this paper: MBT, mid-blastula transition; ZTA, zygotic genome activation.

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dramatically distinct body plans and developmental programs across the metazoan kingdom may be in part specified by differences in timing of zygotic gene expression (Fernandez-Tresguerres *et al.*, 2010, O'Farrell *et al.*, 2004).

Xenopus frogs as a model system for chromatin and transcriptional regulation

Frogs of the genus *Xenopus* are commonly used vertebrate model systems for studying development, cell cycle, cancer, chromatin, and many other biological phenomena. *Xenopus laevis* are evolutionarily close to mammals in comparison to other model organisms like yeasts, *C. elegans,* or *Drosophila,* with substantial homology and complementation among proteins, making them a compelling model for biology as well as human health and disease. *Xenopus* frogs have been used as a laboratory experimental system for a long time and have a history of producing crucial observations in many fields of biology, including being the first vertebrate successfully used for somatic-cell nuclear transfer (Gurdon, 1962). Frogs lay thousands of large eggs, from which cell free extract can be readily prepared that is capable of recapitulating most molecular phenomena in a test tube. The extract can be fractionated, its cell cycle state altered, and drugs and proteins can be added and removed to modulate activities (Costanzo and Gautier, 2004, Costanzo *et al.*, 2004, Hutchison *et al.*, 1988, Tutter and Walter, 2006). *Xenopus* cell-free egg extracts recapitulate most biological phenomena in a biochemically dissectible form.

The *Xenopus* model system is an unrivaled system for testing hypotheses as biochemical and biological studies can be conducted in parallel. *Xenopus* biology is exceedingly conserved with mammals and many seminal discoveries have been made with frogs with substantial implications for human biology. Furthermore, the bias of cleavage-stage eggs and embryos towards rapid replication is a major benefit for chromatin and transcriptional regulation studies, as these amplified conditions serve to enhance the phenotypes. Additionally, somatic cell nuclear transfer to reprogram adult frog or mammalian nuclei in *Xenopus* oocytes, eggs, and cell-free extracts have promoted essential understanding of the roles of transcription factors and chromatin function in global transcriptional reprogramming (Gurdon, 1962, Hansis *et al.*, 2004, Simeoni *et al.*, 2011). Our laboratory has made extensive use of these cell free extracts for probing chromatin assembly, histone modifications, and transcriptional regulation (Banaszynski *et al.*, 2010, Nicklay *et al.*, 2009, Onikubo *et al.*, 2015, Shechter *et al.*, 2009a, Shechter *et al.*, 2009b, Wang *et al.*, 2014, Wang and Shechter, 2016, Wilczek *et al.*, 2011).

Xenopus development and transcriptional regulation

Post-fertilization embryonic *Xenopus* cells are transcriptionally silenced and rapidly alternate between S-phase and M-phase (Laskey, 1985). This transcriptional quiescence and rapid cycling allows the embryo to undergo an accelerated and synchronized cell division and represents an epigenetic phenomenon of mitotic inheritance of transcriptional repression. These processes continue until the MBT and concomitant ZGA at developmental stages 8/9 (Fig. 1). Prior to ZGA, maternal stores are the only cellular source of proteins and mRNAs. This suggests that the quantity of maternally stored material, including the histones that are the major protein component of chromatin, is large enough to support the approximately 4,000 cells in stage 9 embryos. Indeed, substantial evidence shows that histone proteins are stored in vast excess in eggs compared to somatic cells (Almouzni and Wolffe, 1993a, Woodland, 1979, Woodland and Adamson, 1977). However, no available evidence indicates whether stored histone proteins are in adequate abundance for complete chromatin assembly through ZGA or if new histone translation from stored mRNAs is necessary for development. New mass spectrometry technologies and highly specific antibodies may allow precise determination of the mass of histones present.

Chromatin in the frog early embryo

The fundamental repeating unit of chromatin is the nucleosome, in which DNA is wrapped around an octamer of the four core histones, H2A, H2B, H3, and H4 (Luger *et al.*, 1997, Van Holde, 1989). Linker histones and other protein components further condense and arrange chromatin (Ausio, 2006, Woodcock *et al.*, 2006). Transcription assays *in vitro* have shown that nucleosomes efficiently block transcription and that transcriptional activation requires structural alteration of nucleosomes (Venkatesh and Workman, 2015). Post-translational modifications of histones, along with deposition of variant and linker histones, form a "histone code" regulating the usage of the underlying DNA (Strahl and Allis, 2000). Since at least some of these modifications can be retained across cell divisions, these histone modifications may serve as a memory for transcriptional states, forming the basis of epigenetic gene regulations and establishment of cell type-specific transcrip-

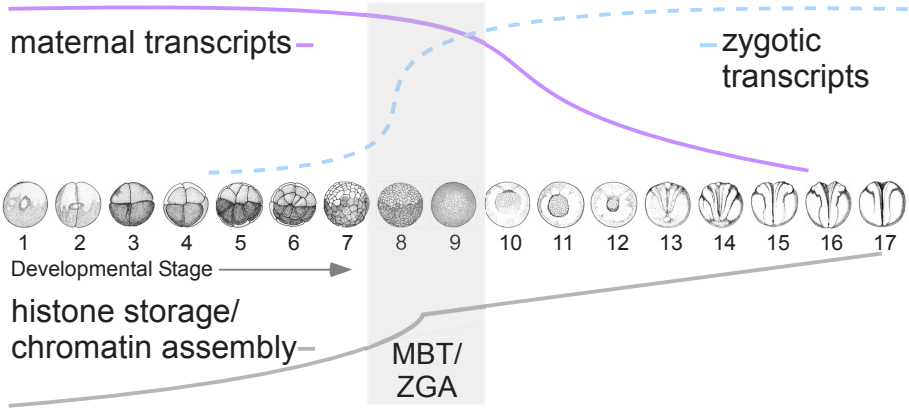

Fig. 1. Cartoon of early development and the timing of transcription and chromatin assembly. *During* Xenopus laevis *early development (morphological stages 1-17 shown), maternally deposited mRNA transcripts are consumed (purple) while new zygotic mRNA transcripts (blue) are first synthesized during the developmental transition of the MBT/ZGA (Mid-blastula transition / zygotic genome activation). Histone chaperones store maternal histones and deposit them during the exponential chromatin assembly (gray) of new zygotic DNA prior to the MBT.*

tome (Ramachandran and Henikoff, 2015).

We previously showed that dynamic changes in deposition of histone variants and alterations of post-translational modification patterns occur throughout the life of a multicellular organism and vary in each cell type (Nicklay *et al.*, 2009, Shechter *et al.*, 2009a, Shechter *et al.*, 2009b, Wang *et al.*, 2014). There are likely several proximate regulatory regimes overseeing this significant developmental switch. We favor the hypothesis that specific repressive components of chromatin suppress assembly and activation of RNA Polymerase II transcriptional complexes (Almouzni *et al.*, 1990b, Almouzni *et al.*, 1991, Dimitrov *et al.*, 1993, Hair *et al.*, 1998, Prioleau *et al.*, 1995, Prioleau *et al.*, 1994, Wolffe *et al.*, 1993) as our experimental work in this issue also supports this model in which chromatin assembly suppresses transcription in egg extract while nucleation of transcription by pre-binding of TBP stimulates transcription (Wang and Shechter, 2016). In this hypothesis, as the nuclear-to-cytoplasmic ratio decreases approaching the MBT and the chromatin assembly process changes with the diminution of maternal chromatin components, transcription may then commence, subject to normal signaling, transcription factors, and histone modification pathways (Fig. 1). Other hypotheses may also be valid: for instance, exogenous TBP protein can induce transcription on a reporter plasmid in cell-free egg extract (Almouzni and Wolffe, 1995, Hair *et al.*, 1998, Prioleau *et al.*, 1994), suggesting that the recruitment of the polymerase machinery may be compromised in the early embryo.

Chromatin assembly, the deposition of histones onto chromatin to form nucleosomes, is highly efficient in *Xenopus* eggs, early embryos, and in egg extract. Any DNA incubated in egg extract is rapidly assembled into a histone containing complex (Almouzni *et al.*, 1990a, Laskey *et al.*, 1977). In embryos, fertilization and during subsequent early embryo cell cycles prior to the MBT, histone deposition and chromatin assembly of newly replicated DNA is robust and rapid (Amodeo *et al.*, 2015, Collart *et al.*, 2013). In *Xenopus*, embryos reach the 12th cell cycle in five hours post fertilization, and the chromatin formed during these stages are enriched for histone variant, modifications, and linker histones specific to the embryo. In particular, we previously showed that *X. laevis* embryos have elevated incorporation of histone H2A.X.3 (previously called H2A.X-F), a variant frequently associated with the cell cycle checkpoints, and a unique maternal linker histone called H1.M (previously B4) (Shechter *et al.*, 2009a, Wang *et al.*, 2014). H1.M is essential for mesodermal competence during frog development (Steinbach *et al.*, 1997). An open question remains as to the specific role of these histone variants and how they are deposited into chromatin, although a reasonable hypothesis for H2A.X.3 is that it suppresses aberrant checkpoint activation during the rapid early cell cycles (Shechter *et al.*, 2009a). Maternal linker histones H1.M are differentially deposited in oocyte and egg extracts and may correlate with gene expression and repression (Dimitrov *et al.*, 1994, Nightingale *et al.*, 1996, Sera and Wolffe, 1998).

Associated with ZGA is rapid incorporation of the zygotically expressed somatic histones (Wang *et al.*, 2014). We favor the hypothesis that the rapid rate of histone deposition and the presence of maternal histones and histone post-translational modifications is, in part, responsible for the suppression of zygotic transcription (Wang *et al.*, 2014, Wang and Shechter, 2016). This is supported by the evidence that the nuclear-to-cytoplasmic ratio and the depletion of maternal histones are important for this regulation (Amodeo *et al.*, 2015).

These processes are directly dissectible in oocytes and eggs, which are transcriptionally active and repressed, respectively. Previous analyses of chromatin assembly, nuclear assembly, DNA replication and transcription in oocyte and egg extracts were performed over twenty years ago and revealed clear differences. Oocyte extract was found not to be able to replicate DNA or assemble nuclei on sperm chromatin (Cox and Leno, 1990). Wolffe, Almouzni and colleagues showed essential activities in cell free extracts for assembling chromatin through many seminal papers (Almouzni *et al.*, 1990a, Almouzni *et al.*, 1990b, Almouzni *et al.*, 1991, Almouzni and Wolffe, 1993a, Almouzni and Wolffe, 1993b). These activities are also essential for reprogramming of somatic nuclei in cell-free egg extract (Miyamoto *et al.*, 2007), although further chromatin rearrangement through mitosis appears to be essential (Ganier *et al.*, 2011, Lemaitre *et al.*, 2005).

In *Xenopus* sperm chromatin, protamines (Sperm Specific Basic Proteins 1-6) are assembled in place of core histones for extreme compaction, although much H3-H4 remains in the mature spermatids (Frehlick *et al.*, 2007, Katagiri and Ohsumi, 1994, Shechter *et al.*, 2009b). After fertilization, or in pronuclear assembled on sperm chromatin in egg extract, the highly compacted sperm chromatin is decondensed with concomitant deposition of maternally-stored, chaperone-complexed H2A-H2B (Philpott *et al.*, 1991), establishing the paternal component of the new embryonic chromatin.

Histones and histone chaperones: key function and insight from *Xenopus*

Nucleosome assembly in a cell is a highly regulated process involving many factors, including histone chaperones, which bind histones and escort them onto DNA, and ATP-dependent chromatin remodelers, which form *bona fide* nucleosomes (Burgess and Zhang, 2013). A recent thorough review of histone chaperones provides an overview (Gurard-Levin *et al.*, 2014). Histone H3-H4 is passed down a collection of molecular and histone chaperones, from Hsp70, Hsp90, NASP (formerly called N1 in *Xenopus*), Asf1, and to HIRA or CAF1 for deposition (Campos *et al.*, 2010, Mattiroli *et al.*, 2015). CAF-1, the H3-H4 chaperone used during replication, is critical for tight coupling of chromatin assembly to DNA replication in *Xenopus* (Quivy *et al.*, 2001). Histone H2A-H2B are chaperoned by Nucleoplasmin family members (Npm1, Npm2, and Npm3), Nap1, and FACT (Gurard-Levin *et al.*, 2014). Much of the elucidation of these pathways was accomplished in *Xenopus* cell-free egg extracts, with identification of Nucleoplasmin (Npm2) in the 1970s (Earnshaw *et al.*, 1980, Laskey *et al.*, 1978), the first protein to be called a chaperone, and the identification of N1/N2 (likely proteins from alloalleles that we now denote as *N1a* and *N1b*, homologous to mammalian NASP) in the 1980s (Kleinschmidt *et al.*, 1985).

Npm2 was originally discovered in egg extract as a protein that neutralized the positive charges of histones and assembled nucleosomes on a plasmid DNA under physiological salt concentrations *in vitro* (Laskey *et al.*, 1978). This charge attenuation by Npm was identified as the critical factor that inhibited histone aggregation and promoted the proper histone deposition onto DNA and led to the concept and the term “molecular chaperones” to describe Npm and its function (Earnshaw *et al.*, 1980). We and others have shown that Npm contains many post-translational modifications, including phosphorylation, glutamylation, and arginine methylation, and these

modifications were found to regulate the chaperoning functions of Npm. In particular, phosphorylation on the short and intrinsically disordered N-terminal tail was found to promote sequestration of histones *in vitro* and suggested that the histone storage function of Npm is also activated through phosphorylation in this region (Onikubo *et al.*, 2015). Further work on Npm histone storage and the identification of the interaction sites necessary for histone sequestration will shed light on the molecular mechanism of histone binding and release. Such knowledge will also provide significant insights into the molecular mechanisms of how the switch between histone assembly and disassembly is regulated with multi-functional somatic histone chaperones such as Nap1 and Asf1. It is also of interest that Npm presence continues long after ZGA, but its hyperphosphorylation, a characteristic feature of Npm during the early stages of development, is lost, while retaining other modifications such as glutamylation and arginine methylation. This observation suggests that Npm may have additional roles, such as linker histone exchange. Ongoing study using the *Xenopus* model will continue to be a significant tool for studying histone chaperone functions with direct application to other eukaryotes and human biology.

The complexity of histone chaperone networks is seen in the fact that many of these proteins are involved in multiple and often opposite functions. Nucleoplasmin, when heavily phosphorylated and otherwise modified in the egg, decondenses sperm chromatin (Philpott *et al.*, 1991) yet binds and sequesters core histones (Onikubo *et al.*, 2015). NASP participates in H3-H4 dimer assembly in the cytoplasm (Campos *et al.*, 2010), stores H3-H4 in the egg (Finn *et al.*, 2012), and also directly transfers linker histones into the nucleus (Finn *et al.*, 2008). Asf1 is a histone chaperone responsible for H3-H4 import into nucleus, but Asf1 has also been implicated in the disassembly of nucleosomes during DNA replication and during transcription (English *et al.*, 2006). Interestingly, Asf1 is dispensable for de novo histone deposition in *Xenopus* egg extracts (Ray-Gallet *et al.*, 2007). The reason for this is not clear, but is perhaps due to its function in replicational stress histone buffering (Groth *et al.*, 2005). Nap1 is a histone chaperone responsible for histone H2A-H2B transport into the nucleus and is implicated in H2A-H2B deposition during DNA replication, while it also chaperones linker histones and is implicated in nucleosome disassembly during transcription. Importantly, post-translational glutamylation of Nap1 significantly altered linker histone dynamics on mitotic chromosomes in *Xenopus* egg extract (Miller and Heald, 2015). FACT is an H2A-H2B and H3-H4 chaperone involved in transcriptional regulation, but intriguingly was recently shown to be responsible for the assembly of H2A.X.3 into *Xenopus* mitotic chromosomes in a reconstituted system, suggesting that many disparate roles exist for chaperones (Shintomi *et al.*, 2015). HIRA, a specialized chaperone for the transcriptionally-activating histone variant H3.3, is present in *Xenopus* oocytes and eggs and is essential for replication independent nucleosomal assembly (Ray-Gallet *et al.*, 2002), required for proper gastrulation and H3.3 deposition (Szenker *et al.*, 2012), and transcriptional reprogramming in nuclear transfer to oocytes (Jullien *et al.*, 2012).

The multiple functions of each histone chaperone and redundant functions among histone chaperones have made the functional analyses of histone chaperones difficult using direct genetic approaches in cells. This is evidenced by a surprising observation that no histone chaperones have been shown to be essential in mammalian cells or in yeast despite their involvement in essential biological functions, such as DNA replication and transcription. Therefore, the molecular mechanisms of the functional switch between nucleosome assembly and disassembly during DNA replication and transcription currently remain unknown and provide unique opportunity for continued investigation in *Xenopus* cell-free extracts in which they can be biochemically probed - by immunodepletion/add-back experiments - and in the absence of ongoing transcription.

Transcription and reprogramming in the early embryo

The mechanism by which the pre-MBT global transcriptional block occurs is a long-standing and unanswered question. Cell free extracts have been invaluable for deciphering this regulation of transcription during early development. *Xenopus* oocyte extracts are capable of transcribing any reporter plasmid with a strong promoter (i.e. CMV, adenovirus), while laid egg extracts are only capable of transcribing the reporter plasmid if it has been pre-incubated with the TATA-binding protein TBP, a component of the TFIID basal transcriptional apparatus (Dimitrov and Wolffe, 1995, Toyoda and Wolffe, 1992). This phenomenon was hypothesized to occur due to a race between chromatin assembly and transcriptional complex assembly (Almouzni *et al.*, 1990b), and may explain the global transcriptional quiescence in the egg. Our new experiments directly demonstrate these phenomena (Wang and Shechter, 2016).

Egg extracts have also been used to test mechanisms of reprogramming the transcriptional state, and perhaps by reverting to the global transcriptional quiescence found in the egg, insights into pluripotency can be further elaborated (Hansis *et al.*, 2004, Jullien *et al.*, 2010, Lemaitre *et al.*, 2005, Simeoni *et al.*, 2011). An ATP-dependent reaction catalyzed by the nucleosomal remodeling component ISWI was responsible for active remodeling of somatic nuclei in the egg. This activity was assayed in part by the loss of TBP protein from somatic nuclei incubated in egg extract, and also by the acquisition of a number of embryonic-specific components, including Npm, TFIIFα, and histone B4 (Kikyo *et al.*, 2000). Consistently, the ATPase Brg1 was critical for mammalian nuclear reprogramming in cell-free egg extract (Hansis *et al.*, 2004). We have identified other egg protein components that are acquired by remodeled sperm and somatic chromatin: proteins involved in nuclear pore and nucleolar assembly as well as the embryonic linker histone B4 (Fig. 1F in ref. Shechter *et al.*, 2009b). Significantly, acquisition of B4 is an early step in somatic cell reprogramming upon injection into *Xenopus* oocytes (Jullien *et al.*, 2010). These observations are consistent with an active remodeling process in the egg. Since maternal chaperones store the histones that comprise potential key components of the transcriptional competence of egg-reprogrammed nuclei, evidence demonstrating that exogenous Nucleoplasmin enhances the efficiency of reprogramming (Betthauser *et al.*, 2006, Huynh *et al.*, 2016) suggests that future studies on maternal histone chaperones will provide new research directions for stem cell research.

Conclusions

The relationship between chromatin, chromatin assembly, and transcriptional regulation is an essential component of the development of multicellular organisms. *Xenopus laevis* has been a crucial model organism and tool for the study of these events and still holds tremendous potential for further dissection of these processes. In particular, the role of specific aspects of chromatin

assembly and histone PTMs in the relationship with the onset of zygotic transcription is unclear. Since histone chaperones are a large class of proteins responsible for key aspects of chromatin metabolism, they lie at the fulcrum of important current areas of research. Future studies, especially in *Xenopus* cell-free extracts, hold great promise in further elucidation of mechanisms of these critical biological pathways.

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